

A method for estimation of circulating immune complexes after oral challenge with ovalbumin

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Summary

Sera from forty-two children orally challenged with ovalbumin were analysed for the presence of IgG-ovalbumin immune complexes. The technique used is based on the adsorption of the IgG of the complex to immobilized protein-A and detection of the ovalbumin of the complex on the solid phase, using isotope-labelled, pepsin-digested antibodies to ovalbumin. Children with high serum levels of antibody to ovalbumin had significantly higher concentrations of IgG-ovalbumin complexes than children with low antibody levels. In both groups the appearance of the complexes in the serum was accompanied by a significant decrease of the complement factor C3 ($P < 0.05$). The duration of the decrease in C3 was correlated to the amount of IgG-ovalbumin complexes, suggesting a consumption of C3 by these immune complexes.

Introduction

Determination of food proteins in serum is an attractive approach to studies of the absorption of antigens through the mucous membranes of the gastrointestinal (GI) tract. Free antigen and antibody as well as products of antigen-antibody interactions in the circulation can be measured by sensitive radioimmune assay (RIA) (Dannaeus *et al.*, 1979). Under certain conditions immune complexes with the potential to activate the complement system might be formed. It has been suggested that such mechanisms may contribute to the symptoms in GI disorders (Shiner, Ballard & Smith, 1975; Editorial, 1977).

In a previous study of healthy children and children with various GI disturbances (Dannaeus *et al.*, 1979), the resorption of orally administered ovalbumin (OA) was found to be increased in all children with coeliac disease and in some children with atopic disorders and food allergies. Many of these children had high levels of antibody to OA in the serum. The serum concentration of OA after challenge was usually low in the presence of antibodies, indicating the formation of immune complexes. The aims of the present study were to develop a method for detection of circulating IgG-OA

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immune complexes, to study the formation of such immune complexes in relation to the serum concentration of OA, IgG-OA antibody and complement factors, and to follow the appearance of such complexes after oral OA loading.

Materials and methods

Preparation of references

A positive IgG-OA immune complex reference serum was prepared by mixing a human serum containing a high concentration of IgG antibodies to OA diluted 1/5 in BSA buffer (0.05 M phosphate, pH 7.4, 0.15 M NaCl, 0.1% NaN_3 , 0.2% bovine serum albumin (BSA) and 0.1% Tween 20) and an equal volume of a 1 mg/ml OA solution in BSA buffer representing an approximately 10-fold antigen excess as judged from gel precipitation. The mixture was allowed to form IgG-OA complexes for 24 hr at 4°C. The preparation was further diluted in a human serum devoid of detectable amounts of OA and antibodies to OA diluted 1/10 in BSA buffer, in order to maintain a constant gammaglobulin concentration in each standard specimen. A negative control reference serum was prepared in the same way from a serum lacking detectable IgG antibodies to OA. Both reference sera were stored after preparation at -20°C until used, being frozen and thawed on several occasions. The most concentrated reference standard was taken as representing 1000 arbitrary units of IgG-OA immune complex. The method was considered to allow measurements of 1 unit or more of IgG-OA complex. This cut-off level represents approximately 1.5 times to twice the activity of the negative reference serum.

Preparation of $F(ab')_2$ fragments of rabbit anti-OA

Three millilitres of Na_2SO_4 -precipitated rabbit anti-OA serum (Behringwerke, Marburg Lot. no. 2476 Q) was pepsin-digested and gel-filtered as described by Johansson & Inganäs (1978). A small amount of protein A-Sepharose CL-4B (Seph-pA) (Pharmacia Fine Chemicals, Uppsala) was added to the pooled and concentrated $F(ab')_2$ preparation in order to absorb any residual intact gammaglobulin. After 30 min at room temperature the anti-OA solution was applied to a 3.5 ml Sepharose-OA immunosorbent column (OA Miles, Twice crystallized, 20 mg OA coupled to each g of CNBr-activated Sepharose 4B) (Pharmacia Fine Chemicals, Uppsala). After 1 hr at room temperature, unbound protein was washed out with 0.05 M phosphate buffer, pH 7.2, until the optical density (280 nm) was below 0.01. The OA-specific $F(ab')_2$ fragments bound to the immunosorbent were desorbed with 0.1 M Glycine-HCl buffer, pH 3.0, containing 0.5 M NaCl, immediately dialysed against 0.1 M Tris-HCl, pH 7.2, concentrated to approximately 1 mg/ml and stored at -20°C.

The antibody preparation was labelled with ^{125}I , using the Chloramine-T method as described by Johansson, Bennich & Berg (1971). Less than 0.3% added activity was directly bound to Seph-pA, as compared with 60–70% for labelled intact rabbit gammaglobulin.

The immunoreactivity of the labelled $F(ab')_2$ anti-OA preparation was calculated to be 15%, using the technique described by Johansson, Kjessler & Sherman (1976).

IgG-OA immune complex test

One hundred microlitres of serial dilutions of the positive and negative reference preparations and serum samples diluted 1/10 in BSA buffer were added in duplicate to

polystyrene test tubes (Cerbo, Sweden). Half a millilitre of a 1.6 mg/ml suspension of Seph-pA in BSA buffer was added to each test tube. The tubes were orbitally rotated for 3 hr at room temperature and the samples were then washed five times with 2.5 ml of saline containing 0.1% Tween 20 by repeated centrifugation and aspiration. The samples were further incubated overnight with 100 μ l of ^{125} I-F(ab')₂-anti-OA in BSA buffer, containing 0.01% normal rabbit gammaglobulin (NRG), corresponding to approximately 200,000 ct/min. After another five washings as above, the remaining radioactivity was determined by a Searle 1285 automatic gammacounter. The mean of the two duplicates was compared with the reference and expressed as units of IgG-OA complex (Fig. 1).

The interassay variation was found to be approximately 12% (1 s.d. in per cent of the arithmetic mean concentration) and the intraassay variation 10% (1 s.d. in per cent of the mean of the count rates).

Complement factors C3 and C4 were determined by a single radial immunodiffusion technique as previously described (Johansson, Högman & Killander, 1968), using commercial antisera (Behringwerke lot. nos. 5109A, A2398A, 975C).

OA was determined by a sandwich type of RIA using ^{125} I-labelled anti-OA and OA-specific antibodies by ^{125} I-labelled OA and Seph-pA as described in detail elsewhere (Dannaus *et al.*, 1979).

Performance of OA loading tests

Forty-two children with varying degrees of malabsorption were tested. After receiving an egg-free diet for at least 12 hr and fasting overnight, the children were given OA orally. The OA (Miles, once crystallized), dissolved in saline to a concentration of 100 mg/ml, was given in a dose of 100 mg per kg body weight. Blood samples were drawn before the administration of OA and after 2 and 8 hr. During the first 2 hours none of

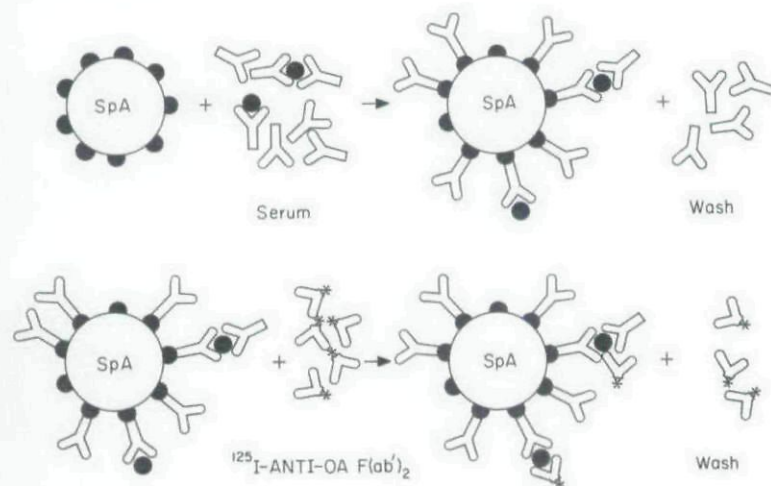


Fig. 1. Assay principle. Diluted serum is added to a suspension of Seph-pA and incubated for 3 hr. IgG-OA complexes are adsorbed via the Fc portion of IgG to the adsorbent. After thorough washing, the adsorbed complexes are incubated overnight together with pepsin-digested, labelled antibodies to OA. Excess amounts of the labelled antibodies are washed away and the remaining radioactivity is detected in a gamma-counter. The more radioactivity remaining in the sample, the more IgG-OA immune complexes present in the serum.

the children received any food, and when food was given later egg was strictly avoided. The children were divided into two groups according to the levels of IgG antibodies to OA in the sample taken before the OA loading (Dannaeus *et al.*, 1979). One group had titres exceeding 10% (high-Ab group) and the other less than 10% (low-Ab group) of the reference.

Statistics

Student's *t*-test for dependent and independent data was used in all calculations for testing the significance of differences.

Results

Factors influencing the specificity of the IgG-OA immune complex test

Serial dilutions of the positive and negative reference sera were tested with Seph-pA and Sepharose as adsorbents. When protein-A was present in the test system, higher isotope binding was obtained with the positive than with the negative reference serum. (Fig. 2).

When protein-A was omitted from the test system, the isotope binding obtained with the positive reference serum decreased to the level in the negative reference serum (Fig. 3). Obviously high binding of labelled anti-OA only occurred if both IgG-OA immune complexes and protein-A were present in the test system.

Some non-specific isotope binding was observed in reference samples containing high amounts of OA, probably due to penetration of the Sepharose beads by free OA. However, this was only significant for OA levels above 5 $\mu\text{g/ml}$, levels which did not occur in any patient serum sample. Thus the results of the serum samples were

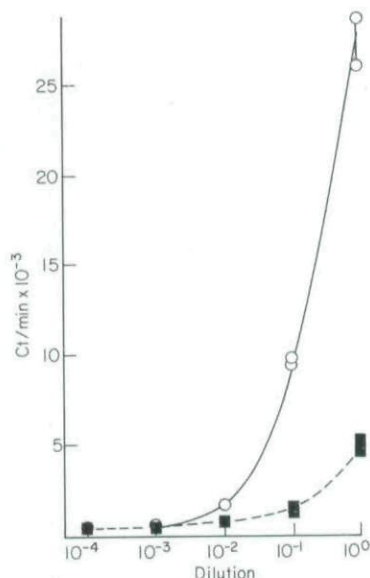


Fig. 2. Serial dilutions of anti-OA positive (○—○) and anti-OA negative (■—■) reference serum preparations (10⁰ dilution = 1000 IgG-OA complex units) tested for the presence of IgG-OA complexes, using Seph-pA as adsorbent.

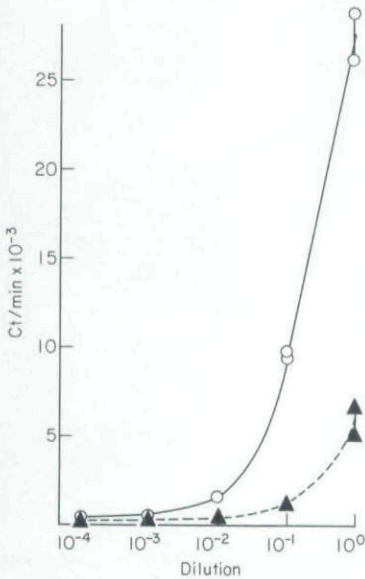


Fig. 3. The anti-OA positive reference serum was tested for the presence of IgG-OA complexes using Seph-pA (○—○) and Sepharose as adsorbents (▲—▲).

evaluated from a 'net' reference curve obtained by subtracting the unspecific isotope binding due to free OA from the total counts, leaving the count rate caused by true IgG-OA immune complexes.

IgG-OA complexes and complement factors in serum from children orally challenged with OA

Low concentrations of IgG-OA complexes were found in most (16/21) pre-challenge samples from the high-Ab group but only in some (8/21) in the low-Ab group. The average concentration of IgG-OA complexes was significantly higher in the former than in the latter group, both in samples taken before and in those taken after the administration of OA. However, in both groups the concentration of IgG-OA complex was significantly elevated in the 2- and 8-hr samples as compared with the initial values (Fig. 4).

A significant ($P < 0.05$) decrease of the C3 concentration occurred in both groups 2 hr after the intake of OA and persisted in the high-Ab group in the 8-hr sample. The C4 concentration did not vary significantly during the sampling period (Table 1).

The relation between OA concentration, OA antibody level and formation of IgG-OA complexes

One child was chosen from each of the two groups to illustrate the importance of the concentration of antibodies for the formation of IgG-OA complexes in serum. In one child with moderate levels of antibodies to OA, representing the high-Ab group (Fig. 5), the appearance of OA in the serum led to consumption of antibodies to OA and simultaneous formation of IgG-OA complexes. In the child representing the low-Ab group, with undetectable antibodies to OA (Fig. 6), no information on IgG-OA

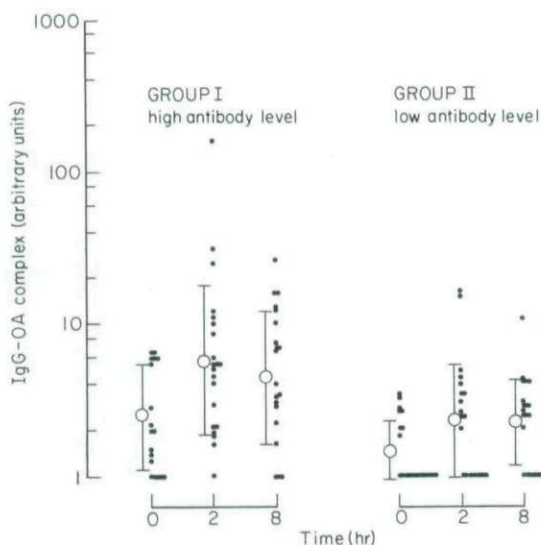


Fig. 4. The concentration of IgG-OA complexes in serum after oral administration of OA to children with high serum OA antibody levels (high-Ab group), and low OA antibody levels (low-Ab group). Significant differences were observed between the two groups in all three samples (0 hr, $P < 0.005$; 2 hr, $P < 0.005$; 8 hr, $P < 0.02$); and within the two groups (Group I: 0/2 hr, $P < 0.01$; 0/8 hours, $P < 0.02$; Group II: 0/2 hr, $P < 0.01$; 0/8 hr, $P < 0.001$) during the observation period. The vertical lines indicate the geometric mean ± 1 s.d.

Table 1. Serum concentration (arithmetic mean ± 1 s.d. mg/100 ml) of complement factors C3 and C4 in children orally challenged with ovalbumin

		0 hours	2 hours	8 hours
C3	High-Ab group	89 $\pm$ 19	86 $\pm$ 20†	86 $\pm$ 19†
	Low-Ab group	95 $\pm$ 17	91 $\pm$ 16†	92 $\pm$ 17
C4	High-Ab group	26 $\pm$ 7	26 $\pm$ 8	26 $\pm$ 8
	Low-Ab group	30 $\pm$ 11	31 $\pm$ 11	31 $\pm$ 12

† $P < 0.05$ compared with the initial level.

complexes was obtained, even though the peak OA concentration in the serum was high.

Discussion

Circulating immune complexes have been reported in a variety of diseases (Masson, 1976) and seem to play a pathogenetic role in disorders of organs such as the kidneys, lungs, blood vessels and joints (Wilson & Dixon, 1975; Geha, 1977; Cochrane & Koffler, 1973; Zvaifler, 1973). *In vitro* methods for the detection and quantification of immune complexes have been developed in attempts to find a correlate for clinical symptoms and the presence of immune complexes. In autoimmune disorders such as systemic lupus erythematosus, a good correlation has been found between the clinical status and *in vitro* findings (Cano *et al.*, 1977). The accuracy of *in vitro* tests varies with

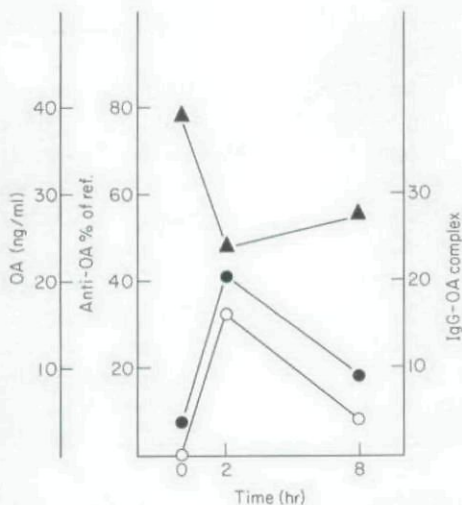


Fig. 5. The relation between the concentration of OA (○—○) and IgG-OA complexes (●—●) in the serum of a child with moderate levels of IgG antibodies to OA (▲—▲) after oral OA intake.

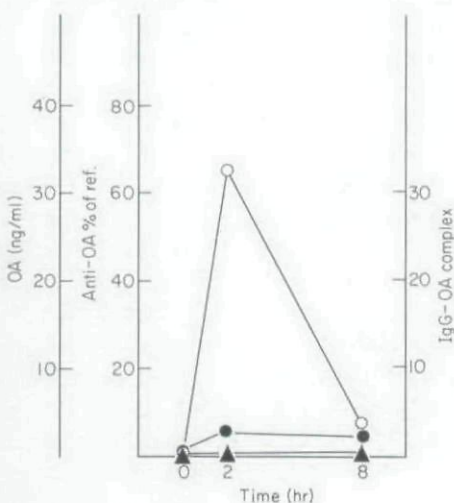


Fig. 6. The relation between the concentrations of OA (○—○) and IgG-OA complexes (●—●) in the serum of a child lacking detectable IgG antibodies to OA (▲—▲) after oral OA intake.

the method used and is often low due to heterogeneity of the antibody, antigen and complement factor composition (Russel *et al.*, 1978). To be able to detect different types of immune complexes it is therefore necessary to use test principles with different specificities (Maini & Holborow, 1977). Complement binding complexes can be detected by methods based upon the binding of isolated, ^{125}I -labelled human Clq to aggregated IgG, (Zubler *et al.*, 1976) or upon the inhibitory effect of immune complexes on the agglutination of IgG-coated latex particles by monoclonal rheumatoid factor or human Clq (Lurhuma *et al.*, 1976). Endogenous complement bound to immune complexes can be detected by bovine conglutinin with specificity for human

C3 (Eisenberg, Theophilopoulos & Dixon, 1977) or by the use of cells with complement receptors (Theophilopoulos, Wilson & Dixon, 1976).

Protein-A from *Staphylococcus aureus* is an interesting alternative reagent of radioimmunoassay systems for the detection of specific IgG antibodies to common antigens (Johansson *et al.*, 1974; Foucard & Johansson, 1976). Protein-A binds to the Fc portion of human IgG1, IgG2 and IgG4 (Kronvall & Williams, 1969). Kessler (1975) used protein-A bearing Staphylococci as an adsorbent for antibodies that had reacted with radiolabelled antigens from cell lysates. His finding that immune complexes were preferentially adsorbed, in contrast to free antibodies, indicated that solid phase bound protein-A was also an excellent immunosorbent for adsorption of immune complexes. Instead of intact Staphylococci, we have been using purified protein-A covalently bound to Sepharose as a solid phase. By means of pepsin-digested, radiolabelled antibodies to a known immune complex forming antigen, a sensitive and reproducible method for immune complex assay was developed. Besides its extreme sensitivity (antigen in the immune complex can be detected down to nanogram quantities per millilitre), the method clearly defines both the antibody and the antigen in the complex, a feature which is lacking in most immune complex assays. In our application of the general principle, the non-specific response due to excess amounts of free antigen was negligible below a concentration of 5 µg antigen/ml (Figs 2 and 3). Thus, the maximum antigen concentration tolerable in this test system was far beyond the concentrations likely to be reached when exogenous antigens enter the circulation (Danneaus *et al.*, 1979).

The presence of specific antibodies to common food antigens in serum from healthy individuals has been shown to be a normal and common phenomenon in infancy and childhood (Rothberg & Farr, 1965). However, the antigenic load may increase if the gut barrier function is impaired, allowing antigens to penetrate the mucosa and enter the circulation, whereby immune complexes may be formed locally (Shiner, Ballard & Smith, 1975) or in the circulation (Delire, Cambiaso & Masson, 1978).

In a previous study we detected OA in sera from children loaded with OA, indicating that the gut mucosa is permeable to this immunologically reactive antigen (Danneaus *et al.*, 1979). However, the presence of antibodies to OA in the serum had a crucial effect on the measurable OA concentration (Danneaus *et al.*, 1979), i.e. the higher the antibody titre to OA, the lower the OA concentration. In addition, when analysing the antibody titres to OA, we noted that consumption of antibodies after OA administration was a general phenomenon, which further indicated the possibility that immune complexes were formed during the loading test.

Using the IgG-OA immune complex test described here, we found that the concentrations of complexes changed significantly during the sampling period both in a group of children with a high and in a group with a low concentration of antibodies to OA (Fig. 4). Significantly higher concentrations were observed in samples before and after OA challenge from the high-Ab group than in the low-Ab group. The findings also indicate that small amounts of IgG-OA complexes were already present in sera from the high-Ab group before the loading, despite the fact that an egg-free diet had been taken for at least 12 hr prior to administration of OA. Raised levels of IgG-OA complexes even in the sample drawn 8 hr after OA administration suggest that the immune complexes may remain in the circulation for a considerable length of time. The observed decrease in C3 in both groups during the test period indicates that the IgG-OA complexes might act as activators of the complement system. This hypothesis

is also supported by the fact that the decrease persisted for a longer time in patients who developed high serum levels of immune complexes after OA-intake.

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