Subpopulations of Circulating B Cells and Regulatory T Cells Involved in In Vitro Immunoglobulin E Production in Atopic Patients with Elevated Serum Immunoglobulin E

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ABSTRACT The B lymphocyte subpopulations producing immunoglobulin (Ig)E and the regulatory T cells modulating this IgE production in normals, and in atopic patients with respiratory allergy, atopic dermatitis, and markedly elevated serum IgE levels (>5,000 ng/ml), were investigated. Peripheral blood lymphocytes (PBL) were separated into T and B cell fractions and the ability of B cells to produce IgE in the presence or absence of pokeweed mitogen (PWM) and/or T cells was determined. The patients had a circulating population of cells which spontaneously produced up to 6 ng of IgE in vitro (per 4×10^5 non-E-rosetting cells) in the absence of T lymphocytes and PWM. PBL from normals did not possess such cells. This IgE synthesis occurred primarily (>75%) over the first 72 h of culture. There was a wide range in their activity between patients and from the same patient studied on repeated occasions (from <300 to 6,000 pg per culture). This spontaneous IgE production was inhibited by PWM (mean inhibition, 37%) or normal T lymphocytes (mean inhibition, 42%). The patients lacked T lymphocytes capable of inhibiting this spontaneous IgE synthesis in 7 of 13 experiments.

Functionally distinct B cells were identified in the patients and normals that responded to PWM with IgE production in vitro and required T-helper cell activity. Patients had normal PWM-responsive B cell IgE biosynthetic activity and T-helper function for these B cells. Suppressor T cell activity for PWM-driven IgE synthesis was also evaluated. Both the normals' and the patients' T lymphocytes provided similar levels of T cell suppressor function for PWM-driven IgE production. Patients with elevated serum IgE possessed these inhibitory T cells at times when the T lymphocytes which suppressed spontaneous IgE production were absent from their PBL.

INTRODUCTION

Immunoglobulin (Ig)E is a class of antibody molecules that is intimately associated with human immediate hypersensitivity disease states (1). Serum levels of IgE are often elevated in the atopic conditions of allergic rhinitis, allergic asthma, and atopic dermatitis, as well as parasitic infections and a variety of other disorders (2). Indeed, the range of polyclonal levels for serum IgE is many times greater than any other immunoglobulin class, ranging from <10 to >100,000 ng/ml.

The regulatory events controlling the synthesis of IgE have been extensively studied in rodent models using in vivo and in vitro systems that employ inbred strains and experimental immunization. These studies have shown the overall T cell dependence of IgE antibody production and the homeostatic role of T helper and T suppressor lymphocytes (3–5). The previous development of in vitro systems for analysis of human total and antigen-specific IgG, IgM, and IgA has led to both an increased understanding of normal human immune cell function as well as insight into the pathophysiologic mechanisms leading to diseases of immune deficiency, autoimmunity, and lymphoproliferative states (6). Recently several laboratories, including our own, have adopted in vitro systems for the analysis of production of human IgE in vitro.

Data from these laboratories have yielded what appear to be conflicting results in regard to the role of T lymphocytes and pokeweed mitogen (PWM)[1] in stim-

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[1] *Abbreviations used in this paper*: BSA, bovine serum albumin; FCS, fetal calf serum; hyper-IgE, hyperimmunoglobulinemia; PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline, 0.04 M; PFC, plaque-forming cells; PWM, pokeweed mitogen.

ulating and regulating B cell IgE production. However, the different investigations have used normals, atopics during their allergy season, and patients with hyperimmunoglobulinemia E.

Our laboratory has recently demonstrated the in vitro identification of functional subpopulations of Ig-producing lymphocytes that differ in their requirements for PWM and T cells (7, 8). In this study we have undertaken an evaluation of IgE-producing B cell subsets in atopic patients with elevated serum levels of IgE and have shown that one subpopulation is found in the blood of these patients, whereas a second subpopulation is shared by both normals and patients. The existence of these subpopulations of B cells and various regulatory T cells affecting their activity clarifies some of the differences seen in vitro IgE synthesis systems.

METHODS

Donors. Blood was obtained from normal donors between the ages of 21 and 35 hr. All normal donors had serum IgE levels <100 IU/ml. Patients with hyperimmunoglobulinemia E (hyper-IgE) were chosen as having serum levels >2,500 IU/ml. All these patients suffered from atopic dermatitis in addition to respiratory allergy. None of the patients had recurrent staphylococcal infection or other findings suggestive of "Hyper-IgE or Buckley Syndrome" (9) and none had received immunotherapy (desensitization).

Lymphocyte preparation and separation procedures. Human peripheral blood lymphocyte (PBL) suspensions were prepared by Ficoll-Hypaque (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) differential sedimentation (10) of heparinized blood. T and B lymphocyte fractions were separated by density sedimentation of spontaneous rosettes formed by T lymphocytes and sheep erythrocytes. The procedure was modified in that the sheep erythrocytes had been pretreated with 2-aminoethyl-isothiouronium bromide (11). These procedures are reported in detail elsewhere (12).

Lymphocyte cultures. Either unfractionated, fractionated, or fractionated and recombined PBL were cultured in RPMI 1640 medium buffered with $NaHCO_3$ and supplemented with 1-glutamine (10 mM), gentamicin (0.04 mg/ml), and 15% heat-inactivated fetal calf serum. All cultures were done in a final volume of either 1.0 or 1.5 ml in 13 × 100-mm plastic tubes (Falcon 2027, Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.). The tubes were incubated in a humidified atmosphere at 37°C with 5% CO_2 for 1–7 d. Experiments with PWM (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) used a final concentration of 1:100 (vol/vol) because this was found to give optimal stimulation of IgE in previous studies (13).

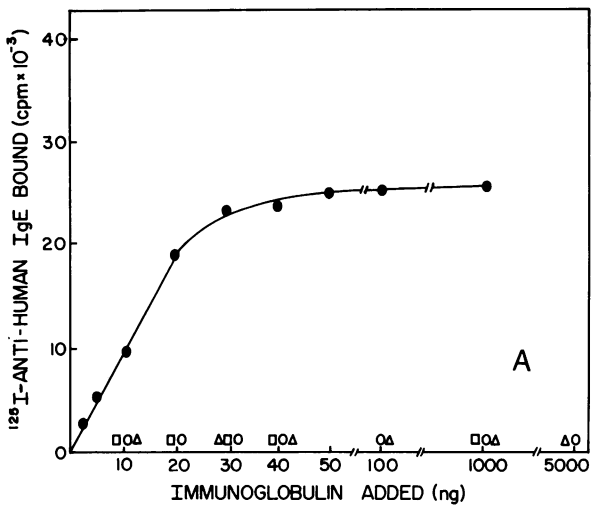
Radioimmunoassay. The quantitative radioimmunoassay for IgE was performed in microtiter plates (14). For measurement of IgE produced in culture, microtiter plate wells were filled with monospecific goat anti-human IgE (Research Products International Corp., Elk Grove Village, Ill., Lot 105) at a concentration of 0.5 mg antibody/ml. After incubation in a humidified chamber at 23°C overnight, the coating antibody solution was removed and saved for reuse. The wells were washed individually three times with 1% bovine serum albumin (BSA) in phosphate-buffered saline, 0.4 M (PBS) and then 10% BSA in PBS was added to the wells for 1 h to saturate any remaining protein-binding surface. After another wash with 1% BSA in PBS, samples to be assayed (350 μl) were added to wells and allowed to incubate overnight in a humidified chamber at 23°C. The following day the samples were removed and discarded. After three more washes with 1% BSA in PBS an IgG fraction of monospecific ^{125}I-labeled (15) goat anti-human IgE (Meloy Laboratories, Inc., Springfield, Va.) was added to each well in a final volume of 300 μl and allowed to incubate at 23°C for 5 h in a humidified chamber. Thereafter the plates were washed three times 1% BSA in PBS and eight times in running tap water. The individual wells were cut apart and the bound radioactivity was determined. For each radioimmunoassay a seven-point standard curve of IgE was performed in parallel with the culture samples on each plate. The lower limit of sensitivity of this assay is 50 pg per sample or 300 pg per 1.5 ml culture.

The IgE standard was a myeloma protein PS (16), which was purified from serum by DEAE-52 column chromatography. After elution of IgE in 0.005 M borate buffer, pH 8.0, the IgE was eluted in 0.025 M borate buffer. This was then rechromatographed on Sephadex G-200 with the protein eluting as one sharp peak. Only the leading half of this peak was used as purified IgE protein (17). Analysis by radioimmunoassay showed no IgG, IgM, or IgA present and sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a single protein of 180,000–200,000 mol wt. This IgE protein was standardized in our radioimmunoassay against World Health Organization (WHO) IgE standard NCl 68/341 (kindly supplied by Dr. Gerald Gleich, Mayo Medical School and Mayo Foundation, Rochester, Minn.). Simultaneous and identical standard curves were performed using IgE protein PS and the WHO standard. The curves were not identical but gave parallel plots on semi-log paper. This discrepancy could be due to some anti-PS idiotype binding by the antisera (raised against protein PS) or differences in the assays for protein concentration. We took the WHO standard as the current value to make our results concordant with other investigative work and adjusted the PS standard results accordingly.

The IgE binding capacity of the anti-IgE coated plates was determined by incubating triplicate wells with increasing amounts of purified IgE. Fig. 1A demonstrates that increasing quantities of IgE added in the first incubation resulted in a subsequent increased binding of the ^{125}I-anti-IgE second antibody with saturation being reached between 35 and 40 ng of IgE. IgM-, IgG-, and IgA-purified proteins were not detected when up to 5,000 ng of purified polyclonal IgG or monoclonal IgM or IgA were added.

The binding of ^{125}I-anti-human IgE to the wells during the second incubation could be inhibited by the addition of purified human IgE but not with IgG, IgM, or IgA (Fig. 1B). 50 ng of IgE were added to each anti-IgE coated well and incubated overnight to saturate the wells. Immediately before the addition of the radiolabeled anti-IgE, increasing amounts of either competitor was added to triplicate wells. Increasing quantities of the IgE competitor gave progressive inhibition of binding of the second antibody to the wells with 50% inhibition being achieved with 143 ng of IgE added. No inhibition of binding was observed when up 5,000 ng of the IgG, IgM, or IgA competitors were added. Similar lack of inhibition occurred when IgG or IgM was added to 2 ng of IgE (12,540 cpm).

The possibility that nonimmunoglobulin serum factors were detected was assessed. Fetal calf serum, goat serum, and calf serum were assayed at a 1:5 final dilution and compared to 5% BSA and the IgE standard. Background level with 50% BSA was 879 cpm, whereas 0.1 and 1.0 ng of IgE gave 1,622 and 10,763 cpm, respectively. Fetal calf serum (FCS) gave 943 cpm, goat serum 1,046 cpm, and calf serum 970 cpm. A human serum deficient in IgE (kindly provided by Dr. Gerald Gleich) was also assayed and found to give binding of only 1,003



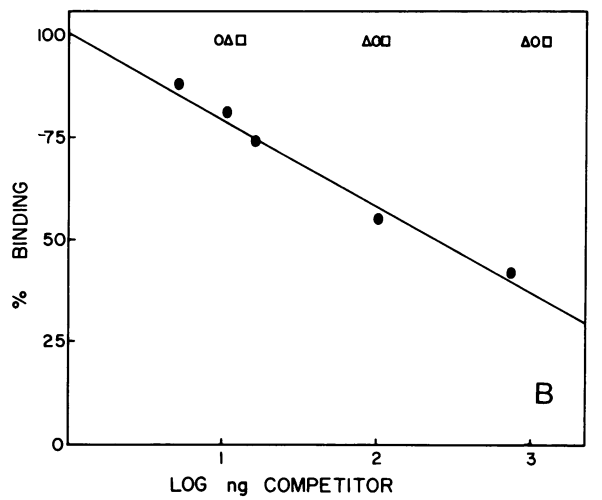


FIGURE 1 Radioimmunoassay for IgE. A. Direct Binding of immunoglobulin. Increasing amounts of IgE (●), IgM (△), IgG (○), or IgA (□) were added to triplicate wells coated with anti-IgE. After 16 h incubation, ^{125}I-anti-IgE was added and the amount of radioactivity bound determined. B. Competition by immunoglobulin with the second antibody. Increasing amounts of IgE (●), IgM (△), IgG (○), or IgA (□) were added to wells at the time of addition of the ^{125}I-labeled anti-IgE antibody. Competition for detection of the IgE was achieved only with IgE. The 100% binding value was 27,682 cpm for 35 ng of IgE.

cpm. This serum was diluted to a final concentration of 1:100 to achieve levels of IgG, IgM, and IgA similar to those produced in culture (100–10,000 ng/ml).

Serum IgE levels were determined using the Prist technique (Pharmacia Fine Chemicals).

Velocity and density sedimentation of PBL. PBL (1×10^8) were separated by 1 g sedimentation on BSA gradients ranging from 1.0 to 2.0%, as described by Miller and Phillips (18). After 3 h, 15-ml fractions were recovered and the cells/fraction quantitated. The cell fractions were then placed in 1-ml cultures at 37°C and assayed for IgE production at various times. The sedimentation values are for human tonicity at 5°C. Density separation was performed using a Percoll (Pharmacia Fine Chemicals) gradient. Percoll, 5.5 ml, was mixed with 5.5 ml of 2 × PBS. This mixture was centrifuged at 26,000 *g* at 23°C for 30 min in a 13-ml cellulose nitrate tube. Cells to be separated ($40–50 \times 10^6$) were resuspended in 1.0 ml of minimal essential medium and gently placed on top of the established gradient. The cell gradient was then centrifuged at 1,000 *g* for 5 min at 23°C. Thereafter, 1-ml fractions of the gradient-cell mixture were removed, starting at the top of the tube. Each fraction was then washed once in minimal essential medium, counted, resuspended at 1.33×10^6 cells per ml in culture medium, and tubes established for IgE production in vitro.

RESULTS

Synthesis of IgE by B cells alone without T cells or PWM. The peripheral blood mononuclear cell fraction obtained from the normal individuals after T lymphocyte depletion of E-rosetting cells synthesized only very low levels of IgE in vitro either in the presence or absence of PWM over days 1–7. This is in agreement with our earlier reports. However, identically derived and cultured B cell fractions from the patients demonstrated that these patients' B cells would spontaneously produce IgE in vitro. The kinetics of the appearance of IgE in these B cell cultures was examined by initiating cultures of B cells at day 0 and harvesting them on days 1–7 (Fig. 2). These experiments showed that the majority of the IgE (>75%) was produced in the first 3 d. Subsequent experiments were harvested on day 3. As can be seen in Table I, over the 6 mo that

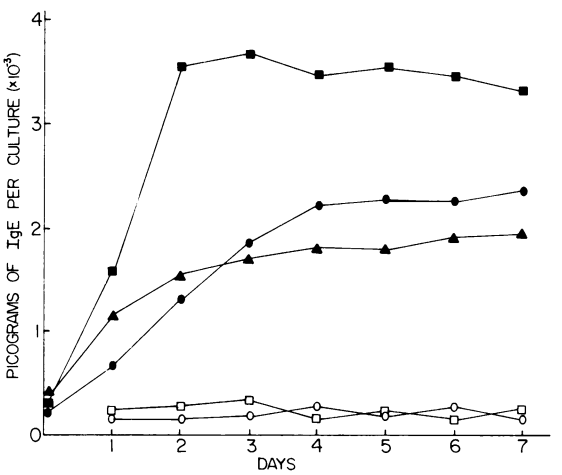


FIGURE 2 Kinetics of IgE production in vitro by patients' unstimulated B cells. B-fraction cells (0.4×10^6/culture) were placed in culture and harvested on days 1–7 and the amount of IgE produced assayed. (■ patient 1, ▲ patient 2, ● patient 5). Identical cultures were also performed in the presence of 50 μg/ml of cycloheximide (□,○).

TABLE I

IgE Produced by B Cells Alone (0.4 × 10^6) in the Absence or Presence of PWM at Day 3

	Serum IgE	B Cells alone	B Cells + PWM
	ng/ml	*pg IgE/culture*	
Patient			
1	8,988	<300	<300
1	—	1,508	840
1	—	300	420
1	—	3,194	2,715
1	—	1,043	469
2	12,831	1,009	666
2	—	3,766	1,506
2	—	841	681
2	—	<300	<300
3	15,036	1,893	1,138
4	6,290	5,721	2,346
5	11,403	4,896	3,378
6	5,891	6,075	4,010
6	—	2,306	1,752
6	—	721	498
Normal donor			
1	132	<300	300
1	61	360	420
2	288	<300	330
3	181	300	<300
4	101	<300	<300
4	—	<300	<300
4	—	<300	<300
5	399	<300	<300
6	187	330	<300

these experiments were performed individual patients showed a marked degree of variability in the amount of spontaneous B cell IgE production observed. Because the patients possessed high serum levels of IgE, we undertook a series of experiments to demonstrate that IgE measured in vitro was synthesized in culture and not cytophillic IgE passively carried over on the surface of basophils or lymphocytes with Fc receptors for IgE (19). Identical numbers of nonrosetting cells as used in the experiments shown in Table I and Fig. 2 were rapidly frozen and then thawed three times in liquid nitrogen at day 0. The tubes were then centrifuged at 300 *g* and the supernate assayed for IgE. None of these supernates revealed >500 pg of IgE. (Range, 220 to 480 pg.) It is possible that in the frozen-thawed cell lysates, basophil IgE might remain bound to cell membrane fragments and thereby not be measured. In cultures this cell-bound IgE might be slowly released in vitro over days 0–3. Therefore, cultures of patients' B fraction cells were also established in the presence of 50 μg/ml of cycloheximide, a known protein synthesis inhibitor, and harvested on days 1–7 (Fig. 2). IgE failed to rise in these cultures.

The effect of PWM on this spontaneous IgE production was next assessed. In each case where >300 pg of IgE was produced at day 3 without PWM, the addition of PWM caused a fall in the amount of IgE produced by the B cells (mean inhibition of 37%) (Table I). There was no correlation between the percentage of inhibition by PWM and the level of IgE produced in its absence.

Suppressor T cells for spontaneous IgE production. The effect of T lymphocytes on spontaneous IgE production was also evaluated. On eight separate test occasions T lymphocytes (1.6 × 10^6/culture) were added to B fraction cells (0.14 × 10^6/culture) in the absence of PWM and the cultures harvested on day 3 (Table II). The T lymphocytes were derived from either that patient (autochthonous), another patient, or a normal donor (both allogeneic). Furthermore, parallel cultures were established with irradiated (3,000 rad) T lymphocytes because this treatment has been shown to remove the majority of suppressor T lymphocyte activity (20, 21). The addition of normal T lymphocytes (seven experimental cultures) consistently inhibited spontaneous IgE production by patients' B cells (Table II). (Mean 42% and median 40% inhibition with a range of 34 to 60% and a standard deviation of 7.8%.) Irradiation of the normal T lymphocytes removed this suppressor activity (mean 2% inhibition with a range of −23 to +22%). As a group, patients' cells showed significantly less suppression than the normals' ($P < 0.01$ by two-tailed *t* test and $P < 0.02$ by Wilcoxon rank sum test) with a mean of 22.5% and a median of 25% inhibition with a range of −8 to 42% and a standard deviation of 15.9%. This was a result of the fact that in 7 of the 13 experimental combinations, T cells from the patients were significantly less effective (<2 SD) in inhibiting spontaneous IgE production (Table II).

As can be seen in Table II, this failure to suppress occurred in both autochthonous and allogeneic cell combinations. Where suppression did occur with patients' T cells, it was sensitive to irradiation as was the suppression mediated by the normal T lymphocytes. Those occasions when patients' T cells failed to demonstrate inhibition of B cell spontaneous IgE production did not correlate with the level of increased spontaneous in vitro IgE synthesis by those patients' cells. Conversely, this type of inhibitory T lymphocyte activity was found in the PBL from patients on occasions when large amounts of IgE were spontaneously produced by their B cells.

A possible explanation for this inhibition of IgE production by T cells would be that the IgE was being passively absorbed to Fc receptors on T cells which have been recently reported by Yodoi and Ishizaka (15). This was excluded by incubating one to four million untreated T cells with 5,000 pg of ^{125}I-labeled IgE for up to 3 d and measuring the amount of radiolabeled IgE re-

TABLE II

Effect of T Lymphocytes (1.6 × 10^6) on Hyper IgE Patients' Spontaneous IgE Production at Day 3

Test no.	T Cell source	B Cells alone	B + T	B + T^{Irr}*	Suppression‡
			pg IgE/culture		%
1	Allogeneic normal	1,893	1,249	1,770	34 (+)
	Autochthonous patient		1,852	2,094	2 (−)
	Allogeneic patient		1,206	1,801	36 (+)
2	Allogeneic normal	3,194	1,278	2,984	60 (+)
	Autochthonous patient		3,077	3,206	4 (−)
	Allogeneic patient		2,124	3,331	34 (+)
3	Allogeneic normal	2,306	1,361	2,075	41 (+)
	Autochthonous patient		1,337	1,913	42 (+)
	Allogeneic patient		1,730	1,888	25 (−)
4	Allogeneic normal	1,508	905	1,630	40 (+)
	Autochthonous patient		1,628	1,593	−8 (−)
	Allogeneic patient		1,392	1,564	8 (−)
5	Allogeneic normal	2,995	1,856	2,866	38 (+)
	Autochthonous patient		1,794	2,641	40 (+)
6	Allogeneic normal	4,216	2,775	4,927	34 (+)
	Autochthonous patient		3,766	3,979	11 (−)
7	Allogeneic normal	2,763	1,603	2,707	42 (+)
	Autochthonous patient		1,851	2,819	33 (+)
8	Autochthonous patient	2,081	1,831	1,807	18 (−)
	Allogeneic patient		1,269	1,972	39 (+)

* Irr, irradiated 3,000 rad.

‡ Positive suppression was defined as a >26% decrease in IgE production as the mean for the normal T cell cultures was 41% with a range (mean±2 SD) of 26 to 57%. See text.

maining in the supernate at the various times (Table III). Other points that support this lack of passive absorption are that it failed to occur with irradiated T cells (Table II) and was not dose dependent in that 4.0 × 10^6 normal T cells failed to inhibit IgE production > 1.6 × 10^6 cells.

TABLE III

*Lack of Absorption of IgE by Normal T Cells**

T cells added (×10^6)	Day harvested	Counts per minute remaining in supernate
		%
0	0	100
0	2	92
0	3	95
1	2	94
1	3	87
1.6	2	103
1.6	3	94
4.0	2	91
4.0	3	97

* IgE protein was ^{125}I-labeled at 1,693 cpm/ng by lactoperoxidase technique. 5 ng were incubated in 1.5 ml of culture medium in triplicate with or without T cells at 37°C in a humidified atmosphere. At days 2 and 3, aliquots of the supernates were harvested and the amount of radioactivity determined.

Because the B cells that produce Ig after PWM activation appear to be functionally distinct from spontaneous Ig-producing cells (7), we examined the ability of patients' B cells to synthesize IgE in vitro after PWM stimulation. PBL from normal donors can be stimulated to synthesize IgE in the presence of normal T (autochthonous or allogeneic) lymphocytes (13). IgE production occurred to some degree in the presence of medium containing fetal calf serum but was enhanced 4–10-fold by the presence of PWM. Normal and patients' PBL B-fraction cells (0.4 × 10^6) were placed in 1.5 ml triplicate cultures with or without 1:100 vol/vol PWM. Identical triplicate cultures were also established with untreated or irradiated normal T lymphocytes from 0.4–4.0 × 10^6 cells/culture. Fig. 3 shows the kinetics of the IgE production over a 7-d time-course for two such experiments. The normal B cell/T cell cultures showed little IgE production without PWM, whereas with PWM IgE synthesis increased from days 4–7. The B cells from the patients had increased IgE production over days 1–3 in the absence or presence of PWM (Fig. 3A). This early synthesis was somewhat diminished by the addition of normal T cells (Fig. 3B). IgE production by the patient B and normal T cell cultures rose in

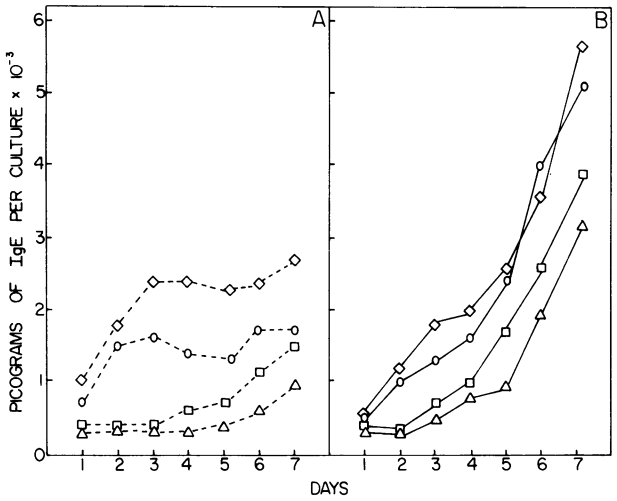


FIGURE 3 Kinetics of IgE production in vitro by PWM-stimulated normal (□,△) and patients' (○,◇) B-fraction cells (0.4×10^6) in the absence (panel A) or presence (panel B) of normal T lymphocytes (1.6×10^6). B-fraction cells were placed in culture, harvested on days 1–7 and the amount of IgE produced assayed.

parallel to the normal controls from days 4–7 in the presence of PWM. Similar experiments (total 11) were performed on six patients with serum IgE levels > 5,000 ng/ml. In each case the combination of patients' B cells and normal T cells plus PWM yielded stimulation of IgE synthesis from days 4–7. However, the level of IgE produced over days 1–3 was quite variable, ranging from 5 to 60% of the total IgE produced by day 7. This early synthesis was directly related to the amount of spontaneous IgE that patients' B cells were producing at the time as measured by cultures of B cells or B cells plus PWM at day 3.

Regulatory T lymphocytes for PWM-induced IgE production. Normal T lymphocyte subpopulations provide both an enhancing and inhibitory effect on normal B cell PWM-driven IgE production (13). It was of interest to see whether the patients with elevated IgE levels possessed normal or altered T regulatory activity for this PWM-driven IgE synthesis. This was investigated by initiating paired titrations of normal B cells with patients' or normals' untreated and irradiated T lymphocytes plus PWM. Fig. 4 shows the results of one such experiment. The normal B cells produced similar amounts of IgE in the presence of irradiated patient or normal T lymphocytes demonstrating equivalent helper T cell function over the range of cells examined. When untreated T lymphocytes (suppressor activity intact) were used for the titration, IgE production was reduced in parallel with both patient and normal T cells. The amount of IgE produced with 1.6×10^6 irradiated T cells was taken as a defined measure of IgE help (22). The amount of IgE produced by the identical B cells in the presence of 1.6×10^6 of the same T cells that had not been irradiated then yields a measure of the irradiation sensitive T suppressor influences present. A suppressor index can be calculated as IgE produced with untreated T cells divided by IgE produced with the irradiated T cells (Table IV). The greater the suppression, the smaller the index number. The mean index for the normal T cells was 0.63 whereas the mean index for the patients' T cells was 0.64. Two culture pairs (normal subject 4 and patient 7) failed to show any inhibition. The significance of this finding is unknown but we have occasionally observed this previously in the normal population (13).

Characterization of the spontaneous IgE-producing B cells. The kinetics of the IgE synthesis by the spontaneous IgE-producing B cells suggested that they were activated and therefore might be large "lymphoblastoid" B cells. Furthermore, we had previously observed spontaneous IgG antibody-forming cells to be such large lymphocytes (7). PBL from patients and normals were separated by 1 *g* velocity sedimentation and individual fractions cultured for 7 d. Aliquots (300 μl) were removed on day 3 from each culture for quantitation of IgE production. This was replaced with an equal volume of fresh medium. When calculating total IgE produced at day 7, this dilution effect was taken into account. The majority of PBL sedimented with a mean velocity of 3.7 mm/h. However, the cells responsible for the spontaneous IgE production sedimented at a mean velocity of 6 mm/h. (Fig. 5). In other experiments the spontaneous

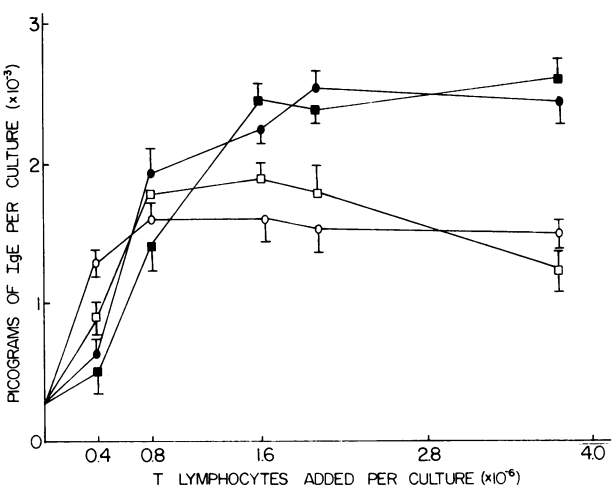


FIGURE 4 Effects of T lymphocytes on B cell PWM-driven IgE synthesis in vitro. Normal (●,○) or patients' (■,□) T lymphocytes were added in increasing numbers to normal B cells (0.4×10^6) in the presence of PWM. T lymphocytes were either irradiated (●,■) or untreated (○,□). Culture supernates were assayed on day 7 for IgE.

TABLE IV

Effect of T Cells vs. Irradiated T Lymphocytes on Normal B Cell PWM-Driven IgE Synthesis

Experiment no.	Normal		Patient		Suppressor index‡
	T^{Irr}*	T	T^{Irr}	T	
	pg IgE produced/culture				
1	8,765	5,695	—	—	0.65
1	—	—	7,645	5,580	0.73
2	4,450	2,230	—	—	0.50
2	—	—	5,050	2,830	0.56
3	3,160	1,770	—	—	0.56
3	—	—	3,520	1,620	0.46
4	6,875	7,080	—	—	1.03
4	—	—	8,230	5,100	0.62
5	2,360	1,770	—	—	0.75
5	—	—	1,990	1,275	0.64
6	4,965	2,930	—	—	0.59
6	—	—	5,610	3,100	0.57
7	8,645	5,445	—	—	0.63
7	—	—	7,905	8,380	1.06
8	9,140	3,385	—	—	0.37
8	—	—	8,055	4,755	0.59
9	1,675	1,005	—	—	0.60
9	—	—	2,490	1,370	0.55

* Irr—irradiated 3,000 rad.

‡ Suppressor index = ratio of $\frac{\text{IgE with T}}{\text{IgE with } T^{Irr}}$.

IgE-producing cells were seen in a broader band of fractions extending into the faster sedimenting cells from 4 mm to >11 mm per h. Results from 7-d cultures showed similar patterns to 3-d cultures and usually contained a little more total IgE in each fraction. Normal PBL did not show any cell fraction that made IgE at day 3. Similar experiments were performed but PBL were separated on the basis of density utilizing a Percoll gradient (Fig. 6). The majority of IgE produced was found in the fractions that were lighter than the majority of PBL.

DISCUSSION

Although several laboratories have reported in vitro production of human IgE, the conditions leading to enhanced or inhibited production appear to conflict. Buckley and colleagues (23) originally reported PWM to stimulate IgE production by normal PBL, but in a more recent report found a variable effect on normal IgE production (24). Yet, in patients with elevated serum IgE levels PWM usually inhibited IgE production, although in a number of instances where IgE production was low without PWM, addition of the mitogen increased IgE production as measured on day 7 (25). Furthermore, these investigators went on to demonstrate that in the absence of PWM, normal individuals' T cells had an inhibitory effect on this IgE production by the patients' B cells while the patient's own T cells often failed to exhibit this suppressor influence (24, 25). Tjio et al. (26) examined the ability of nonatopic individuals to produce IgE in vitro and failed to detect production in the presence or absence of PWM over 6 d. However, in atopic individuals with a geometric mean IgE serum level of only ~100 IU/ml, they detected up to 14,762 pg of IgE-produced per 10^6 cells. In these individuals, PWM had an inhibitory effect on total and specific IgE production. However, in contrast to Buckley's studies the majority of IgE production occurred in the first 4 d of culture. Furthermore, these studies were conducted in the ragweed season and the ragweed-sensitive patients produced far more IgE than grass-sensitive individuals. Followup studies reported that the in vitro total and specific IgE production by these patients declined markedly after the ragweed season (27). Finally, our laboratories studied IgE production in vitro by nonatopics and atopics with IgE levels <400 IU/ml in California. We demonstrated PWM stimulation of IgE production in both groups and the requirement for T-helper cells as well as T cell suppressor influences in final IgE production (13).

The data in the paper present evidence for at least two functional populations of B cells that may produce IgE in vitro. The existence of spontaneous immunoglobulin-secreting lymphocytes in the peripheral blood of experimental animals and humans is well documented. Sorkin and Landy (28) demonstrated that within the 1st wk after immunization of rabbits with polysaccharide from *Salmonella enteritidis*, circulating lymphocytes capable of generating plaque-forming cells (PFC) in vitro against this antigen appear transiently in the blood. Frost et al. (29) identified the release into the efferent lymph of antigen-specific PFC 72 to 96 h after administration of antigen. The

TABLE V

Effect of T Cells and PWM on Different B Cell IgE Production

	T	T^{Irr}	PWM
Spontaneous B cells	↓*	—	↓
PWM B cells	↑ or ↑‡	↑↑	↑

* The T cells involved in inhibition of spontaneous B cell IgE production are probably functionally distinct from those that inhibit PWM-driven IgE synthesis (see text).

‡ Contains both helper and suppressor effects but helper effect predominates under experimental conditions used.

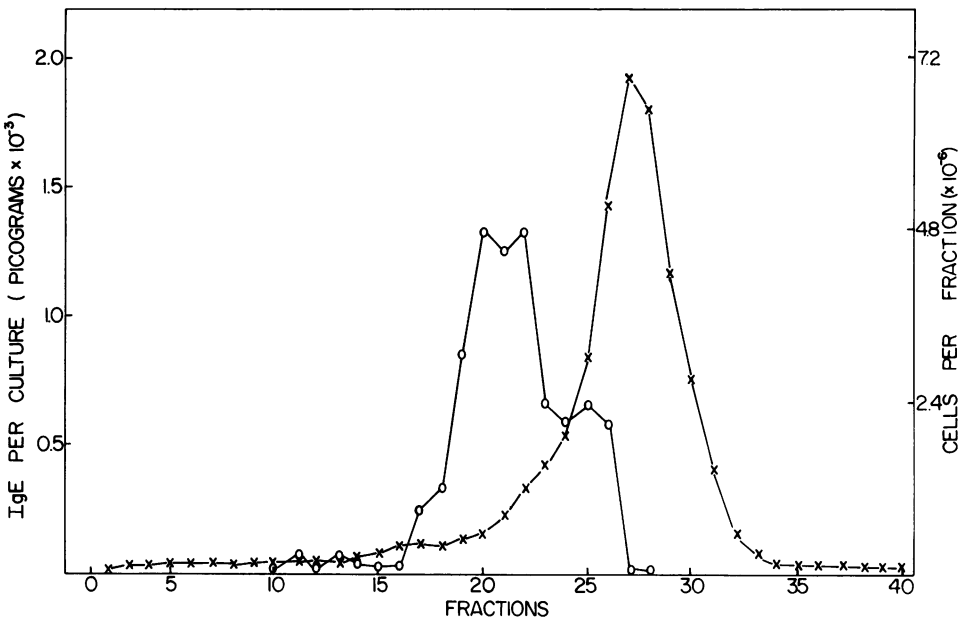


FIGURE 5 Velocity sedimentation of PBL. Cells (1×10^8) were loaded into 600 ml of a 1–2% gradient of BSA in minimal essential medium. After 3 h at 5°C 15-ml fractions were collected and the distribution of cells determined (×). Larger cells are found in the lower number fractions. Cultures were established with 1 ml of culture medium without PWM. At day 3, 300-μl aliquots were removed from each culture and the IgE produced per culture determined (O).

release of these lymphoblastoid cells declined sharply 8–10 d after administration of the antigen.

Similar circulating populations of immunoglobulin and antibody-secreting cells have been described in humans. Eby et al. (30) and later Ginsburg et al. (31) detected circulating nonantigen-specific PFC in the PBL of normal individuals not receiving experimental immunization. 8–10 d after immunization with *Salmonella typhi*, Thompson and Harris (32) detected circulating PFC for salmonella lipopolysaccharide. These studies indicate that circulating PFC are a common occurrence in humans and may be correlated with a recent immune response. Such cells may also be increased in the blood in disorders of altered immune homeostasis such as systemic lupus erythematosis (33), chronic graft vs. host disease (personal observation), and immunodeficiency with hyper-IgM (34). We have previously shown that in normal individuals after booster immunization with soluble tetanus toxoid, cells arise in the circulation that are capable of synthesizing appreciable quantities of IgG-anti-Tet without PWM stimulation or T cell help (7). Indeed PWM and un-irradiated T lymphocytes exerted an inhibitory effect on antibody production by these spontaneous antibody-producing cells. Such cells could be functionally distinguished from PWM reactive cells for both IgG and IgM antibodies.

We purposely chose atopic patients with very high serum levels of IgE to see if their PBL contained analogous cells producing IgE. Our data indeed showed the spontaneous production of IgE by these patients' B cells. Furthermore, these cells could also be inhibited by (normal) T cells and PWM. Our failure to detect these cells in our atopic population previously studied may have resulted from the fact that those patients (*a*) had much lower serum IgE levels, (*b*) were receiving immunotherapy and repeated parenteral immunization blocks the appearance in the blood of lymphoblastoid B cells (personal observation)[2] (*c*) differ from the patients in the central and eastern United States as our patients are exposed to essentially continuous allergen-pollen exposure in southern California. We noted marked variations in the presence of these cells even in our chosen population with elevated serum IgE levels (Table I). This probably relates to in vivo events that are stimulating these patients' IgE production but are not understood at this time. By using a PFC assay, Lawrence et al. (35) were unable to demonstrate spontaneous IgE-producing cells in normal PBL but did detect such cells in 5 of 11 normal human bronchial lavage fluids.

The presence of such spontaneous IgE-producing cells in in vitro culture would explain the observations of Tjio et al. (26, 27) of increased production by patients undergoing in vivo IgE immune response (ragweed) as well as its inhibition by PWM. Furthermore, the

[2] Manuscript in preparation.

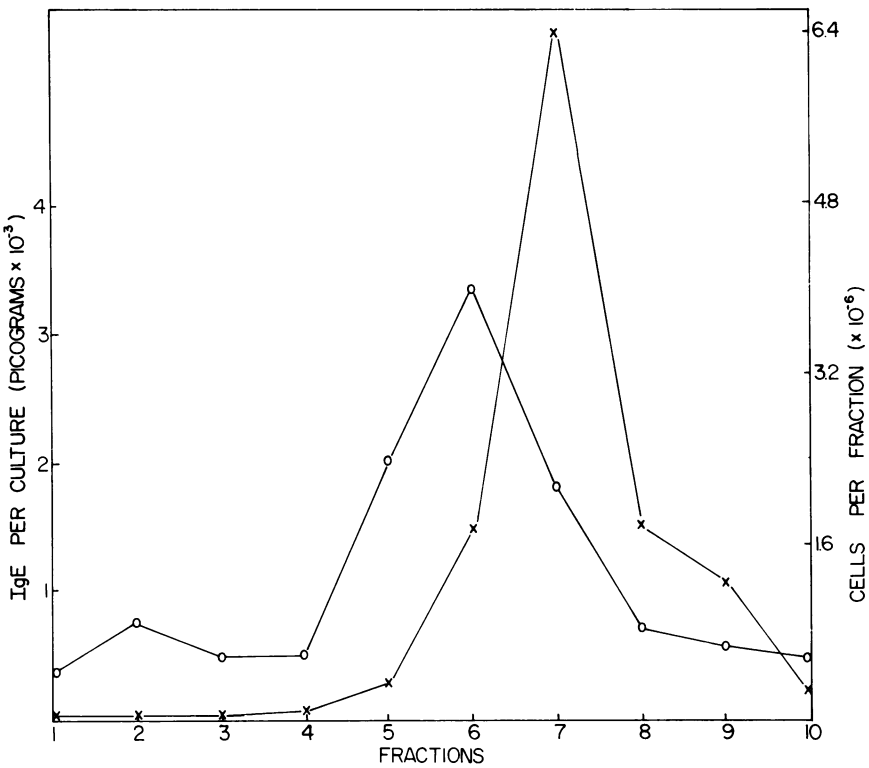


FIGURE 6 Density separation of PBL. Cells (50×10^7) were loaded onto a preformed Percoll density gradient. After centrifugation at 1,000 *g* for 5 min at 23°C, 1-ml fractions were removed. Fraction 1 represents the uppermost fraction and fraction 10 the lowermost fraction removed. Lighter cells are found in the upper fractions. Cell distribution was determined (×) and cultures were initiated in 1 ml of medium. At day 3, 300-μl aliquots were reserved and the IgE produced per fraction determined (○).

kinetics of IgE production they reported fits that known to occur with peripheral blood spontaneous immunoglobulin producing cells. Indeed, the first report of IgE production in vitro by human PBL by Patterson et al. (36) probably represented the activity of such cells as it occurred in two patients with serum levels of 100,000 and 40,000 IU/ml, respectively, and the IgE appeared in the first 2–3 d in culture. The existence of IgE lymphoblastoid B cells also helps to put in perspective the results of Buckley et al. (23–25) where PWM stimulated some normals' unfractionated PBL to produce IgE but usually inhibited IgE production in the high serum IgE patients.

A second subset of IgE-producing B cells that we have been able to consistently detect are those that respond in the presence of PWM and T-helper effect. We have previously reported on the regulation of these cells in vitro and showed that they are PWM-activated as well as being responsive to T-helper and T cell suppressor influences (13). Other investigators have not been able to consistently define the presence of these cells. At first Buckley et al. (23) reported stimulation of IgE by PWM in normals. Recently they concluded that PWM did not stimulate normals' IgE production in vitro when they took the mean IgE production of stimulated vs. unstimulated cultures. Analysis of their data reveals that 7 of the 10 normal subjects originally reported had a >50% increase in IgE with PWM and one patient had an 11-fold increase in IgE production. In their latest report (24), 5 of 15 had a >50% increase with one patient having a 13-fold increase. However, as they averaged stimulated, unchanged, and inhibited cultures, the net result was no difference. In a total of 13 of 25 cultures PWM stimulated IgE by >50%. We would interpret this to suggest that they are achieving PWM stimulation in some normals but not consistently as we have. One fact which may explain why we have achieved this is that we have screened all lots of FCS to use only those that provide little or no stimulation of immunoglobulin synthesis on their own while supporting vigorous Ig production in the presence of PWM. With this culture system, we achieve 20-fold increases in IgG and IgM with PWM stimulation (23) and 4-fold stimulation of IgE (13). Fiser and Buckley (24) showed only 2.4–7.3-fold stimulation of IgM by PWM in normals in their

system. Although all FCS appears to have some mitogenic effects, FCS with more pronounced activation of immunoglobulin may well obscure or even compete with PWM activation. We feel it is crucial to demonstrate the lack of stimulation of IgG, IgM, and/or IgA without PWM and vigorous responses in its presence before concluding that one is examining solely PWM and not primarily FCS effects.

We can only speculate why Tjio et al. (26) did not observe this effect. It may well have been because of their use of flat-bottomed culture dishes which would limit cell to cell contact (37). Such conditions would prevent the required cell interactions between the rare B and T cell for PWM-induced IgE synthesis where the B cell precursor frequency is very low, ~1:50,000 B cells (38) while not affecting the spontaneous IgE production by lymphoblastoid B cells.

The combination of the IgE production by these two B cell populations leads to the biphasic IgE synthesis we observed with patients' B cells plus T cells with FCS as shown in Fig. 2. PWM lowers the initial IgE (days 1–3) but increases the later IgE synthesis. Our hyper-IgE patients did have PWM-responsive B cells similar to those in the normal individuals as evidenced by the increased IgE production with PWM from days 4–7.

The in vivo role of these PWM-responsive PBL responsive to B cells remains obscure. PWM-driven IgG antibody responses appear to represent a subset of recent memory B cells arising in the blood soon after antigen exposure whereas PWM-driven IgM antibody responses may not require this stimulation. Because IgE responses in experimental animals and humans are more like IgG T-dependent responses, it is likely that the PWM IgE response also represents a form of recent or short term memory. Thus the patients as well as the normals appear to be able to generate equal "amounts" of this form of potential memory. However, there are clearly differences in the quantitative in vivo IgE responses of normals and the patients we studied who had very high serum IgE levels. This may well relate to the presence of the spontaneous IgE-secreting B cells which have been shown to arise at the time of experimentally induced antibody synthesis in other studies (7, 32) and appear to represent the dissemination of an ongoing immune response (39).

The studies reported herein also provide evidence for at least three functionally distinct populations of T lymphocytes that can regulate in vitro IgE production. One set of PBL T lymphocytes is capable of inhibiting spontaneous IgE production by B cells. These T cells were found uniformly in the normals but their activity was diminished in 7 of 13 of the experimental cultures with patients' T cells. The variable decrease in these cells' function at times from the patients' PBL would explain the variability observed by Buckley et al. (23–25) in suppression of IgE production by T cells in cultures without PWM. Our data demonstrated that the presence of this form of suppressor T activity in the patients' PBL did not correlate with the absence of the spontaneous IgE-producing B cells and vice versa. This is not surprising in that suppressor T cell activity for spontaneous IgG antitetanus activity is present simultaneously with the active B cells in PBL after experimental immunization(s). However, the loss of such suppressor T cell function may be an important reflection of events in lymph nodes or other lymphoid tissue where these lymphoblastoid cells arise and where cellular interactions must occur to regulate their production. Thus the decrease in this form of inhibitory activity in vitro may have important implications for the development of the enhanced IgE in vivo. We do know that normals will not continually regenerate antigen-specific IgG lymphoblastoid cells after repeated immunizations (unpublished observation). Some patients with elevated IgE levels appear to possess isotype E-specific B lymphoblastoid cells in the blood a large proportion of the time. Failure to normally suppress their development could be secondary to loss of the appropriate inhibiting T cell subset.

As in normals, the patients could be demonstrated to have both helper and suppressor T cells that would regulate PWM-induced IgE production. Furthermore, the activity of these separable T cell effects were equivalent in both normals and patients. These results can be understood in the light of our definition of the PWM system as it relates to recent antigen exposure. The PWM-responsive T helper and T suppressor cell subsets arise after the main production of the antibody response in vivo (15) and appear to relate to regulation of some form of possible short term memory. Thus normal activity of these cells would not affect the initial IgE antibody response in the hyper-IgE patients.

The suppressor T cells for PWM-driven IgE synthesis are functionally distinct from the T suppressors of spontaneous B cell IgE synthesis. This was evidenced by the fact that on seven occasions, PBL from patients with hyper-IgE failed to possess the latter type of suppressors but possessed the former.

Thus we have been able to define at least two functional B cell and at least three functional T cell populations that contribute to IgE production in vitro with human PBL (Table V). The possible role of each one of these subsets has to be taken into account when analyzing data on in vitro IgE synthesis. Furthermore, it must be appreciated that under varying in vitro culture conditions, expression of the activity of these B and T cell subpopulation may vary widely.

Definition of the exact in vivo role of these subsets remains to be determined as does the effect of various therapeutic modalities, such as immunotherapy (de-

sensitization) and steroids. However, studies in a number of laboratories have been rapidly defining these parameters for immunoglobulin and antibodies other than IgE. These observations promise to provide important insights that can be extended to the IgE system. Similarly, use of the in vitro IgE model to dissect the mechanisms in the altered immune (immune deficiency) state leading to increased IgE responses in vivo should yield information regarding both disease pathophysiology and normal basic immune homeostasis.

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