

***In vitro* stimulation of IgE production at a single precursor level by human alloreactive T helper clones**

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SUMMARY

Human alloreactive proliferating T cell clones were isolated by a combination of allogeneic stimulation and interleukin 2-dependent growth. When cultured with B cells and macrophages bearing the appropriate alloantigens, the helper clones induced the production of high levels of IgE, while in the absence of the appropriate stimulation B cells produced undetectable IgE levels. Allogeneic peripheral blood T cells did not provide any help, but rather suppressed the IgE production induced by the alloreactive clones. The development of culture conditions where the help provided by specific T cell clones is non-limiting and suppressor-cytotoxic T cells are absent allowed the set up of limiting dilution experiments to determine the frequency of the B cells activated to high rate IgE production. One in 2,200–6,000 B cells in the peripheral blood of normal donors was activated to produce measurable amounts of IgE. This experimental system will allow the clonal analysis of the IgE antibody repertoire, in different physiological and pathological conditions.

Keywords T cell clones allogeneic effect IgE

INTRODUCTION

The possibility of inducing IgE production *in vitro* by human peripheral blood mononuclear cells (PBM) has been investigated in different experimental systems with conflicting results. While there is a general agreement that PBM of atopic patients may spontaneously synthesize low amounts of IgE in culture (Fiser & Buckley, 1979; Tjio, Hull & Gleich, 1979; Saxon, Morrow & Stevens, 1980b; Romagnani *et al.*, 1980), there is still considerable debate as to whether antigens or mitogens may trigger IgE production by PBM of normal donors (Fiser & Buckley, 1979; Saxon & Stevens, 1979; Romagnani *et al.*, 1980; Nonaka *et al.*, 1981). More recently it has been reported that some IgE synthesis can be induced in normal B cells by soluble helper factors produced by T cells from patients with high serum IgE levels (Saryan, Leung & Geha, 1983; Romagnani *et al.*, 1983). Some of the above difficulties, as well as the generally low amounts of IgE produced could be explained not only by the insufficient provision of helper signals to the B cells, but also by the triggering of suppressive circuits in cultures that contain activated T cells with different functional properties. Indeed the IgE response has proved particularly sensitive to suppression, at least in the mouse system (Ishizaka, 1976; Katz, 1978).

In the present report we have investigated the possibility of inducing a polyclonal IgE response using alloreactive T cells. Although it is known that high levels of IgE can be produced *in vivo* during an acute graft versus host reaction (Geha *et al.*, 1980; Ringdén, Persson & Johansson, 1983),

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previous reports showed that co-culture of allogeneic PBM results in an actual suppression of IgE production (Fiser & Buckley, 1979; Saxon *et al.*, 1980b; Geha *et al.*, 1981). The recently developed technology of long term culture of specific T cells (reviewed by Paul, Sredni & Schwartz, 1981) allows the development of strictly defined culture conditions where optimal help is provided by specific helper T cell clones in the absence of any activated suppressor-cytotoxic T cell (Lanzavecchia, Ferrarini & Celada, 1982).

In this paper we demonstrate that when B cells of normal donors are cultured with specific alloreactive T cell clones, high levels of IgE are produced. Furthermore, limiting dilution experiments suggest, that the IgE production induced by alloreactive clones results from the activation of single B cell precursors to proliferation and differentiation into clones producing nanogram amounts of IgE.

MATERIALS AND METHODS

Cell separation. PBM were isolated from heparinized venous blood of healthy donors by Ficoll-Hypaque density centrifugation. When used as a source of stimulator cells PBM were irradiated with 2,500 rad from a ^{50}Co source. PBM were rosetted with neuraminidase treated sheep erythrocytes and T and non-T cells were separated on Ficoll-Hypaque as described (Lanzavecchia *et al.*, 1982). Non-T cells contained approximately 50% B cells as detected by a direct immunofluorescence staining with a fluorescein labelled goat anti-human F(ab')₂ antiserum.

Media and supernatants. The medium used throughout was RPMI 1640 (Flow Laboratories, Irvine, Scotland) supplemented with 2 mM L-glutamine, 1% non-essential amino acids, 1% pyruvate, 50 µg/ml gentamycin (Flow), 5×10^{-5} M 2-mercaptoethanol and 15% fetal calf serum (FCS).

To support the growth of alloreactive T cell clones a supernatant of phytohaemagglutinin activated tonsil cells (PHA-SUP) containing high levels of interleukin 2 (IL-2) was prepared as previously described (Lanzavecchia *et al.*, 1982).

Isolation of alloreactive T cell clones. Peripheral blood T cells were stimulated in a primary MLR with irradiated allogeneic PBM. After 6 days the T blasts (Tb) were isolated on percoll gradients (Lanzavecchia *et al.*, 1983) and restimulated in secondary MLR with the original stimulator. After 4 days the Tb so obtained were cloned by limiting dilution at 0.3 cells/well in U bottom 96 well microplates containing 3×10^4 irradiated PBM from the original stimulator and 20% PHA-SUP. After 2 weeks the culture wells showing visible cell growth were transferred to 24 well flat bottom Costar plates and expanded in the presence of irradiated stimulator cells and PHA-SUP. When a sufficient cell number was available, the Tb were washed and tested for their capacity to proliferate in response to the original stimulator. Tb (5×10^4 /ml) were cultured with irradiated non-T cells (2×10^5 /ml) in 0.2 ml microcultures in the absence of exogenous IL-2. After 48 h the cultures were pulsed with 0.5 µCi ^3H -thymidine (Amersham, specific activity 5 Ci/mM), harvested after an additional 16 h and the radioactivity incorporated counted by liquid scintillation.

Quantitation of IgE. IgE levels were measured with an enzyme immunoassay developed using as a solid phase a monoclonal antibody (E-45, kindly provided by Dr G. Damiani, Department of Biochemistry, University of Genova) and as a second antibody a conventional antiserum obtained in rabbits by immunization with purified myeloma IgE and absorbed extensively with cord serum and purified immunoglobulins. Briefly, polystyrene microplates (Greiner) were coated with 20 µg/ml purified E-45 in 20 mM phosphate buffer, pH 7.5, overnight. After washing the culture supernatants and standard IgE diluted in PBS containing 5% FCS, were added for 2 h at room temperature. The plates were washed again and rabbit anti-human IgE (1/5,000 in PBS-FCS) was added for 1 h, followed by a goat anti-rabbit antibody coupled to alkaline phosphatase. After a final wash the enzyme activity bound to the wells was measured using 1 mg/ml *p*-nitrophenylphosphate (Sigma Chemicals, St Louis, Missouri, USA) in 10% diethanolamine buffer pH 9.8. The absorbance was read with an automated photometer (Flow). A standard curve was constructed using a standard serum as well as purified IgE myeloma protein. The sensitivity of the assay was 0.3 ng/ml IgE. Purified IgM, IgG and IgA (10 µg/ml) were not detected.

Helper assay for IgE production. Non-T cells (2×10^5 /ml) were cultured with alloreactive Tb

(5×10^4 /ml) in 1.5 ml RPMI FCS in flat bottom Costar plates (macrocultures). After 12 days the supernatants were harvested and tested for total IgE levels. To determine the frequency of cells producing IgE amongst total B cells, limiting numbers of surface Ig positive B cells were stimulated in 150 μ l volumes in U bottom microplates by an alloreactive clone (2×10^4 /ml) in the presence of irradiated autologous PBM (3×10^5 /ml) as a source of stimulator cells. Sixty replicate microcultures were set up for any number of B cells tested and total IgE were measured in any culture supernatant after 12 days.

Limiting dilution analysis. The logarithm of the fraction of IgE negative cultures was plotted against the number of B cells per culture. A straight line was fitted through the origin by the least square method. The single hit kinetics was tested by the chi squared method as described by Lefkovits & Waldmann (1979). The frequency of cells producing IgE was calculated from the 37% interpolation of the regression line which, according to the Poisson distribution, indicates the number of cells containing a single precursor.

RESULTS

Alloreactive proliferating T cell clones were isolated by limiting dilution from a secondary MLR between two HLA incompatible donors. Twelve clones were obtained with a cloning efficiency of 60%. Out of these, six were able to proliferate when cultured with irradiated PBM from the original stimulator, in the absence of exogenous IL-2. Two clones proliferated only in response to the original stimulator, but four recognized alloantigens coded by a single parental haplotype of the stimulator, which could also be found on some unrelated healthy donors and were thus selected for further experiments. The specificity of the clones will be reported elsewhere.

Alloreactive Tb were cultured with non-T cells from the original stimulator or from unrelated normal donors possessing the relevant alloantigens. Cell proliferation was measured after 40 h and total IgE levels were determined in the 12 day culture supernatant. A representative experiment

Table 1. Stimulation of IgE synthesis by alloreactive T cell clones

Cells in culture*		³ H-thymidine incorporation† (ct/min)	IgE produced‡ (ng/ml)
Tb (clone)	Non-T cells (donor)		
MA-1	A	42,600	12.5 ± 2
MA-1	F	41,500	10 ± 2
MA-1	P	39,200	28.0 ± 2
MA-1	M	120	< 0.5
MA-1	A(Rx)§	31,200	< 0.5
MA-8	A	31,950	20.0 ± 2.5
MA-8	F	33,500	45.5 ± 4
—	A	50	< 0.5
—	F	80	< 0.5
—	P	110	< 0.5

* Cloned alloreactive Tb from donor M anti-A (5×10^4 /ml) were cultured with non-T cells (2×10^5 /ml) from donors A, F and P possessing the relevant alloantigens or from donor M (control).

† Thymidine incorporation was measured in microcultures after 48 h.

‡ IgE levels were measured in the 12 day supernatant of macrocultures. Data represent mean of triplicate determinations.

§ Non-T cells were γ -irradiated (2,500 rad).

Table 2. Inhibition of IgE production by allogeneic peripheral blood T cells

Cells in culture*		
T cells (donor M)	Non-T cells (donor)	IgE produced (ng/ml)†
<i>Expt 1</i>		
—	A	< 0.5
PB-T	A	< 0.5
MA-1	A	33.8 ± 4
MA-1 + PB-T	A	2.0 ± 1.5
<i>Expt 2</i>		
—	A	< 0.5
PB-T	A	< 0.5
MA-2	A	13.5 ± 2.5
MA-2 + PB-T	A	4.2 ± 1.5

* PB-T from donor M (10^6 /ml) or alloreactive Tb from clone MA-1 (5×10^4 /ml) were cultured with non-T cells from donor A.

† Total IgE levels were measured as in Table 1.

reported in Table 1 shows that, when allorecognition occurred, IgE production was also induced by the alloreactive clones. Appropriate controls showed that IgE were not produced spontaneously by non-T cells or when these were γ -irradiated, indicating that IgE production was radiosensitive and required B cell proliferation.

We next investigated whether also peripheral blood T cells (PB-T) could trigger IgE production. Notably, PB-T not only failed to stimulate IgE production, but suppressed IgE production when co-cultured with non-T cells and a helper T cell clone (see Table 2). These data indicate that peripheral blood T cells may contain, in addition to alloreactive helper T cells, also suppressor cytotoxic precursors that inhibit the IgE response.

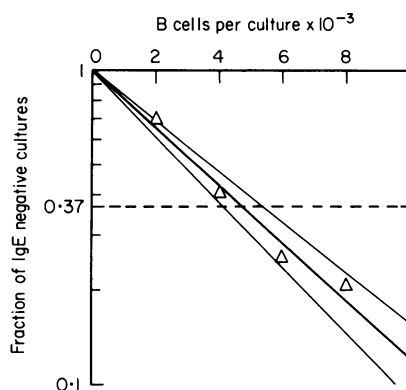


Fig. 1. Limiting dilution analysis for the frequency determination of the B cells activated to IgE production by alloreactive T cell clones. Graded numbers of B cells (donor A) were stimulated in the presence of autologous irradiated PBM by clone MA 1 in 60 replicate cultures. After 12 days the supernatant of each culture was tested for the presence of IgE. The natural logarithm of the fraction of IgE negative cultures is plotted against the number of B cells. The 37% interpolation of the slope indicates a precursor frequency of one in 4,700 B cells (1 in 4,200–1 in 5,400, 95% confidence limits).

Table 3. Frequency of B cells activated to high-rate IgE production by alloreactive clones

Tb (clone)	B cells (donor)	Frequency of IgE producing B cells*
MA-1	A†	1 in 4,500; 1 in 3,500; 1 in 5,000
MA-1	T	1 in 6,000
MA-8	F†	1 in 4,500; 1 in 2,500
MA-8	P	1 in 2,200

* Determined in plots of the logarithm of the fraction of non-responding cultures against the number of SIg⁺ B cells as the number of B cells yielding 37% non-responding cultures.

† Determined in separate experiments over a period of 4 months.

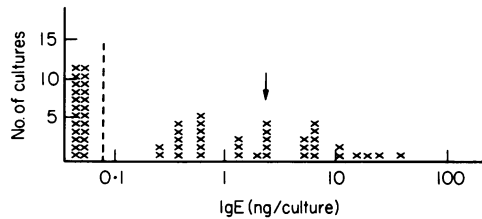


Fig. 2. Amount of IgE produced by single activated B cell precursors. B cells were stimulated in limiting dilution condition as in Fig. 1. The amount of IgE produced in 60 replicate cultures is indicated. At this B cell input the positive cultures, which statistically contained a single IgE B cell precursor, produced from 0.2 to 40 ng IgE. The arrow shows the median value for positive cultures (2.5 ng). The dotted line indicates the sensitivity of the assay.

To investigate whether the IgE produced result from the activation of single B cells to proliferation and differentiation into a clone producing measurable amounts of IgE, limiting numbers of B cells were stimulated in 60 replicate microcultures by alloreactive T cell clones. The results of a representative experiment reported in Fig. 1 demonstrate that cultures producing detectable amounts of IgE were found with a frequency that increased with the number of B cells cultured. By plotting the number of B cells per culture versus the fraction of IgE negative cultures straight lines were obtained indicating that a single B cell was limiting for IgE production. According to the Poisson statistics the precursor frequency could be calculated from the 37% interpolation of the regression line. The frequency of B cells activated to high-rate IgE production in the peripheral blood of four normal donors is summarized in Table 3 and varied from 1 in 2,200 to 1 in 6,000 B cells. The amount of IgE produced by single activated B cell precursor is shown in Fig. 2. In this experiment a culture in which only a single IgE precursor was calculated to be activated produced from 0.2 up to 40 ng of IgE (median 2.5 ng).

DISCUSSION

The present experiments demonstrate that B lymphocytes from normal donors can be activated to high rate IgE production by alloreactive helper T cells. The amounts of IgE produced in the present experiments are much higher (at least 100 times on a per B cell basis) than reported in previous publications using PWM, antigen or T cell supernatants for stimulation (Fiser & Buckley, 1979;

Saxon, Kaplan & Stevens, 1980a; Nonaka *et al.*, 1981; Sampson & Buckley, 1981; Zuraw *et al.*, 1981; Saryan *et al.*, 1983; Romagnani *et al.*, 1983). Furthermore in cultures stimulated by alloreactive T helper clones it was found that the IgE production results primarily from the *in vitro* activation of a low number of B cells to proliferation and differentiation into clones producing measurable amounts of IgE.

Several reports have demonstrated that T cells can activate B cells to antibody production as a consequence of allorecognition (reviewed by Katz, 1977 and Chiorazzi, Fu & Kunkel, 1979). On the other hand the allogeneic stimulation *in vitro* by peripheral blood T cells has generally failed to activate IgE production as demonstrated by Fiser & Buckley (1979) and also by the present experiments. It is likely that some of these difficulties as well as the general inefficiency in the activation of IgE responses using mitogens could be explained by the activation of suppressive T cells in cultures containing a mixture of T cells with different functional properties (Brenner & Munro, 1981). This is also clearly demonstrated by the fact that the IgE production induced by an alloreactive helper clone was inhibited by the addition of allogeneic peripheral blood T cells. The nature of this inhibition is not clear. Although it is not possible to formally rule out an overcrowding effect of the PB-T, recent data from our laboratory indicate that the alloreactive clones provide help also for the activation of CTL precursors (manuscript in preparation). This fact may suggest that one possible mechanism for the inhibition of IgE production is the induction of specific cytotoxic T cells. Indeed allogeneic PB-T are far more effective as inhibitors than PB-T autologous to the responder B cells (data not shown). Experiments are in progress in order to characterize the mode of the suppression of IgE production by T cells. Thus the cloning of alloreactive helper T cells is required in order to obtain culture condition devoid of activated suppressor cytotoxic T cells.

The nature of the B cells triggered to IgE production by alloreactive clones is not yet clear. Previous findings in the mouse system (Katz, 1977) as well as more recent experiments using human T cell clones (Lanzavecchia *et al.*, submitted for publication) suggest that the allogeneic stimulation may preferentially trigger a pool of memory B cells. It will be therefore particularly important to know whether the IgE production in cultures stimulated by alloreactive clones results from a switch in B cells previously producing another isotype or from the activation of B cells that were already committed *in vivo* to IgE production. By depletion experiments it has been recently demonstrated that the spontaneous IgE production *in vitro* by atopic patients is due to surface IgE⁺, IgM⁻ B cells (Romagnani *et al.*, 1982), whereas PWM inducible, IgE producing B cells have both surface IgM and IgE (Saxon *et al.*, 1980a). Cell separation experiments according to surface markers, as well as the analysis of the specificity of the IgE antibody produced will be instrumental to clarify this issue and are currently underway in our laboratory.

On the whole the experiments reported suggest that alloreactive clones can be used as a tool for the activation and the clonal analysis of the IgE antibody repertoire in healthy and atopic donors.

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