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In vitro production of IgE by human peripheral blood mononuclear cells

I. RATE OF IgE BIOSYNTHESIS

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SUMMARY

IgE protein and grass-specific IgE antibodies were detected in the supernatants of 7-day cultures of unstimulated and pokeweed mitogen (PWM) stimulated human blood mononuclear cells from non-atopic and grass pollen-sensitive individuals. Significant amounts of IgE protein were detected in culture supernatants of grass-sensitive individuals and, even at lower levels, in those of non-atopic subjects. In contrast, detectable amounts of grass-specific antibodies were found only in the culture supernatants of grass-sensitive subjects. The mean values of total and grass-specific IgE detected in the supernatants of unstimulated and PWM-stimulated cultures did not differ statistically. Time sequence studies showed that IgE concentrations, measured in the 7-day supernatants, were due to a continuous release from the cells of IgE quantities progressively decreasing up to days 7 or 8. Comparison of the IgE protein and IgE antibody found in the 7-day culture supernatants to those released from initial cell pellets by treatment with acid buffer or freezing and thawing, showed that the IgE detected in 7-day supernatants could result, in part, from the release of cytophilic IgE bound to basophil or other cell types and in part also from the release of preformed lymphocyte cytoplasmic IgE into the supernatant fluids during the course of culture. In most non-atopic subjects and in some grass-sensitive patients the preformed IgE accounted virtually for the total IgE detected in the 7-day culture supernatants. However, the increase of IgE above the levels measured in the initial cell pellets, which was found in most grass-sensitive subjects, clearly reflected newly synthesized IgE. Both cycloheximide and puromycin were capable of reducing significantly the IgE concentration in culture supernatants when it was greater than the amount found in the initial cell pellets. The treatment of cells with mitomycin C was also able to decrease significantly the amount of IgE released in the supernatant after day 3 of culture.

INTRODUCTION

Several studies have described *in vitro* models of IgE production by peripheral blood lymphocytes (Patterson *et al.*, 1975, 1976; Buckley & Becker, 1978; Tjio, Hull & Gleich, 1979; Fiser & Buckley, 1979). These *in vitro* models could potentially represent an important tool for the study of control mechanisms which regulate the IgE synthesis in non-atopic and atopic individuals. However, until

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now conflicting results have been reported with regard to the type of lymphocyte donors and the time curve of IgE detection. Unlike studies of *in vitro* production of IgE reported by Patterson *et al.* (1975, 1976), where the subjects had markedly elevated levels of serum IgE protein and the concentration of IgE in the supernatant was maximal at 24 hr after initiation of culture, more recent studies showed that patients who had only slightly increased or normal serum IgE levels were also capable of forming IgE protein *in vitro* and peak supernatant IgE concentrations were found in 6-day (Tjio *et al.*, 1979) or 7-day (Buckley & Becker, 1979) cultures. Thus, further studies are necessary to establish whether the detection of IgE protein in the supernatant of lymphocyte cultures could permit investigation of the *in vitro* control of IgE production by T cell subsets or specific allergen.

The purpose of this paper is to describe our observation of *in vitro* production of IgE protein and antigen-specific IgE antibodies with special reference to the rate of biosynthesis.

MATERIALS AND METHODS

Cell donors. Blood was obtained from fifty-three individuals, twenty-nine of whom were grass pollen-sensitive (mean serum IgE 511 ± 70 iu/ml; range 17 to 1,400) and twenty-four non-atopic (mean serum IgE 105 ± 18 iu/ml; range 11 to 380).

Cell preparation and culture techniques. Ficoll–Hypaque-enriched mononuclear cells were washed and suspended in RPMI 1640 (Gibco) tissue culture medium containing 10% foetal calf serum (FCS; Gibco), 100 u/ml penicillin G, 100 μg/ml streptomycin, and 2·0 mM L-glutamine. The cell suspension was placed in 17-mm-diameter round-bottomed tissue culture tubes (Falcon, Oxnard, California) at a concentration of 1×10^6/ml of medium. Experiments were performed in the presence and absence of pokeweed mitogen (PWM; Gibco) shown to be able to stimulate antibody synthesis of IgM and IgG class (final dilution 1/200). Cultures were harvested at different time intervals. The supernatants were centrifuged, lyophilized and reconstituted in 1/10 distilled water.

Measurement of IgE protein. A sensitive solid-phase sandwich test specific for IgE protein and capable of measuring as little as 100 pg/ml of IgE was used to detect IgE protein in the tissue culture medium. Briefly, solid-phase anti-IgE was prepared by reaction of CNBr-activated Sepharose 4B (Pharmacia, Uppsala) with a purified gammaglobulin solution obtained by precipitation with 18% sodium sulphate from an anti-human Fc(ε) sheep antiserum. The solid phase (0·1 ml) was incubated for 48 hr at room temperature with 0·1 ml of the IgE under test, washed and then incubated again for 48 hr at room temperature with a ^{125}I-labelled anti-IgE antibody (Phadebas Rast, Pharmacia). A calibration curve was always prepared with the help of appropriate dilutions of a reference IgE serum containing 2,000 ng of IgE/ml.

Measurement of grass-specific IgE. Grass-specific IgE antibody activity was measured by the calibrated radioallergosorbent test (Rast) as previously reported (Romagnani *et al.*, 1976). Briefly, CNBr-activated paper discs were coated with a mixed grass extract (Lofarma, Milan) and incubated for 24 hr with either grass-specific IgE-rich serum or with concentrated cell culture supernatants. The discs were washed and incubated with ^{125}I-labelled immunosorbent-purified rabbit anti-IgE for an additional 24 hr. After other washings the discs were counted in a gamma counter. Results were expressed as Rast score as detailed elsewhere (Romagnani *et al.*, 1976).

Drug treatment of cells. Cycloheximide (10 μg/ml) and puromycin (10 μg/ml) were added to certain mononuclear cell cultures. Other cultures were prepared with mononuclear cells which had been incubated for 30 min at 37°C with mitomycin C (10 μg/ml/10^6 cells). Mitomycin-treated cells were washed three times with medium before culturing. Cell viability was evaluated by exposure of the cells to 0·1% eosin and determination of percentages of cells which excluded the dye. Viability of cells at the beginning of culture was consistently greater than 95%.

Treatment of cells with acid pH. IgE were dissociated from basophils using the technique described by Conroy, Adkinson & Lichtenstein (1977). Briefly, aliquots of day 0 cell pellets were resuspended in 1 ml of acetate buffer, pH 3·7, and incubated in an ice bath for 10 min. After

centrifugation, the supernatant solution containing IgE was collected, dialysed against medium and lyophilized for later measurement of IgE levels.

RESULTS

Detection of IgE protein and grass-specific IgE in supernatants of unstimulated and PWM-stimulated cultures from non-atopic and grass-sensitive subjects

Total and grass-specific IgE were measured in the supernatants of 7-day cultures of mononuclear cells from twenty-four non-atopic and twenty-nine grass-sensitive subjects. As shown in Table 1, IgE protein could be detected in supernatants of unstimulated mononuclear cell cultures from normal subjects in concentrations ranging from 250 to 2,600 pg/ml with a mean concentration of 1,390 ± 193 pg/ml. In PWM-stimulated cultures, IgE could be detected in concentrations ranging from 250 to 4,600 pg/ml with a mean concentration of 1,706 ± 242 pg/ml. On the other hand, IgE was detected in culture supernatants from grass-sensitive patients in concentrations ranging from 725 to 5,000 pg/ml with a mean concentration of 2,285 ± 184 and in the PWM-stimulated cultures in concentrations ranging from 875 to 5,200 pg/ml with a mean of 2,348 ± 197 pg/ml.

No significant amounts of grass-specific IgE antibodies could be detected in the 7-day supernatants of non-atopic individuals (mean value of RAST score 0·11 ± 0·02; range 0·001 to 0·48). In contrast, in the grass-sensitive group the RAST score ranged from 0·01 to 9·76, with a mean value of 1·54 ± 0·37. Addition of PWM did not induce significant changes in the amount of grass-specific IgE recovered in the supernatants of 7-day cultures (Table 1).

Detection of IgE protein in 0-day cell extracts and acid buffer eluates of mononuclear cell suspensions

To establish whether preformed IgE could account for the IgE detected in the culture supernatants, mononuclear cells taken from eighteen grass-sensitive subjects were cultured for 7 days and the amount of IgE was measured either in the 7-day supernatant or in the 0-day cell pellet after freezing and thawing the entire cell suspension five times. The possibility that preformed IgE had merely been released from basophil Fc receptors was also tested. For this purpose, the 0-day pellets of eight out of eighteen grass-sensitive subjects were also treated with a pH 3·7 elution buffer, known to release IgE from such receptors. The results of these experiments are summarized in Fig. 1 and Table 2.

A significant amount of IgE protein could be detected in the 0-day cell extracts of all subjects and it was at least in part due to IgE detectable in acid eluates. In eleven out of eighteen subjects tested the amount of IgE protein detected in the 0-day cell pellets was consistently lower than the amount found in the supernatants of 7-day cultures, but in seven subjects there were no significant differences (less than 100% increase) between the amount of IgE protein detected in the 7-day supernatants and that measured in the corresponding 0-day cell pellets.

Detection of IgE protein and grass-specific IgE in cell extracts and in supernatants at different culture time intervals

In order to determine the rate of IgE release from the cells into the supernatant, mononuclear cells from four grass-sensitive individuals were cultured for 8 days and both total and grass-specific IgE were measured in the cell pellets and in the supernatants at 24-hr intervals. As shown in Fig. 2, the amount of IgE protein increased in the supernatant from time 0 until day 5 of culture, with a plateau thereafter. On the other hand, a significant amount of IgE was still detectable in extracts from the lysed cells harvested at day 5. Similarly, peak levels of grass-specific antibodies in the supernatants were reached on day 5 and significant amounts of specific immunoglobulins were still detectable in the cell extracts until day 5 (data not shown).

In another set of experiments, mononuclear cells from two out of four grass-sensitive subjects mentioned above were divided into ten equal aliquots. At 24-hr intervals, an aliquot of cells was centrifuged, the supernatant discharged, the cells washed three times and then cultured again for 24 hr in the presence of a new medium. Total and grass-specific IgE were then measured in all 24-hr supernatants and in the last washing medium until day 10. No measurable concentrations of IgE

Table 1. Detection of IgE protein and grass-specific IgE antibodies in the supernatant of 7-day cultures of unstimulated and PWM-stimulated mononuclear cells from non-atopic and grass-sensitive subjects

Subjects	No. of cases	IgE protein (pg/ml)		Grass-specific antibodies (RAST score)	
		Unstimulated	PWM-stimulated	Unstimulated	PWM-stimulated
Non-atopic	24	1,390 ± 193	1,706 ± 242	0·11 ± 0·02	n.d.
Grass-sensitive	29	2,285 ± 184	2,348 ± 197	1·54 ± 0·37	2·04 ± 0·39

n.d. = Not done.

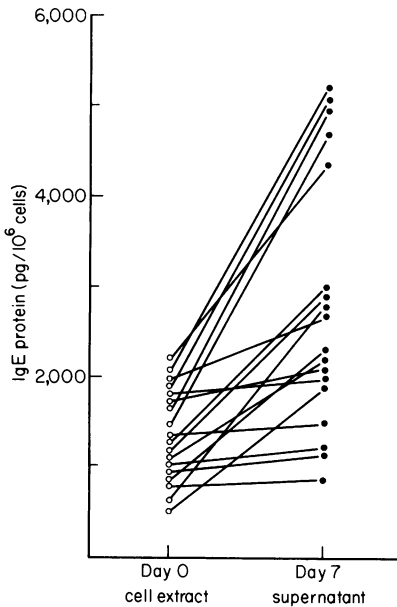

Fig. 1

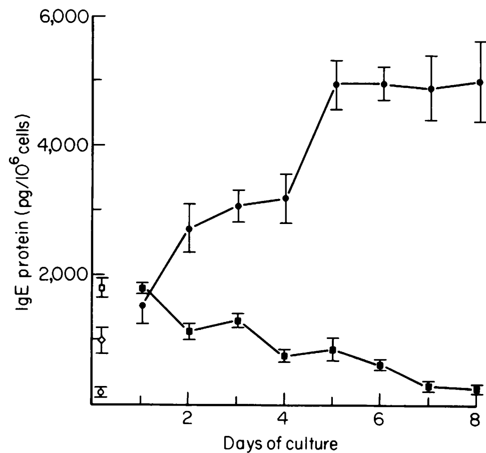

Fig. 2

Fig. 1. Detection of IgE protein in 0-day cell extracts and in 7-day culture supernatants of eighteen grass-sensitive individuals. Cell extracts were obtained by freezing and thawing the entire 0-day cell suspension (1×10^6 cells/ml) five times. After centrifugation, the supernatant was lyophilized and reconstituted with 1/10 distilled water. IgE protein was then measured in the supernatants of either 0-day cell extracts or 7-day cultures.

Fig. 2. Detection of IgE protein in cell extracts (■—■) and supernatants (●—●) examined at different time intervals after the initiation of culture. IgE protein was also measured in the last washing medium (○), in the acid buffer 0-day cell eluate (◇) and in 0-day cell extract obtained after freezing and thawing (□). The results represent the mean value ± s.e. of four separate experiments.

protein were found in any of the last washing media, whereas a significant amount of IgE was detected in all the 24-hr supernatants from day 1 until day 6 of culture. The maximum amount of IgE was found in the 24-hr supernatant of the 2nd day of culture. Rate of release of grass-specific IgE paralleled that of IgE protein (Fig. 3).

Effect of inhibitors of protein synthesis on the detection of IgE in culture supernatants

In order to furnish direct evidence that IgE protein and grass-specific IgE antibodies detected in the culture supernatants were, at least in part, actively synthesized by cells, we studied the effect induced

Table 2. Detection of IgE protein in time 0 cell acid eluates, in time 0 frozen-thawed cell pellets and in supernatants of 7-day cultures of mononuclear cell suspensions from grass-sensitive patients

	IgE protein (pg/10^6 cells)		
Patient	0-day acid eluate	0-day cell pellet	7-day supernatant
D.N.	770	970	2,750
L.G.	620	880	2,040
T.S.	900	1,200	4,500
T.A.	350	1,700	1,800
C.R.	320	1,950	2,700
M.E.	990	2,250	4,200
L.R.	850	1,800	5,640
L.G.	435	580	2,750
Mean value ± s.e.	654 ± 92	1,416 ± 208	3,297 ± 472

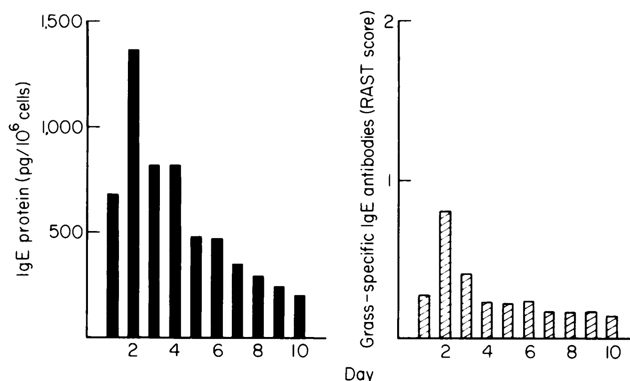

Fig. 3. Detection of IgE protein (■) and grass-specific IgE antibodies (▨) released into the culture supernatant during 24-hr periods. Mononuclear cells from grass-sensitive individuals were divided into equal aliquots. At 24-hr intervals, an aliquot of cells was centrifuged, the supernatant discharged, the cells washed three times and cultured again for 24 hr in the presence of a new medium. IgE protein and grass-specific IgE were then measured in all 24-hr supernatants until day 10. The results represent the mean value of two separate experiments.

by the addition of inhibitors of protein synthesis, such as cycloheximide and puromycin, in culture. As expected, both cycloheximide and puromycin did not induce any change in the amount of IgE detected in the culture supernatants whose values were not significantly different from those measured in the corresponding 0-day cell pellet extracts. However, when IgE concentrations detected in the supernatants were significantly higher than in the corresponding 0-day cell extracts, the addition of either cycloheximide or puromycin significantly reduced the amounts of IgE in the supernatants (Fig. 4).

Effect of inhibition of DNA synthesis on the detection of IgE in culture supernatants

To determine whether cell division was necessary for IgE synthesis *in vitro*, mononuclear cells from two grass-sensitive subjects were treated with mitomycin C and both total and grass-specific IgE were detected in the supernatant after 3 days of culture. Other aliquots of cells from the same donors were cultured for 3 days, washed and incubated again in new medium until day 10. The amount of IgE detected in the supernatants on days 3 and 10 was compared with the amount found in the

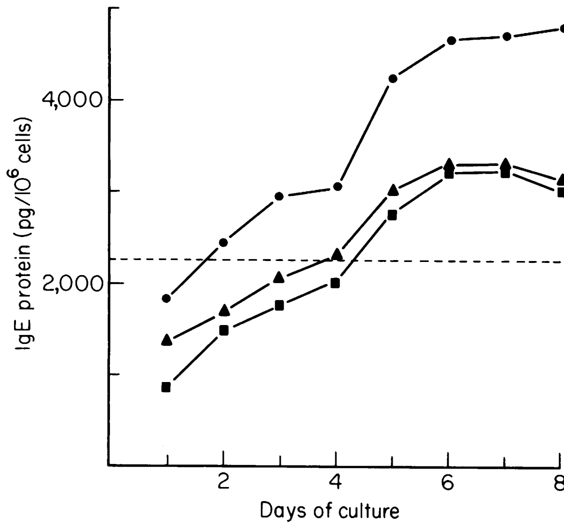

Fig. 4. Effect of treatment with cycloheximide and puromycin on the detection of IgE protein in the supernatant of mononuclear cell cultures from grass-sensitive individuals. Before initiation of culture, mononuclear cells were treated with cycloheximide (10 μg/ml) (▲—▲) or puromycin (10 μg/ml) (■—■). IgE protein was then measured in the culture supernatant at 24-hr intervals and compared to the IgE concentration found in the corresponding culture supernatants of untreated cells (●—●). Dotted line indicates the amount of IgE protein detected in the 0-day cell extracts. The results represent the mean value of two separate experiments.

Table 3. Effect of treatment with mitomycin C on the detection of IgE protein in the supernatants of 0–3- and 3–10-day cultures of mononuclear cells from grass-sensitive subjects

		IgE protein (pg/10^6 cells)	
Supernatant	Inhibitor	Expt 1	Expt 2
0-day pellet	—	820	1,730
0–3-day culture	—	1,500	3,560
	Puromycin	940	2,120
	Mitomycin C	1,300	3,860
3-day pellet	—	800	1,820
3–10-day culture	—	1,100	3,350
	Puromycin	740	1,620
	Mitomycin C	860	1,500

supernatants of corresponding cultures of untreated cells and of cells incubated with puromycin. As shown in Table 3, a significant amount of IgE was detected in the supernatant of either 0–3- or 3–10-day untreated cell cultures. The addition of puromycin was able to reduce significantly the amount of IgE detected in the supernatants of either 0–3- or 3–10-day cultures. In contrast, the treatment of cells with mitomycin C reduced the amount of IgE detectable in the supernatants of 3–10-day cultures, but it did not alter significantly the amount of IgE detectable in the supernatants of 0–3-day cultures.

DISCUSSION

Previous investigators have reported formation of IgE protein and allergen-specific IgE antibodies *in vitro* (Patterson *et al.*, 1975, 1976; Buckley & Becker, 1978; Tjio *et al.*, 1979; Fiser & Buckley,

1979). The present study confirms and extends the previous results in that IgE protein and specific IgE antibody were elevated in supernatants from mononuclear cell cultures. However, unlike results reported by Patterson *et al.* (1975) and Buckley & Becker (1978) where the subjects had much increased amounts of serum IgE protein, our subjects had only slightly elevated or normal serum IgE levels. Furthermore, in the study of Patterson *et al.* (1975) the highest IgE formation was in the first 24 hr of culture and it was no longer measurable after 48 hr of culture. In contrast, in our time sequence studies the amount of IgE protein and grass-specific IgE increased in the supernatant until 5–7 days of culture with a plateau thereafter. In this respect, our results agree apparently with those of Buckley & Becker (1978) and Fiser & Buckley (1979) showing maximum production of IgE after 6 or 7 days of culture. In agreement with the results of these workers, we also found that PWM, a known polyclonal B cell activator, did not significantly increase and, sometimes, decreased the IgE concentration detected in cell culture supernatants.

On the other hand, when we tried to measure the amount of IgE released in the supernatant from the cells day by day, the highest amount of IgE was found in the 24-hr supernatant of day 2 of culture and declined progressively thereafter. These data suggest that IgE concentrations measured in the supernatants of 6- or 7-day cultures were due to a continuous release from the cells of IgE quantities which were progressively decreasing from day 2 to days 6 or 7. In view of the precocity of IgE released in the supernatant, we considered it important to exclude the hypothesis that the IgE being detected in our culture supernatants were merely cytophilic IgE released from basophil Fc receptors and/or from other cell types present in the mononuclear cell populations which have also been shown to bind IgE protein (Gonzalez-Molina & Spiegelberg, 1977; Yodoi & Ishizaka, 1979). For this purpose, IgE concentrations were measured in eluates from acid buffer-treated mononuclear cell pellets. Unlike results reported by Fiser & Buckley (1979), we found detectable amounts of IgE protein in the acid eluates of time 0 cell pellets from either normal or atopic individuals. These IgE amounts accounted for 12–30% of the IgE being detected in the supernatant at 7 days. A still higher concentration of IgE protein and grass-specific IgE antibody was found in the time 0 frozen-thawed cell extracts, which in some cases virtually accounted for the total amount of IgE being detected in the supernatants of 7-day cultures. In contrast, in other grass-sensitive patients the IgE concentration measured in 7-day culture supernatants was significantly higher (three to five times) than the amount of IgE recovered in time 0 frozen-thawed cell supernatants. These data suggest that the IgE concentration measured in 7-day supernatants could result in part from the release of cytophilic IgE bound to basophils or other mononuclear cells and in part also from the release of preformed lymphocyte cytoplasmic IgE into the supernatant fluids during the course of culture. In some cases, the preformed IgE accounts for the total amount of IgE detected in 7-day culture supernatants. However, in other cases the increase of IgE above the levels measured in frozen-thawed cell supernatants strongly suggests that a significant proportion of IgE has been formed *in vitro*. This possibility was confirmed by the results of experiments with metabolic inhibitors. Both cycloheximide and puromycin were capable of reducing the concentration of IgE detected in the culture supernatants, but only when it was significantly higher than the amount found in 0-day cell pellets. These data suggest that, at least in these cases, a significant proportion of IgE protein and of grass-specific IgE, detected in 7-day supernatants, is dependent on protein synthesis and therefore clearly represents newly synthesized IgE. In addition, we have also shown that the amount of IgE, released after day 3 of culture, is reduced by treatment of the cells with an inhibitor of DNA synthesis. Thus, it is probable that a proportion of IgE detected in the supernatants of 7-day cultures may also be due to the activity of proliferating cells.

Another important question is the selection criterion of lymphocyte donors to be tested for the study of IgE production *in vitro*. In our study, we never found values of synthesized IgE as high as those reported by Buckley & Becker (1978) and Fiser & Buckley (1979). This could be due to the fact that our cases had not been selected and they presented only slightly increased or normal serum IgE concentrations. The IgE values found by us in the 7-day culture supernatants from mononuclear cells of grass-sensitive patients were rather similar to those reported by Tjio *et al.* (1979). These workers showed that ragweed-sensitive subjects are capable of secreting higher quantities of IgE antibody than grass-sensitive individuals. In other experiments we were able to demonstrate that mite-sensitive patients are able to produce higher quantities of IgE protein than grass-sensitive

patients (data not shown). This finding, as well as the results reported by Tjio *et al.* (1979), could be related to the seasonal period of the study. In fact, most of our experiments were carried out some months after the grass pollination period and during the peak period (end of the summer to the beginning of autumn) of the number of mites recovered from house dust samples (Voorhorst, Spieksma & Varekamp, 1969). Thus, it is possible that the greatest quantity of IgE antibodies is secreted in the cell culture supernatants as a consequence of *in vivo* stimulation of IgE antibody-producing cells during or slightly after the exposure of atopic subjects to specific allergen(s). This possibility is now under study in our laboratory.

Finally, further work is necessary in order to establish the nature of cells which are responsible for the production of IgE antibody *in vitro*. In preliminary experiments we showed that spontaneous IgE biosynthesis *in vitro* cannot be reduced by depletion of mononuclear cell suspensions in surface IgM- and/or IgD-bearing lymphocytes. This finding, as well as the finding of the precocity of IgE release in the culture supernatant and of the unsuccessful stimulation by PWM, strongly suggests that spontaneous IgE antibody production *in vitro* represents the result of an activation which has occurred *in vivo* and that, therefore, surface IgE-bearing B memory lymphocytes or surface immunoglobulin free plasma cell precursors are the cells mainly implicated in this IgE antibody production. Whether or not these IgE antibody-producing cells can still be influenced by *in vitro* manipulation remains the most critical area for future investigations.

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