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Do IgE–IgG complexes occur in the circulation?

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SUMMARY

IgG anti-IgE autoantibodies in human allergic sera have been investigated using an enzyme immunoassay on microplates coated with anti-human IgE MoAb. After incubation with serum, the plates were developed with peroxidase-labelled anti-human IgE MoAb for the determination of total IgE levels, or with anti-IgG MoAb for evaluating IgG anti-IgE autoantibodies. Using this methodology, no correlation was found between total IgE and IgG anti-IgE levels in groups of sera of allergic individuals. Although the results obtained with enzyme-labelled anti-IgG are often interpreted as indicative of IgE–IgG complexes captured from the serum, molecular sieving on gel columns as well as direct ultrafiltration experiments through 300-kD membranes demonstrate that such complexes do not occur preformed in the circulation, but arise *de novo* on the anti-IgE-coated solid phase during *in vitro* incubation with human serum. It is suggested that IgG anti-IgE autoantibodies react with IgE only after the latter has undergone a conformational change, either by colloidal manipulation or after reaction with allergen.

Keywords IgE IgG immune complexes

INTRODUCTION

It has been well established that the blood serum of (atopic) allergic individuals contains IgG-(auto-) antibodies directed against the Fc portion of immunoglobulin E [1–4]. Although several authors have suggested a role for these IgG anti-IgE antibodies in the pathology of allergic disease, low levels of such autoantibodies are also found in the serum of normal individuals [5–10]. Attempts to demonstrate a correlation between the levels of IgG anti-IgE and total IgE have produced conflicting results. Some authors reported a positive correlation [2,5,6,11], whereas in other studies no clear-cut association was found [4,9,12]. As an explanation it has been considered that IgG anti-IgE antibodies might hamper the correct determination of IgE levels [13], although other data indicate that the IgG autoantibodies do not interfere with the detection of total IgE in solid-phase assays [9].

It is often assumed that immune complexes of IgG anti-IgE and IgE occur preformed in the circulation. For example, Inganäs *et al.* [1] have shown that heating the serum of allergic patients augments the level of detectable IgG anti-IgE antibodies, and interpreted this effect as being due to the disruption of IgE–IgG complexes, thereby releasing free IgE. Studies by gel chromatography and high performance liquid chromatography (HPLC) subsequently indicated that most of the IgG anti-IgE antibody occurred in complexed form with a molecular size in the range 350–600 kD, while IgE immunoglobulin was detected in both the free and complexed forms [8,9,12,13].

Essentially, two methods have been employed for the estimation of IgG anti-IgE, i.e. determination of free IgG anti-IgE antibodies captured by means of purified IgE adsorbed to a solid phase [2,4,14], or evaluation of IgG anti-IgE already in complex with IgE (IgG anti-IgE/IgE) by using immobilized anti-IgE antiserum [13]. In several previous studies, IgG autoantibodies were measured as free IgG anti-IgE antibodies [1,2], precluding a true assessment of the occurrence of preformed IgG anti-IgE/IgE aggregates. However, using anti-IgE-coated microtitre plates for detection, Swainson *et al.* [12] reported IgG anti-IgE antibodies in eluates of HPLC columns in both the complexed and monomeric forms.

In the present work we have attempted to localize possibly preformed IgG–IgE complexes in the blood serum by means of gel filtration and ultrafiltration. Also, by using identical methodologies for determining the levels of both IgG–IgE complexes and total IgE, the relationship between these two parameters has been reinvestigated.

PATIENTS AND METHODS

Blood sera and antibodies

Blood samples of patients with various clinical symptoms of type I allergy were obtained from hospitals in Spain and The Netherlands. A pool of sera comprising equal volumes from more than 50 individual samples was employed for the gel

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filtration experiments. Sera were also obtained from adult patients with atopic dermatitis. Serum samples were kept frozen at −20°C until use. Human IgG-bearing B lymphocytes were isolated and supplied by the Immunology Department of the University Hospital, Utrecht, The Netherlands, and were cultured to produce IgG immunoglobulins. The supernatant of proliferating human B lymphocytes cultured with cytokine IL-4 in combination with Epstein–Barr virus (EBV) was used as a source of IgE. IgG1 κ-chain paraprotein and polyclonal IgG purified by means of DEAE-cellulose ion-exchange chromatography were also supplied by the above Immunology Unit. Aggregated IgG from human gammaglobulin (Laboratorios LETI, Barcelona, Spain) was prepared by heating at 62°C for 30 min.

Assay of IgE–IgG complexes

Microtitre plates (Nunc-Immuno Poly Sorp Labclinics, Barcelona, Spain) were coated overnight at room temperature with mouse monoclonal anti-human IgE (Janssen Biochimia, Beerse, Belgium) at 2 μg/ml in PBS. The plates were washed with a solution containing 0·04% v/v Triton X-405 (Sigma, St Louis, MO). Serial dilutions 1:2 to 1:256 of the sera to be tested (100 μl) were made up in PBS/Tween-20 (0·5%) containing 1% human serum albumin (HSA) and were left with shaking in 100-μl aliquots per well for 30 min at room temperature. After subsequent washing steps, 100 μl of peroxidase-labelled mouse monoclonal anti-human IgG (Janssen Biochimia) at 0·5 μg/ml in PBS/Tween–HSA were added per well and the plates were shaken for 30 min at room temperature. After further washing steps bound peroxidase was evaluated by incubation with a mixture of tetramethylbenzidine (Boehringer, Mannheim, Germany) and hydrogen peroxide. The enzymatic reaction was stopped by the addition of 100 μl 2 N sulphuric acid. Non-specific background fixation of IgG to the wells was checked on non-coated microtitre plates. Optical densities were finally read at 450 nm in a microplate reader (Titertek Multiskan, Finland) and were plotted against log serum dilution. The value obtained at 1:2 dilution was taken as an estimate of the concentration of IgG considered to be in complex with IgE.

Control for possible IgE anti-IgG activity

Vinyl microtitre plates (Costar, 96 wells) were coated overnight at room temperature with mouse monoclonal anti-human IgG (Janssen Biochimia) at 2 μg/ml in PBS. The plates were washed with PBS–Tween (0·05%). To block non-specific binding, 100 μl bovine serum abumin (BSA) 1% was added to each well and the plates were incubated for 1 h at room temperature and washed. Next, 100 μl of a solution containing IgG immunoglobulins (i.e. IgG from the supernatant of B lymphocyte cultures; polyclonal IgG purified from normal human serum (NHS) by DEAE-cellulose chromatography; IgG captured directly from normal serum; and heat-aggregated IgG) were incubated with the anti-IgG-coated plates and shaken for 2 h at room temperature. The IgG κ-chain paraprotein was coated directly to non-treated microtitre wells at 200 μg/ml because of the non-specificity of the anti-IgG reagent for the κ-chain.

Sequential dilutions 1:2 to 1:256 of a pool of sera in PBS–(1%) BSA were added in 100-μl volumes to each series of wells pretreated with anti-IgG/IgG immunoglobulins. After 2 h incubation at room temperature and subsequent washing steps, the plates were treated for 1 h with HRP-labelled mouse MoAbs to human IgE (Ingenasa, Madrid, Spain) at 1 μg/ml in PBS. Finally, the plates were washed again and developed colorimetrically as described above.

Determination of total IgE

To compare the levels of total IgE and IgE–IgG complexes, microtitre plates (Nunc-Immuno Poly Sorp) were coated with anti-IgE under the same conditions as described for the IgE–IgG complex assay. Also, the serial dilutions of the serum samples subsequently incubated for 30 min at room temperature were the same as before. To detect bound IgE, horseradish peroxidase (HRP)-labelled mouse monoclonal anti-human IgE at 0·1 μg/ml diluted in a blocking solution containing PBS–Tween (0·1%), fetal calf serum (FCS; 25%) and heparin (1%) was added and left for 30 min at room temperature. After renewed washing, the plates were developed as before. The optical densities were plotted against log dilution and the value obtained at 1:4 dilution was used as an estimate of the concentration of total IgE.

Detection of specific IgG

For monitoring IgG during serum fractionation, allergen-specific IgG antibody was determined by enzyme immunoassay. The allergenic preparation of *Lolium perenne* comprised the unfractionated non-dialysable portion (cut-off 10 kD) of aqueous pollen extract (Laboratorios LETI, Barcelona, Spain). A solution of *L. perenne* allergenic proteins at 0·01 mg/ml in sodium carbonate buffer pH 9·4–9·7 was incubated overnight on microtitre plates at 37°C in aliquots of 150 μl/well. After washing with Triton X-405 (0·04%), column fractions diluted in PBS/Triton X-405 (0·01%) were transferred to the wells and left for 30 min at room temperature. After a further series of washing cycles, HRP-labelled monoclonal mouse anti-human IgG (Janssen Biochimia) at 1 μg/ml diluted in PBS–Triton X-405 (0·01%) was added and allowed to react for 30 min at room temperature. Finally, the plates were developed as described in the previous sections.

Chromatographic separation of sera

Samples of 5 ml from a pool of sera of patients allergic to grass pollen were fractionated on a 2·6 × 100 cm column of Sephacryl S300 (Pharmacia Fine Chemicals, Uppsala, Sweden) by elution at room temperature with PBS pH 7·4. Eluates were monitored for 280 nm absorption and fractions of 2 ml were collected and analysed in 1:2 dilution for the determination of IgE–IgG complexes, total IgE and allergen-specific IgG. The column was calibrated in a pre-run using the molecular weight markers fibrinogen (Sigma), glucose oxidase and peroxidase (both from Boehringer).

Ultrafiltration experiments

Ultrafree-PFL filter systems incorporating a polysulphone membrane (cut-off 300 kD) were purchased from Millipore. A volume of 0·5 ml of serum diluted 1:4 in PBS was slowly introduced into the top compartment by means of a syringe. The samples were left overnight at 4°C for complete ultrafiltration by gravity flow. The $\geqslant$ 300 kD material retained on the membrane was taken up in PBS, while the material that passed the membrane was collected directly ($<$ 300 kD portion). The membranes were verified with aggregated IgG (37·5% of

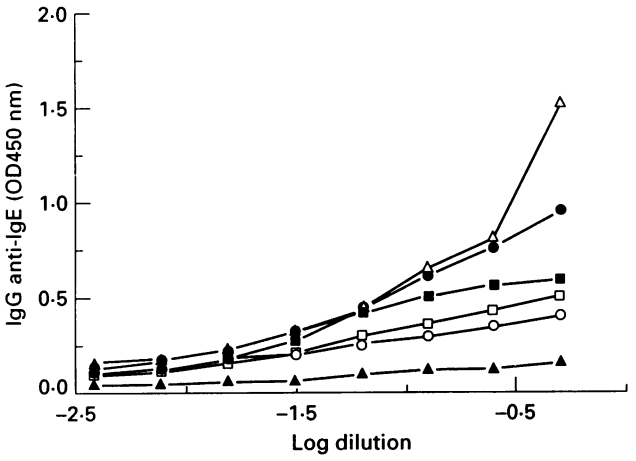

Fig. 1. Examples of the binding to anti-IgE-coated plates of IgG anti-IgE autoantibody or of IgE–IgG 'complexes', using peroxidase-labelled mouse monoclonal anti-human IgG for detection and dilutions of serum samples of individual patients (numbers 1–6) with pollinosis, allergic bronchial asthma or atopic dermatitis. □, Patient 1; ○, patient 2; ■, patient 3; ●, patient 4; △, patient 5; ▲, patient 6.

retention of IgG from a commercial γG preparation and 91·5% of retention after heat aggregation), and with IgE from lymphocyte culture supernatant (no retention).

RESULTS

The IgE–IgG complexes encompass IgG anti-IgE autoantibody

Estimation of the presumed IgE–IgG complexes in the blood serum was achieved by capture on an anti-IgE-coated solid phase and development with HRP-labelled anti-IgG. Figure 1

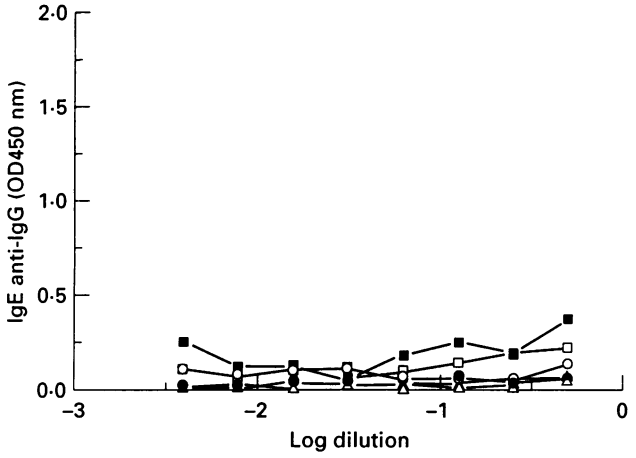

Fig. 2. Results of experiments designed to detect possible IgE anti-IgG antibodies using a pool of allergic sera reacted with IgG immunoglobulins from different sources affixed to anti-IgG-coated wells of microtitre plates, i.e. IgG from the supernatant of B lymphocyte cultures (○); polyclonal IgG purified from normal human serum (NHS) by DEAE-cellulose chromatography (□, 1 μg/ml; ■, 25 μg/ml); IgG captured directly from NHS (●); and heat-aggregated IgG (AGG; ▲). The IgG κ-chain paraprotein (△) was coated directly to non-treated microtitre wells.

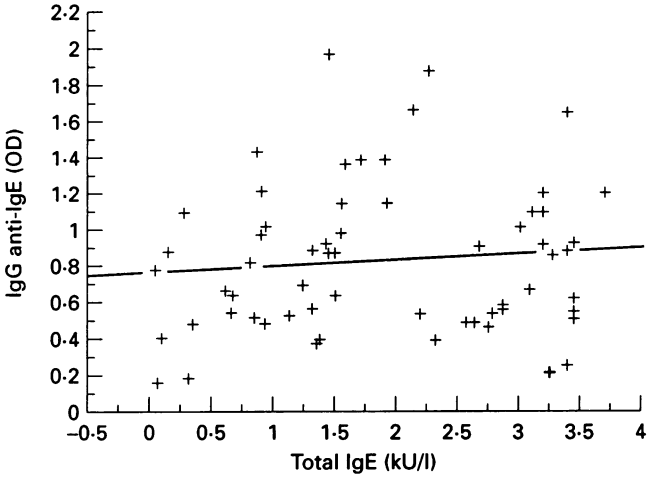

Fig. 3. Scatter diagram of the relation between IgG anti-IgE autoantibodies and the total IgE serum levels estimated under identical conditions. The equation for the regression line was $y = 0{\cdot}04x + 0{\cdot}76$ ($r = 0{\cdot}10$, not significant).

shows the colorimetric readings due to IgG captured onto such plates and therefore fixed to IgE bound from the serum by immobilized anti-IgE.

We have investigated the reverse possibility, in which the IgE within the complex might function as an anti-IgG autoantibody similar to IgE rheumatoid factor (IgE-RF). In these experiments, IgG preparations from different sources were coated on a solid phase, either directly or indirectly, and allowed to react with a pool of allergic sera followed by development with an anti-IgE reagent (see Patients and Methods). As shown in Fig. 2, IgE from human sera failed to react with the various γG-globulins under the conditions of assay. It was therefore concluded that IgE–IgG complexes detected on the plates consisted of IgG anti-IgE bound to γE antigens, whereas the reverse situation did not detectably occur.

Comparison between levels of IgE–IgG complexes and total IgE determined under identical conditions of assay

In order to verify whether total serum IgE levels influence the formation of IgE–IgG complexes, the relationship between these two parameters was reinvestigated. Most investigators have employed different methodologies for determining total IgE levels (e.g. PRIST) and IgG anti-IgE antibodies or IgG anti-IgE/IgE complexes (e.g. radioimmunoassay, solid-phase enzyme immunoassay or dot immunobinding). These divergent technologies may be the reason why the relationship between the two parameters still remains unsettled.

In the present work, the relationship was examined by setting up identical assay conditions for both parameters, using the same anti-IgE-coated solid phase, reagents and incubation times (see Patients and Methods). Under these conditions, no statistical correlation was observed, as shown in the scatter diagram of Fig. 3.

Analysis for IgE–IgG complexes by gel filtration

Experiments were performed whereby aliquots from a pool of allergic sera were separated by molecular sieving and total IgE,

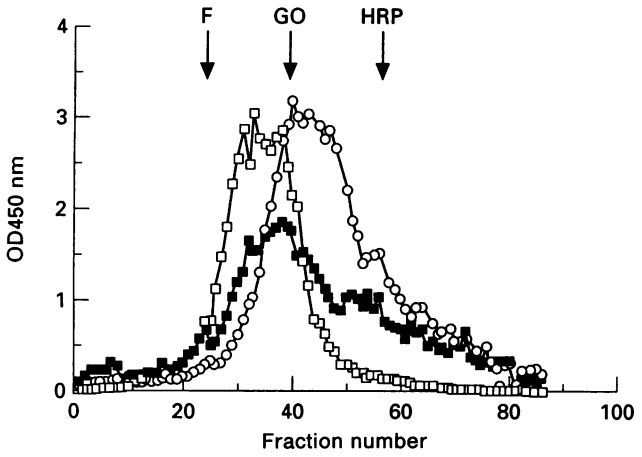

Fig. 4. Molecular sieving of human allergic serum on a column of Sephacryl S300. Eluates were analysed for total IgE (□), grass pollen-specific IgG (○), and IgE–IgG complexes (■). The elution positions of the molecular weight markers fibrinogen (F; mol. wt = 339 kD), glucose oxidase (GO; mol. wt = 150 kD) and horseradish peroxidase (HRP; mol. wt = 44 kD) are indicated.

allergen-specific IgG and IgE–IgG complexes were located by analysis of eluates. A typical elution profile is shown in Fig. 4. Two separated peaks corresponding to IgE and specific IgG were detected. In contrast with the results of some other authors, all IgE was found in a molecular weight position corresponding to the monomeric form. More important, the complexes IgE–IgG were detected at an intermediate elution volume, i.e. precisely at the overlap of the IgE and IgG monomers, indicating that IgE–IgG complexes could be measured only in those fractions containing both immunoglobulins in their free form. These experiments allow two possible conclusions: a possible dissocation of preformed complexes on the column [12], or *de novo* formation of IgE–IgG aggregates on the solid phase during the assay procedure.

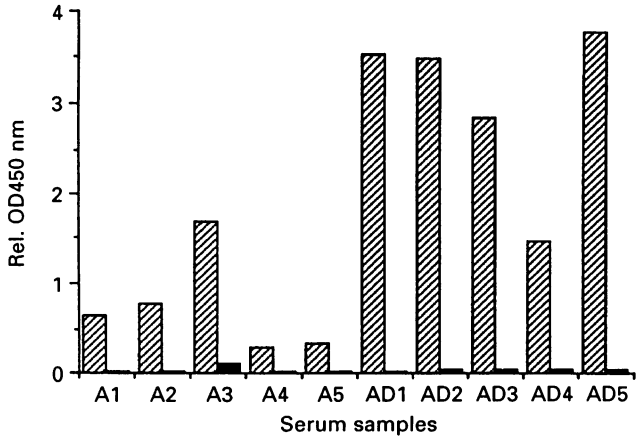

Fig. 5. Distribution of total IgE over fractions higher (■) and lower than 300 kD (▨) observed with 1:64 diluted sera of individual patients with pollinosis/asthma (A) or atopic dermatitis (AD).

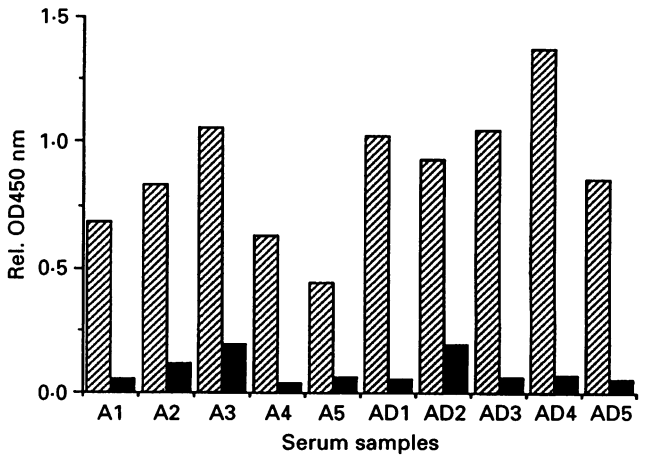

Fig. 6. Distribution of IgE–IgG complexes over fractions higher (■) and lower than 300 kD (▨) observed with 1:8 diluted sera of individual patients with pollinosis/asthma (A) or atopic dermatitis (AD).

Ultrafiltration experiments

The molecular weight reported for IgE–IgG complexes varies from 350 to 900 kD. Aggregates this size would be retained by suitable membranes, and experiments were therefore designed to detect preformed IgE–IgG complexes in serum by ultrafiltration, using membranes with a cut-off of 300 kD. Serum samples were employed of patients with allergic bronchial asthma or atopic dermatitis, since in these diseases the presence of circulating immune complexes has frequently been alleged.

Fractions retained by the membranes ($\geqslant$ 300 kD) and those that passed through (< 300 kD) were analysed for the distribution of total IgE (Fig. 5) and of IgE–IgG complexes (Fig. 6). Figure 5 shows that all the serum IgE was detected in the fractions lower than 300 kD, similar to IgE from supernatants of B cell cultures (see Patients and Methods). Interestingly, the presumed IgE–IgG complexes were likewise detected in the serum portion passing across the membrane and therefore lower than 300 kD. This observation provides firm evidence that the IgE–IgG complexes detected in our assays do not occur preformed, but are being assembled from the monomeric partners on the solid phase during the incubation step with serum. The values for the proposed IgE–IgG complexes observed with the sera of asthmatic or atopic eczema patients were quite similar despite the very high levels of total IgE in the sera of the latter.

DISCUSSION

A substantial number of published investigations suggest that IgG anti-IgE autoantibody occurs in the serum both in the free form and in complex with self IgE, notably in allergic and parasitic manifestations [4,9]. According to current opinion, the complexes are composed of an IgE immunoglobulin and IgG anti-IgE autoantibody, the specificity of which has been established by competitive inhibition with human immunoglobulins of each isotype (IgG, IgA, IgM, IgD and IgE), using purified IgE or isolated myeloma IgE coated to a solid phase [2,9]. Previous workers have indubitably demonstrated that the IgG autoantibody is directed against IgE epitope(s) residing

within the Cε2 and the Cε4 domains of the γE molecule [15]. Using γG proteins of different provenance we have been able to detect—within the sensitivity limits of the assay system employed—only negligible traces of rheumatoid factor-like IgE anti-IgG autoantibodies in the sera of allergic individuals [16]. The discussion may therefore be restricted to IgE–IgG complexes composed of γE antigen and IgG anti-IgE antibodies.

For estimating the presumed IgE–IgG complexes as well as total IgE levels we adopted the technique of antigen capture by means of anti-IgE antibody immobilized on a solid phase. With this methodology and in contrast to our earlier data [11] we were unable to establish a significant statistical correlation between total IgE and the level of IgE–IgG complexes in the serum. The subsequent gel filtration experiments established that IgE and allergen-specific IgG both occur in the free form, and that possibly preformed IgE–IgG complexes did not emerge from the columns at their expected molecular weight position. Instead, they were detected in eluate fractions intermediate between the elution volumes of IgE and IgG immunoglobulins. This observation is entirely in agreement with the results of Swainson *et al.* using fractionation by HPLC, who interpreted the finding as being due to the dissociation of the IgE–IgG complexes on the column, followed by recombination in the eluates [12].

The latter hypothesis is refuted by the ultrafiltration experiments, which were chosen to avoid possible fractionation artefacts. In all sera examined so far, IgE–IgG 'complexes' were consistently detected among the serum proteins with an apparent molecular size < 300 kD. This finding precludes the existence of preformed IgE–IgG complexes in the blood serum, for which dimensions far over 300 kD have to be assumed. Taken together with the gel filtration experiments, our results rather indicate that complexes consisting of native IgE and IgG do not exist in the circulation, but that such conglomerates arise *de novo* during the assay on an anti-IgE-coated solid phase. This conclusion coincides with the earlier observations of Twena *et al.* [9], who reported that the removal of high molecular weight aggregates from allergic sera by ultracentrifugation had no significant effect on the levels of detectable IgG anti-IgE autoantibodies. Their and our data are in conflict with the current assumption that 50–100% of the IgG anti-IgE antibodies circulate in a complexed form [1–3,14].

As a possible explanation of our results it may be inferred that the IgG autoantibodies do not recognize the native IgE antigen, but react with an epitope revealed in the Fcε portion after the γE molecule has undergone some conformational alteration. Such a modification might be envisaged by a change in the colloidal environment, i.e. during isolation of natural or myeloma IgE, by chromatographic manipulation, by ultracentrifugation and other separation techniques, by combining with (monoclonal) anti-IgE antibody (e.g. on the surface of microtitre plates), or perhaps by the reaction of specific IgE antibody with allergen in fluid phase. Experiments in progress indicate that IgE-containing aggregates ⩾ 300 kD indeed form in the serum *in vitro* upon addition of the corresponding allergen. IgE- and IgG-containing aggregates may occasionally (i.e. depending on the patient's clinical status) be observed by ultracentrifugation [17,18], but it remains undecided whether such aggregates are composed of IgE–IgG alone, of circulating allergen–IgE complexes bound in their turn to IgG anti-IgE antibody, or whether they comprise a mixed complex of allergen with the corresponding anti-allergen IgE and IgG antibodies. The experiments of Brostoff *et al.* [19] indicate that complexes formed *in vivo* in the presence of (food) allergen consume haemolytic complement, presumably due to an IgG antibody partner, but it is not possible to decide whether this concerns IgG anti-IgE or IgG anti-allergen. The normal complement levels in the sera of non-challenged allergic patients in any event argue against preformed and circulating complexes composed solely of native IgE antigen and IgG autoantibody [20].

Considering the possible physiological function of IgG anti-IgE autoantibodies, current opinion favours interference with the binding of IgE to CD23 receptors and modulation of IgE biosynthesis [21]. However, the available evidence is also compatible with a role for IgG anti-IgE as a truly 'blocking' antibody which may serve the purpose of clearing allergen–IgE complexes from the circulation.

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