

Detection of antigen-specific IgE-plaque-forming cells from peripheral-blood lymphocytes of ragweed- and grass-allergic patients

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Summary

The reverse haemolytic plaque assay was developed to measure antigen (AgE or rye grass I)-specific IgE-plaque-forming cells from the peripheral-blood lymphocytes of ragweed- and grass-allergic patients. The anti-IgE-developing antisera was shown to be isotype-specific, and the response of the assay was inhibited by 52% by the addition of 10 pg of antigen. In addition, the assay was shown to have a reproducibility (s.d.) of 15%. The blood lymphocytes from all fifteen atopic (grass and ragweed) patients were shown to form antigen-specific IgE-plaque-forming cells during the pollen season (mean value 115 cells) and up to 6 months after the season (mean value 56 cells). Cycloheximide appears to block the formation of the plaque-forming cells. This method appears to be sensitive and reproducible enough to study *in vitro* IgE antibody synthesis of peripheral-blood lymphocytes from atopics.

Introduction

Detection of specific human IgE antibody formation *in vitro* would be of great significance toward the understanding of the mechanisms of IgE synthesis and allergic disease. This has rarely been achieved, but Tijo, Hull and Gleich (1979) detected IgE antibodies to ragweed and grass allergens by RAST in cell supernatants of peripheral-blood lymphocytes (PBL) of pollen allergic patients. The studies were performed during the pollen season, and IgE antibody responses were detected only after 10-fold concentration of these cell supernatants. Moreover, it was reported (Gleich, 1980) that several months after the pollen season, the IgE antibody levels could no longer be detected by RAST. Thus, RAST appears to lack the sensitivity to measure such IgE antibody responses and such studies would be limited to pollen seasons.

The present study was undertaken to modify the reverse haemolytic plaque assay (RHPA) of Cunningham (Cunningham & Szenberg, 1968) for the detection of

antigen-specific IgE antibody plaque-forming cells (PFC) of PBL from ragweed- and grass-sensitive patients. For this purpose, AgE and rye grass in sheep red blood cells were used and all PBL from fifteen patients were capable of producing PFC both during and 3 months after the pollen season.

Methods and materials

Blood was taken from fifteen untreated patients (nine ragweed, three rye grass and three patients allergic to both). Patients were shown to be atopic by history, skin test and RAST. All patients' sera gave 3+ RAST or greater. Blood from 5 non-atopic individuals were used as controls. The study was performed between May 1981 and February 1982.

Peripheral-blood lymphocytes (PBL) were separated from fresh heparinized blood by Ficoll-Paque density gradient centrifugation. After three washes in RPMI 1640, the PBL were resuspended at a concentration of 2×10^6 cells/ml. The cells (at 0.2×10^6 cells/0.2 ml of enriched media) were placed in microtitre plates and cultured for 7 days in a humidified atmosphere of 5% CO₂ and 95% air. The enriched media consisted of 2% glutamine (GIBCO), 100 µg/ml penicillin, 1% streptomycin, 100 µg/ml gentamicin and 10% fetal calf serum (Flow Laboratories). At the end of the 7-day period, the viability of the cells were assessed by ethidium-bromide and acridine-orange stains. The cell count was performed and the cell suspension adjusted to 2×10^6 viable cells/ml. The cells were then assayed by the reverse haemolytic plaque assay (RHPA) as described below.

Reverse haemolytic plaque assay for detection of antigen-specific IgE antibodies

The method of Cunningham (Cunningham & Szenberg, 1968) was modified as follows: prior to performing the RHPA, several reagents were prepared. Sheep red blood cells (SRBC), always obtained from the same animal, were sensitized with AgE or rye grass I allergens. Thus, 0.1% chromic chloride solution was adjusted to pH 5.0, prior to coupling. Equal volumes of SRBC, chromic chloride and antigen (0.5 mg/ml) were incubated for 30 min, then centrifuged (300 g) and washed 3 times with RPMI 1640. The coupled SRBC pellet was diluted 1/7 volume and stored at 4°C up to periods of 2 weeks before use. The guinea-pig complement used in the RHPA was absorbed in fetal calf serum to reduce toxicity, and diluted 1:1 in saline prior to use. The anti-human IgE was obtained from Atlantic Antibody Inc. (IgG fraction of anti-human IgE, 26.5 mg protein/ml) and shown to be free of anti-IgG, -IgA, -IgM, -IgD and light-chain antibodies. Thus, various immunoglobulin and light chain (κ and λ) preparations (1 mg/ml) were separately coupled to paper discs. Whereas 42% of the ¹²⁵I-radiolabelled anti-IgE preparation reacted with IgE coupled disc, this material failed to react (0.5% binding) with the G, A, M, D and light-chain preparations. More important, in a separate study (Beaudoin *et al.*, 1982), 100 µg of these materials failed to inhibit the RHPA with the anti-IgE-developing antisera. For the RHPA, 20 µl of coupled SRBC were mixed with 20 µl of cultured PBL, 20 µl of developing anti-IgE antiserum (diluted 1:100 with RPMI 1640), and 20 µl of guinea-pig complement. The mixture of reagents were pipetted between two microscope slides correctly spaced to form a micro chamber where the cells could extend by capillarity to form a monolayer. The edges of the slides were then sealed by dipping them slightly into a melted mixture (1:1) of paraffin petroleum jelly. The slides were then incubated at 37°C overnight and then observed under 600 times magnification. By appropriately scanning the slide, each plaque was

counted. Only those plaques with a lymphoid cell in the centre were considered positive. Results were expressed as the number of plaque-forming cells (PFC) per 10^6 viable cells. Appropriate controls consisted of substituting the anti-IgE or PBL with RPMI 1640 medium. To assess the reproducibility of the RHPA, the assay was repeated 10 times with PBL of ragweed-allergic patient JL. The number of IgE-specific PFC/ 10^6 viable cells against AgE was found to be 50 ± 10 . Similarly, lymphocytes from allergic patients BL and PB demonstrated 30 ± 7 and 400 ± 53 , respectively. On the basis of these experiments, the plaque assay was assessed to have a standard deviation (s.d.) of 15%. All results reported represent duplicates for each RHPA.

In some experiments the effect of cycloheximide on the RHPA responses were evaluated. For this purpose, an aliquot of 20 μ l of cycloheximide (200 μ g/ml) was added to the mixture of reagents used in the RHPA 18 hr prior to the assay. Results of these experiments are shown in Table 3.

Results

Table 1 summarizes the results of the number of antigen-specific IgE antibody PFC of PBL from ragweed- and grass-pollen-allergic patients. This portion of the study was done during the grass and ragweed pollen seasons. None of the PBLs from the five non-atopic subjects gave positive PFC. On the other hand, all of the PBLs from both the grass- and ragweed-allergic patients manifested positive PFC ranging anywhere from 40 to 400 PFC/ 10^6 viable cells with the mean value of 115 PFC/ 10^6 viable cells. Table 2 shows that PBLs from four patients manifested considerably less PFC 3–6 months after both the grass and ragweed seasons. As the above results were obtained without stimulation of the PBL from the atopic patients, it can be assumed that these cells produce IgE antibody spontaneously by *in vivo* allergen exposure. Thus, it is not

Table 1. Number of PFC in atopics and non-atopics

	Atopics	Non-atopics
IgE PFC/ 10^6 Cells (AgE)	125 ± 17 ($n=12$)	< 20 ($n=5$)
IgE PFC/ 10^6 Cells (rye grass I)	166 ± 23 ($n=6$)	< 20 ($n=5$)
Range	40–400	< 20
Mean	115 ± 16	< 20

Table 2. Number of PFC during and several months after the season

Patient	Antigen	During season*	December-tested
		Ag-specific IgE PFC/ 10^6 viable cells	
MM	AgE	180 ± 24 (August)	40 ± 10
BM	AgE	80 ± 15 (August)	40 ± 10
RP	Rye grass I	220 ± 30 (June)	90 ± 14
LE	Rye grass I	150 ± 20 (June)	60 ± 12

* Months in brackets indicate when tests were performed.

surprising to find a decrease in the level of such cells several months after the season. Table 3 shows that cycloheximide blocks the *in vitro* secretion of IgE antibodies from the PBL of four patients tested.

In order to obtain some indication of the sensitivity of the RHPA used to detect antigen-specific IgE PFC, an AgE inhibition assay was performed. Thus, the addition of various concentrations of AgE to RHPA reagents showed a decrease in the number of PFC (Table 4). Thus, it can be seen that 50 pg of AgE added caused complete inhibition of response whereas the addition of 10 pg resulted in a 52% inhibition. The fact that the addition of as little as 10 pg of AgE caused such a considerable inhibition suggests that the assay must have a sensitivity to detect IgE antibody response within the 1–10 pg range.

Table 3. Effect of cycloheximide on antigen-specific IgE PFC

Patient	Antigen	Cycloheximide	AgE-specific IgE PFC/10 ⁶ viable cells
MM	AgE	—	180 ± 24
		+	20 ± 3
BM	AgE	—	230 ± 27
		+	40 ± 6
JP	AgE	—	95 ± 14
		+	< 20
LP	AgE	—	80 ± 12
		+	< 20

Table 4. Antigen inhibition of plaque formation

Amount of AgE added	PFC IgE/10 ⁶ viable cells (AgE)	% inhibition
pg		
0	130 ± 20	—
1	120 ± 18	7
4	96 ± 14	26
10	63 ± 11	52
50	< 20	100

Discussion

There have been a number of reports on IgE synthesis from PBL of atopic patients (Buckley & Becker, 1978; Tijo *et al.*, 1979; Saxon, Morrow & Stevens, 1980). Of all of these reports, only one has dealt with IgE antibody synthesis in attempting to delineate the cellular mechanisms involved in modulating IgE synthesis. Both double-binding and radiometric assays have allowed the detection of as little as 40 pg/ml of total IgE and the sensitivity of these assays has been useful in studying supernatants from PBL of atopics. The RAST assay has been found relatively insensitive for use and has been only moderately successful after cell supernatants were concentrated 10-fold (Tijo *et*

al., 1979). Furthermore, such studies were limited to seasonal responses where spontaneous IgE antibody responses are maximal. In the present study, the RHPA assay was developed to measure antigen-specific IgE PFC and thus allowing the detection of IgE antibody-producing cells. The results of this study indicate that the RHPA was sensitive enough to detect positive PFC from the PBL of all fifteen patients tested. Furthermore, responses, although diminished, were still positive well after the season. From antigen inhibition assay results of the RHPA, it was estimated that the limit of sensitivity was in the range of 1–10 pg IgE antibody/ml. More recently, this method has been extended to the detection of specific IgE PFC to timothy (Beaudoin *et al.*, 1982), β -lactoglobulin (Parish, 1982) as well as mountain cedar (T. Knicker, J. Dolan & L. Perelmutter, unpublished observations) allergens. In some preliminary studies (Perelmutter & Larocque, 1983), the allergenicity of crude ragweed as well as PEG-modified allergen has been assessed by the RHPA.

In the present study, the anti-IgE preparation used as developing antisera in the RHPA was shown to be isotype-specific by radioimmunoassay methods and in a separate study (Beaudoin *et al.*, 1982) by inhibition assay of the RHPA by various immunoglobulins. In addition, cycloheximide was shown to completely block the secretion of IgE antibody production of the PFC whereas, in a separate study (Perelmutter, Potvin & Hébert, 1984), the addition of low concentration of antigen was capable of stimulating IgE antibody PFC whereas high concentrations suppressed this response. Thus, this technique appears to be reliable and reproducible to study the effect of drugs that will cause stimulation or suppression of IgE antibody response. This method appears to have the capabilities of differentiating the spontaneously active B cells making IgE antibody from those cells that require *in vitro* stimulation, and thus has the capabilities of investigating the modulators that regulate B-cell differentiation for IgE antibody synthesis.

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