

IgD antibodies: *in vitro* blocking activity of IgE mediated reactions

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Summary

Sera from 177 atopic patients treated by immunotherapy were screened in order to detect IgD antibodies against a purified fraction of *Lolium perenne* (fractions C). IgD antibodies were found in 23% of the subjects. Eight of these sera were selected and their IgD isolated by means of immunoabsorption. The *in vitro* biological activity of the IgD antibodies against fraction C was tested by three methods: basophil degranulation, histamine release and RAST inhibition test. In all these assays, the data obtained indicated a possible blocking effect of the IgD antibodies upon the IgE *in vitro* mediated reactions.

Introduction

New activities attributed to IgD have been rarely described in the past few years (Leslie & Martin, 1978). The antigens reacting with IgD are mainly autologous tissue substances and in some cases are associated with drugs or infectious agents.

At the 4th European Immunology Meeting, Budapest, we described specific IgD antibodies against fraction C, a purified fraction of *Lolium perenne* (Lahoz *et al.*, 1978). This fraction is probably the same as group II described by Marsh for rye grass pollen (Marsh, Haddad & Campbell, 1970). As these antibodies were found in the sera of patients suffering seasonal allergic pollenosis treated by immunotherapy, we were interested in the investigation of their possible blocking effect in the *in vitro* anaphylactic hypersensitivity reaction systems.

After isolation of IgD by solid phase immunoabsorption with specific anti-human δ chain antiserum, three methods are employed in order to examine this activity: (a) basophil degranulation (Benveniste *et al.*, 1976), (b) histamine release (Campbell *et al.*, 1970) and (c) RAST inhibition assay (Ceska, Eriksson & Varga, 1972).

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Materials and methods

Patients

Sera from 177 patients with seasonal allergic pollenosis were stored at -20°C in presence of 0.01 M EACA in order to avoid IgD plasmin digestion (Spiegelberg, 1972). Sera were screened for the presence of specific IgE by RAST, and specific IgD and IgG antibodies by a radioimmunoassay described below. Patients were treated by immunotherapy for periods of 2 months to 30 years.

Antigens

Lolium perenne fraction C was obtained from *Lolium perenne* crude extract by batch chromatography in DE-32 cellulose (Whatman Biochemical Ltd, England). The fraction eluted with 0.25 M NH_4HCO_3 was gel filtered through Sephadex G-75 and G-100 (Pharmacia, Uppsala, Sweden) for further purification (Marsh *et al.*, 1970). The allergenicity of the fractions was tested by the Ceska method (Ceska *et al.*, 1972). Fraction C was pure by immunodiffusion against anti-whole crude extract antiserum and its molecular weight was about 15 000 Daltons as determined by SDS-PAGE (unpublished data).

Antisera

Anti-IgD antiserum. Pure human IgD was obtained from IgD myeloma serum by block electrophoresis and Sephadex G-200 gel filtration (Pharmacia, Uppsala, Sweden). Buffers containing 0.01 M EACA were used in every step. Rabbits were immunized as described previously (Garcia *et al.*, 1978) with $100\text{ }\mu\text{g}$ of the purified antigen in FCA. After booster injections, the anti-IgD antiserum was made specific for human δ chains by repeated solid phase immunoabsorption with human light chains of both types, being further absorbed with human gammaglobulin fraction (AB-KABI, Stockholm, Sweden) coupled to CNBr activated Sepharose 4B (Pharmacia, Uppsala, Sweden). No band was observed in Ouchterlony plates when the absorbed antiserum was tested against different dilutions of κ and λ chains. IgG fraction of the absorbed antiserum was obtained by DE-32 cellulose chromatography eluted with 0.01 M phosphate buffer, pH 7.6. Afterwards this fraction was bound to CNBr activated Sepharose 4B.

Anti-IgE and anti-Lolium perenne antisera. These antisera were raised in rabbits. Antigen concentrations were 1 mg of *Lolium perenne* and $100\text{ }\mu\text{g}$ of IgE. Anti-IgE antiserum was absorbed as described previously (Garcia *et al.*, 1978).

Sheep anti-rabbit gammaglobulin antiserum. This was prepared by heat aggregated rabbit IgG injected in Freund's complete adjuvant.

Radioactive labelling

Fraction C and specifically purified antibody anti-human ϵ chain were labelled with ^{125}I (Amersham, England) by the method of Kliman and Taylor (1969).

Radioimmunoassay for IgD antibodies

Radioimmunoassay for IgD antibodies to *Lolium perenne* fraction C was performed by a method modified from Lertora, Gomez-Perez & Leslie (1975): $100\text{ }\mu\text{l}$ of different dilutions of patients' sera in PBS containing 0.1% BSA were incubated overnight at 4°C with $20\text{ }\mu\text{l}$ of ^{125}I -fraction C (20 000 ct/min , specific activity $3\text{ }\mu\text{Ci}/\mu\text{g}$) with gentle

shaking, 50 μ l of rabbit anti-human δ chain antiserum were added and incubated in the same conditions. After 24 h, complexes were co-precipitated with sheep anti-rabbit gammaglobulin antiserum. After washing three times with cold PBS, incorporated radioactivity was measured in a gamma scintillation counter.

Specificity controls. See 'Results'.

Radioimmunoassay for IgG blocking antibodies

IgG blocking antibodies in patient's sera were determined according to the technique described by Sobotka *et al.* (1976). Briefly, ^{125} I-fraction C was incubated with serial dilutions of patients sera using 1/50 normal human serum as diluent. After 24 hr incubation, soluble complexes were precipitated with anti- γ chain antibodies, washed and counted in a gamma scintillation counter.

Basophil degranulation test

Basophil degranulation test was done on isolated human cells after dextran glucose separation: 20 ml of fresh heparinized peripheral blood were mixed with 4 ml of 3% dextran, 3% glucose, 0.9% NaCl over 40 min. The leucocyte fraction was collected and washed with PBS and finally resuspended in Tris-ACM. Five hundred microlitres of cells (46×10^3 basophils/ml in 15×10^6 leucocytes/ml) were incubated for 30 min at 37°C with 100 μ l of serial dilutions of antigen (fraction C). Antigen concentrations ranged from 760 to 76 ng per 100 μ l. Controls containing 100 μ l of PBS instead of antigen were included in the test. After incubation, basophils were stained by the method of Moore & James (1953) and counted in a Fuchs-Rosenthal chamber. Results were given as the percentage decrease with respect to the control basophil count. More than 30% degranulation was considered positive.

Histamine release assay

Histamine release tests were made as for the degranulation test and the histamine released was assayed by the spectrofluorometric method described by Shore, Burkhalter & Cohn (1959). Fluorometric reaction was carried out over 4 min at room temperature (Siraganian, 1976). Results were expressed as a percentage of total histamine released after cell disruption. Each assay included four tubes with serial dilutions of the antigen in Tris-ACM, and control tubes for spontaneous release. Total histamine from cells disrupted by freezing and thawing and blanks was also included.

RAST method

Specific IgE in atopic patients against whole *Lolium perenne* crude extract was measured by the RAST technique (Pharmacia, Uppsala, Sweden). Results were given as a percentage of activity bound by normal sera (Wide, Bennich & Johanson, 1967).

Total serum IgE

Total serum IgE was measured by PRIST (Pharmacia, Uppsala, Sweden).

Results

Specificity controls of the absorbed anti-human chain antisera

As the major intention of this investigation is to describe a new antibody activity of IgD, the following specificity controls were performed.

(a) In order to check the anti-IgD antiserum for chain specificity we did an RIA test

using cold human IgG as inhibitor. One hundred microlitres of an appropriate dilution of anti-IgD antiserum were incubated with 100 μ l of ^{125} I-labelled IgD (10 000 ct/min) for 24 hr at 4°C. Different concentrations of cold IgG, ranging from 0 to 100 μ g were added in order to determine inhibition. After incubation, complexes were co-precipitated with goat anti-rabbit gammaglobulin and counted. No inhibition was obtained even with 100 μ g of IgG.

(b) Serum from a hay-fever patient under immunotherapy possessing high titres of specific IgG and IgD antibodies as measured by radioimmunoassay (IgG anti-fraction C, 1/10⁴, and IgD anti-fraction C, 1/400) was passed through a Pasteur pipette column containing 1 g of CNBr activated Sepharose 4B coupled to specifically purified anti-human γ chain. After absorption through the column, the IgG titre decreased to 1/10³ while the IgD titre remained unchanged. These results took the dilution factors into account.

(c) The antiserum anti-human δ chain previously absorbed with pure IgD gave negative results with the IgD antibodies by radioimmunoassay.

(d) Antiserum anti-IgD was incubated with ^{125} I-fraction C in order to check non-specific precipitation. After co-precipitation no radioactivity was incorporated in the precipitate above the background levels.

(e) Pure myeloma IgD was incubated with ^{125} I-fraction C in order to check non-specific binding of IgD with the antigen by regions other than the antigen-combining sites. After the test, negative results were obtained.

IgD and IgG anti-fraction C

Sera from 177 patients with seasonal allergic pollenosis treated by immunotherapy were tested by radioimmunoassay in order to look for IgD antibodies anti-fraction C. Forty-one of them had IgD antibodies and their titre ranged from 1/50 to 1/400. Eight sera with the highest titres were selected in order to isolate the IgD as described below.

In thirteen cases, IgG blocking antibodies were also determined choosing patients in different stages of treatment.

As shown in Table 1 no correlation was found between the titre of IgG and IgD or with the duration of immunotherapy.

Isolation of human IgD antibody against fraction C

Eight IgD preparations were isolated from positive sera by immunoabsorption. Two millilitres of sera with the highest titres of IgD anti-fraction C antibody were passed through a column containing CNBr activated Sepharose 4B coupled to specific anti-human δ chain antibodies. Fractions eluted with borate-buffered saline, pH 8.0, containing 0.01 M EACA did not show any activity by radioimmunoassay (RIA) to detect IgD antibodies anti-fraction C. After extensive washing of the column, fractions were eluted with 0.01 M EACA, HCl-glycine, pH 3.0, and neutralized with 0.01 M EACA, 1 M Tris. After dialysis and concentration, the IgD antibody activity remained in these fractions as determined by RIA. By immunodiffusion in Ouchterlony plates, the isolated IgD gave no band against anti-whole human serum, and in some instances gave a single line against anti-human δ chain antiserum.

When the fractions containing IgD were passed through an immunoabsorbent of *Lolium perenne* crude extract bound to CNBr activated Sepharose 4B, only fractions eluted at acid pH contained antibody activity as measured by radioimmunoassay. These fractions were used in the inhibition experiments.

Table 1. A representative example of IgD and IgG blocking antibodies

Patients	Years of treatment			Serum	Specific IgE‡
		IgG anti-fraction C*	IgD anti-fraction C*	IgE (U/ml)†	
A	2 Months	1/800	1/160	1000	+++
B	2	Negative	Negative	180	++
C	3	1/400	Negative	900	+++
D	4	1/800	Negative	240	+++
E	4	1/1600	1/100	600	+++
F	6	1/1600	1/100	1000	++++
G	7	1/200	Negative	800	+++
H	7	1/400	1/100	400	+++
I	8	1/400	1/400	500	++++
J	8	1/800	1/400	350	+++
K	10	1/100	1/100	400	+
L	13	1/800	Negative	ND	+++
M	30	1/3200	Negative	ND	+++

* IgG and IgD anti-fraction C antibodies were detected by radioimmunoassay.

† PRIST method.

‡ RAST assay.

ND = not done.

Blocking effect of the IgD antibodies anti-fraction C

The possible blocking effect of the IgD antibodies in the neutralization of *in vitro* IgE-mediated reactions was tested in three different ways: (a) basophil degranulation, (b) histamine release and (c) RAST inhibition. Eight IgD preparations isolated as stated above were tested—all of them in the basophil degranulation, two in the histamine release assay and three in the RAST inhibition assay.

(a) *Basophil degranulation. Inhibition assay.* In order to validate our method of basophil degranulation, we demonstrated in a previous investigation a positive correlation (93.7% agreement) on comparing degranulation and histamine release in patients with allergic pollenosis (Lahoz *et al.*, 1979). The basophil degranulation test was performed as described in 'Materials and methods'. For the inhibition assay, 100 μ l of serial dilutions of fraction C in concentrations ranging from 7.6 to 0.076 μ g/ml were previously incubated with 100 μ l of isolated IgD for 1 hr at 37°C. Afterwards, the patients' cells were added. Each inhibition assay was compared to a basophil degranulation test in which antigen dilutions were incubated with 100 μ l of saline instead of the IgD antibody. The percentage of inhibition was calculated by the formula:

$$\% \text{ inhibition} = \frac{\% \text{ control degranulation} - \% \text{ degranulation in presence of IgD}}{\% \text{ control degranulation}} \times 100.$$

Results are shown in Table 2 and Fig. 1. Three different sets of experiments are expressed as compared with the corresponding degranulation controls. In general it is observed that the percentage inhibition of basophil degranulation is greater as the antigen concentration decreases.

Table 2. Inhibition of basophil degranulation test by IgD antibodies*

Dilutions of fraction C†	Percentage degranulation: control‡	Percentage inhibition§			
		IgD1	IgD2	IgD3	
10 ⁻²	85	83	50	67	
10 ⁻³	85	50	50	100	
10 ⁻⁴	71	40	60	40	
		IgD4	IgD5		
10 ⁻²	77	0	0		
10 ⁻³	88	54	67		
10 ⁻⁴	100	100	100		
		IgD6	IgD7	IgD8	
10 ⁻²	91	36	36	100	
10 ⁻³	91	45	54	67	
10 ⁻⁴	95	82	83	89	

* Three different experiments are shown in this table.

† Undiluted fraction C concentration is 7.6 µg/ml.

‡ Cells from pollinic patients incubated with fraction C.

§ Percentage inhibition produced when fraction C had been incubated with the different IgD preparations prior to the addition of cells. IgD concentrations are: IgD1, 0.25 mg/ml; IgD2, 0.16 mg/ml; IgD3, 0.29 mg/ml; IgD4, 0.13 mg/ml; IgD5, 0.26 mg/ml; IgD6, 0.19 mg/ml; IgD7, 0.24 mg/ml; IgD8, 0.31 mg/ml. Determined by OD at 280 nm.

IgD isolated from myeloma serum did not show any inhibitory activity upon the degranulation reaction when tested as control under the same conditions. From these experiments we conclude that the antibody activity detected by radioimmunoassay in certain pollinic sera may be attributed to the IgD immunoglobulin class.

(b) *Histamine release. Inhibition assay.* One hundred microlitres of IgD3 and IgD7 were previously incubated at 37°C for 1 hr with 100 µl of fraction C in concentrations ranging from 760 to 0.0076 pg in Tris-ACM. Then, 5000 basophils from the pollinic patient were added to each tube. Controls without antigen were included and percentage inhibition was calculated as above. Results are expressed in Table 3. IgD3 reach a 69% inhibition at an antigen dilution of 10⁻⁷ whereas IgD7 produced the total inhibition at the optimal concentration for histamine release. These results are in agreement with those obtained in the basophil degranulation inhibition test, and further indicate the possible IgD blocking effect of the *in vitro* IgE mediated reactions.

(c) *RAST Inhibition assay.* Fraction C was incubated in different concentrations ranging from 32 000 to 1.6 ng/ml with CNBr activated paper discs (Ceska *et al.*, 1972), and the RAST assay was performed with the pollinic serum as positive control. The control counts obtained when the discs were previously incubated with three IgD

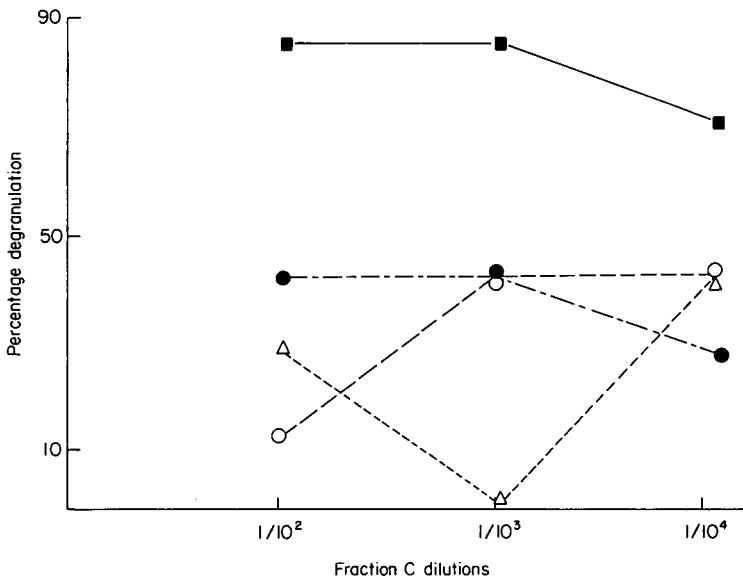


Fig. 1. Comparison between degranulation control and degranulation inhibition produced by three different IgD preparations. ■—■, Control; ○---○, IgD1; ●- - -●, IgD2; Δ-----Δ, IgD3.

Table 3. Inhibition of the histamine release test by IgD antibodies*

Dilutions of fraction C†	Percentage histamine release: control‡	Percentage inhibition: IgD7§
1	0	—
10 ⁻¹	0	—
10 ⁻²	80	100
10 ⁻⁴	20	100
		IgD3
10 ⁻⁵	35	28
10 ⁻⁶	55	36
10 ⁻⁷	65	69
10 ⁻⁸	60	28
10 ⁻⁹	65	29

* Two different experiments are shown in this table.

† Fraction C concentration is 760–0·0076 pg/100 μl.

‡ Control histamine release: percentage of total histamine released by pollinic patients incubated with fraction C.

§ Percentage inhibition produced when fraction C had been incubated with the different IgD preparations prior to addition of the cells. IgD concentrations are the same as in Table 2.

preparations (IgD1, IgD2 and IgD7) are presented in Table 4. Incubation was carried out for 1 hr at 37°C with 100 µl of the IgD preparation. After washing, the discs were incubated with pollinic sera containing specific IgE, and the RAST was performed as usual. The blocking role of IgD, competing with the specific IgE for the antigenic sites in the paper discs, is shown in Table 4. With IgD1 and IgD2, respectively, 33% and 47% inhibition was obtained when the antigen concentration at the disc was the lowest. With IgD7, 15% inhibition was obtained.

Table 4. Inhibition of the RAST assay by IgD antibodies*

Fraction C dilution†	Control (ct/min)‡	IgD1		IgD2	
		ct/min	Percentage inhibition‡	ct/min	Percentage inhibition
50 ⁻¹	4878	4536	7	4458	8
10 ⁻²	5172	4761	7	4346	15
10 ⁻³	5053	4578	9	4594	9
10 ⁻⁴	2726	1944	28	3023	0
10 ⁻⁵	2664	1796	33	1390	47

		IgD7	
		ct/min	Percentage inhibition
10 ⁻²	4379	6291	0
10 ⁻³	4571	4763	0
10 ⁻⁴	4516	3843	15

* Two experiments are shown in this table.

† Fraction C concentration is 32 µg/ml at a dilution of 1/50.

‡ Control RAST: paper discs coupled to fraction C are incubated directly with the pollinic sera.

§ Percentage inhibition of RAST when discs had been incubated previously with different IgD preparations. Concentrations of IgD are the same as in Table 3. Percentage inhibition is calculated according to the formula:

$$\% \text{ inhibition} = \frac{\text{ct/min control} - \text{ct/min IgD}}{\text{ct/min control}} \times 100.$$

Discussion

Most current investigations on IgD are on the role of this immunoglobulin acting as antigen membrane receptor (Rowe *et al.*, 1973), and little is reported on any new antibody activity (Corte *et al.*, 1979). The antigen specificity generally described for IgD is for nuclear antigens (Ritchie & Portland, 1968), insulin (Devey *et al.*, 1970), bovine gammaglobulin (Heiner & Rose, 1970) and penicillin (Levine & Redmond, 1967). Nearly all the cases reported have several features in common. (a) Chronic antigen stimulation such as that produced by nuclear material, insulin-therapy in diabetic patients or recurrent infections. (b) In nearly all the cases, concomitant high IgG antibody levels are present. These two features also appeared in the patients who we investigated, patients with seasonal allergic pollenosis treated by immunotherapy.

The presence of serum IgG blocking antibodies (Lichtenstein, Holtzman & Burnett, 1968; Giessen *et al.*, 1976) is insufficient to account for the improvement of the patients treated by immunotherapy.

Despite the possible clinical irrelevance of blocking antibodies we initiated a study to detect antibody classes other than IgG that may result from immunotherapy. By a radioimmunoassay method, we found IgD antibodies in 23% of treated patients, and no relation was found between the duration of treatment and presence of IgD antibodies against fraction C (Table 1). Our results suggest that the IgD antibodies synthesized by these patients have a blocking effect in the *in vitro* tests of the IgE mediated reactions, i.e. basophil degranulation, histamine release and competition with IgE for the antigenic determinants in the paper discs.

The possibility of non-specific binding of IgD through molecular fragments other than the antigen binding sites, as those found in the binding of bacterial to human IgD (Forsgren & Grubb, 1979), may be ruled out because myeloma IgD had no effect on the radioimmunoassay results. Furthermore, no inhibition was found when this protein was tested in the corresponding assays.

Apart from the immunotherapy-treated patients, we found two normal people with high specific serum IgD levels. They were preparing extracts of pollen grains in the laboratory. Thus specific IgD antibodies may occur in normal persons, and possibly in allergic persons not receiving immunotherapy because on further tests we found two of seven non-treated patients to have specific IgD anti-fraction C.

Lack of skin sensitizing properties of IgD has been demonstrated (Henney *et al.*, 1969; Ovary, 1969), but association between IgD and IgG has been reported in some clinical conditions, as in hyper-IgE syndrome and undue susceptibility to infections (Buckley & Fiscus, 1975), as well as in patients with Nezelof syndrome. Further, when immunoglobulin levels were studied in atopic and non-atopic people, a statistically significant increase in IgD was found in the atopic groups (Kohler & Farr, 1967). Recently, the finding of specific IgD and IgE antibodies was reported for the complex tartrazine-rabbit serum albumin in the sera of patients with clinically diagnosed tartrazine hypersensitivity (Weliky & Heiner, 1980).

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Abbreviations

RAST, radioallergoimmunosorbent test; EACA, ϵ -amino caproic acid; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; FCA, Freund's adjuvant; PBS, phosphate-buffered saline; BSA, bovine serum albumin; Tris-ACM, Tris-albumin cation buffer; OD, optical density.

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