

Peripheral blood based T cell-containing and T cell-depleted culture systems for human IgE synthesis: the role of T cells

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Summary

Background Comparable T cell-containing and T cell-depleted culture systems for human IgE synthesis are currently not available.

Objective This has prompted us to develop peripheral blood mononuclear cell (PBMC) based culture systems for human IgE synthesis in the presence and absence of T cells.

Methods In this paper we describe simplified conditions for *in vitro* synthesis of high levels of IgE by human peripheral blood B cells, both in T cell-containing cultures and in anti-CD40 stimulated T cell-depleted cultures.

Results T cell-depleted cultures released approximately 20 times more IgE [range 410–2220 ng/mL (mean 1270 ng/mL); based on six experiments using cells from three donors] than did T cell-containing cultures [range 23–105 ng/mL (mean 58 ng/mL); based on 15 experiments using cells from three donors]. Reconstitution experiments were performed to investigate the role of T cells on IgE synthesis. Adding T cells back to the anti-CD40 stimulated T cell-depleted cultures resulted in a dose-dependent inhibition of IgE production. In the absence of anti-CD40 low numbers of T cells stimulated, while high numbers suppressed, IgE production: the optimal ratio of T cells to non-T cells for maximal IgE production was found to be 1:1. At this ratio, irradiated (non-replicating) T cells supported a much greater IgE synthesis than did non-irradiated T cells.

Conclusion The development of these systems provides directly comparable T cell-containing and T cell-depleted cultures for human IgE synthesis from peripheral blood, allowing further study of the role of T cells in IgE regulation. These systems will also be of use for determining whether potential modulators of IgE synthesis act on the T cells or on other cell types.

Keywords: allergy, CD40, IgE, IL-4, PBMCs, T cell, TH₁, TH₂

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Introduction

In vitro studies of peripheral blood mononuclear cells (PBMCs) have identified a requirement for at least two

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molecular signals for selective switching to IgE synthesis [1]. The first is IL-4, a 'TH₂-type' cytokine (also released by mast cells and eosinophils) which directs recombination to the constant region of the ϵ -heavy chain [2–3]. This activity has been shown to be down-regulated by interferon (IFN)- γ , a 'TH₁-type' cytokine that is also produced by NK cells [4]. The second signal is CD40

ligand (CD40L or gp39), which is found on activated T cells and many other cell types. The gp39 molecule acts as the counterstructure for CD40 on B cells, and induces IgE producing cells to become plasma cells [5–7]. Such contact-mediated help, delivered by the T cell to the B cell, can be simulated by using an anti-CD40 monoclonal antibody in culture systems containing highly purified human B cells [8].

Given that overproduction of IgE by B lymphocytes plays a key role in the development of allergic hypersensitivity [1], considerable efforts are being made to develop ways of controlling IgE antibody responses. As part of these efforts, it is important to establish whether potential pharmacologic agents (e.g. Cyclosporin A) are acting on T cells or not. Current systems for T cell-dependent and T cell-independent IgE synthesis rely on using PBMCs and tonsillar B cells respectively. Given the diverse cell sources, the two culture systems are not directly comparable and it is not always possible to obtain both populations from the same donor. Infections within the tonsils, leading to high background levels of IFN γ , also make this tissue unsuitable for studying the cellular events leading to immunoglobulin synthesis. This has prompted us to develop peripheral blood mononuclear cell (PBMC) based culture systems for human IgE synthesis in the presence and absence of T cells.

In this paper we describe culture conditions for IgE synthesis with or without T cells. The availability of these two systems has permitted further characterization of the role of peripheral blood T cells in IgE regulation.

Materials and methods

Antibody reagents

Anti-CD40 (G28.5) monoclonal antibody (reactive with CD40 on human resting B cells) was a kind gift from Dr J. Ledbetter (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, USA). The following monoclonal antibodies (MoAbs), which were purchased from Becton Dickinson (Cowley, UK), were used for phenotyping of cells: LeucoGATE (CD45/CD14), Control (g1/g2a), Leu-4/11c + 19 (CD3/CD16 + CD56), Leu-4/12 (CD3/CD19), anti-HLA-DR and anti-CD25 (anti-IL-2R). Anti-IL-4 monoclonal antibody was purchased from Genzyme (West Malling, UK).

Cell culture reagents

Iscove's Modified Dulbecco's Medium (IMDM) (JRH Biosciences, Crawley Down, UK) was supplemented with 10% fetal calf serum (FCS) (Sera Lab, Crawley Down, UK), 2 mM glutamine, 50 U/mL penicillin and

50 μ g/mL streptomycin. Cell culture plates (96 well, U-shaped) were purchased from ICN (Flow) Biomedicals (High Wycombe, UK).

Cytokines and ELISAs

Recombinant human IL-4 was obtained from Genzyme (West Malling, UK) and the Milenia IgE kinetic EIA kits were purchased from Diagnostic Products Corporation (Caernarfon, UK).

Other reagents

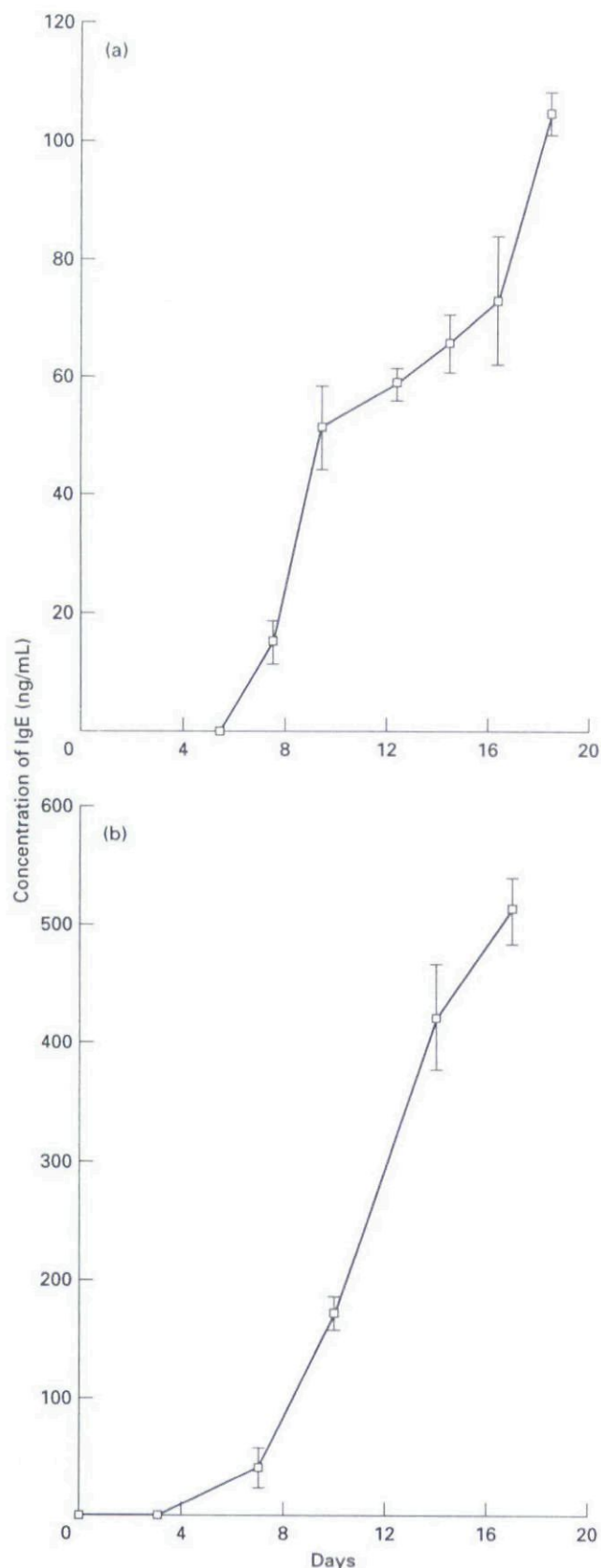
Human holo-transferrin (Tf) and 2-aminoethylisothiouonium bromide (AET) were both purchased from Sigma (Poole, UK). J-prep and sheep red blood cells (SRBCs) were purchased from Techgen International Ltd (London, UK) and TCS Biologicals Ltd (Botolph Claydon, UK) respectively. Recombinant gp39 was kindly provided by Dr G. Le Gros (Ciba-Geigy, Basel, Switzerland).

Cells

Blood specimens were obtained from human volunteers with informed consent and following approval by the Medical School Ethical Committee. PBMCs were isolated from the heparinised blood of non-atopic donors from the Department of Immunology (University Hospital, Nottingham) by buoyant density centrifugation over J-prep (1600 g/20 min). PBMC preparations usually contained 7–14% B cells 51–58% T cells, 13–15% monocytes, 6–15% NK and 5–14% other cells, as judged by FACS analysis (FACScan, Becton Dickinson, Cowley, UK) using anti-CD19, anti-CD3, anti-CD14 and anti-CD16/56 MoAbs respectively.

For IL-4/anti-CD40 stimulated T cell-depleted cultures, T cells were removed from PBMC preparations by two cycles of rosetting with AET-treated sheep erythrocytes [9], followed by centrifugation on J-prep. The non-rosetting cells (referred to as non-T cells) collected from the interface of the second J-prep step (1600 g/20 min) typically contained 20–21% B cells, <0.1% CD3⁺ T cells, 43–52% monocytes, 8–12% NK cells and 15–19% other cells, as determined by FACS analysis using anti-CD19, anti-CD3, anti-CD14 and anti-CD56 MoAbs respectively.

T cells used in the reconstitution experiments were recovered from the pelleted SRBC-T cell complexes following lysis of the erythrocytes with isotonic NH₄Cl. Such preparations typically contained more than 88% CD3⁺ cells, with the remaining cells being of the NK lineage. Irradiation of T cells (to stop replication) was carried out by exposing them to a total of 4500 rads (i.e.



900 rads for 5 min) from a Gammacell Cesium-137 irradiator (Nordon International, Inc., Ontario, Canada).

In vitro culture systems for IgE synthesis

IL-4 stimulated T cell-containing cultures Optimum IgE production was observed when PBMCs (3×10^5 cells/well; 200 μ L/well) were cultured in IMDM/10% FCS [10], containing IL-4 (200 U/mL) and Tf (50 μ g/mL). The cells were cultured in U-shaped microtitre wells in a 5% CO₂ incubator at 37°C for 17 days, supernatants harvested and tested for IgE production.

IL-4/Anti-CD40 stimulated T cell-depleted cultures In the T cell-depleted cultures, optimum IgE production was observed when non-T cells (5×10^4 cells/well; 200 μ L/well) were cultured in IMDM/10% FCS, containing IL-4 (200 U/mL), Tf (50 μ g/mL) and anti-CD40 (1 μ g/mL). The cells were cultured in U-shaped microtitre wells in a 5% CO₂ incubator at 37°C for 17 days, supernatants harvested and tested for IgE production.

Detection of IgE

IgE was measured according to the manufacturer's protocol using the Milenia total IgE kinetic EIA. The lower detection limit of this assay, fully validated in our laboratory [R. Jones, personal communication], was 0.2 IU/mL (0.48 ng/mL). Briefly, supernatants were added to biotin coated wells, along with biotin-labelled anti-IgE. During the initial reaction, endogenous IgE reacted with anti-IgE in the liquid phase. During a second incubation, the addition of a multivalent anti-ligand (avidin) forms a bridge between the IgE/anti-IgE complexes and the biotin coated wells. The plate was then washed and the complexes reacted with horseradish peroxidase (HRP)-labelled monoclonal anti-IgE antibodies. TMB (3,3',5,5'-tetramethylbenzidine) — a substrate reactive with HRP — was added and the rate of colour development was measured at 650 nm.

Fig. 1. (a) Typical time course of IgE production by PBMCs from SJS, a non-atopic female. Cells were cultured (3×10^5 /well in 200 μ L IMDM/10% FCS) with IL-4 (200 U/mL) and Tf (50 μ g/mL). Cultures were terminated at 3–4 day intervals and supernatants were harvested and tested for IgE. Results are expressed as means $\pm$ SEM of duplicate culture wells. (b) Typical time course of IgE production by PBMCs depleted of T cells. Cells were cultured (5×10^4 /well in 200 μ L IMDM/10% FCS) with IL-4 (200 U/mL), Tf (50 μ g/mL) and anti-CD40 (1 μ g/mL). Cultures were terminated at 3–4 day intervals and supernatants were harvested and tested for IgE. Results are expressed as means $\pm$ SEM of triplicate culture wells.

Statistical analysis

Student's unpaired *t*-test was used to assess the statistical significance of differences in levels of IgE synthesis.

Results

Time course of IgE synthesis

In order to determine the stage at which cultures should be terminated, parallel culture systems were performed and terminated at 2–3 day intervals. Supernatants from terminated cultures were stored at -20°C for analysis of IgE levels. Typical time courses of IgE production from both IL-4 stimulated T cell-containing, and IL-4/anti-CD40 stimulated T cell-depleted IgE cultures, are shown in Figs 1a and 1b respectively. When terminated after 17 days, T cell-depleted cultures released approximately 20-fold more IgE [range 410–2220 ng/mL (mean 1270 ng/mL); based on six experiments using cells from three donors] than did T cell-containing cultures [range 23–105 ng/mL (mean 58 ng/mL); based on 15 experiments using cells from three donors]. Individual IgE values are shown in Table 1.

Requirements for in vitro IgE synthesis

In order to determine the importance of IL-4, Tf (an iron transport protein) and anti-CD40 in the T cell-depleted system, cultures with or without these reagents were set up and terminated after 17 days for IgE determination. As expected, IgE production in this system was IL-4, Tf and anti-CD40 dependent (Fig. 2a). Similar results were obtained with the T cell-containing culture system, again

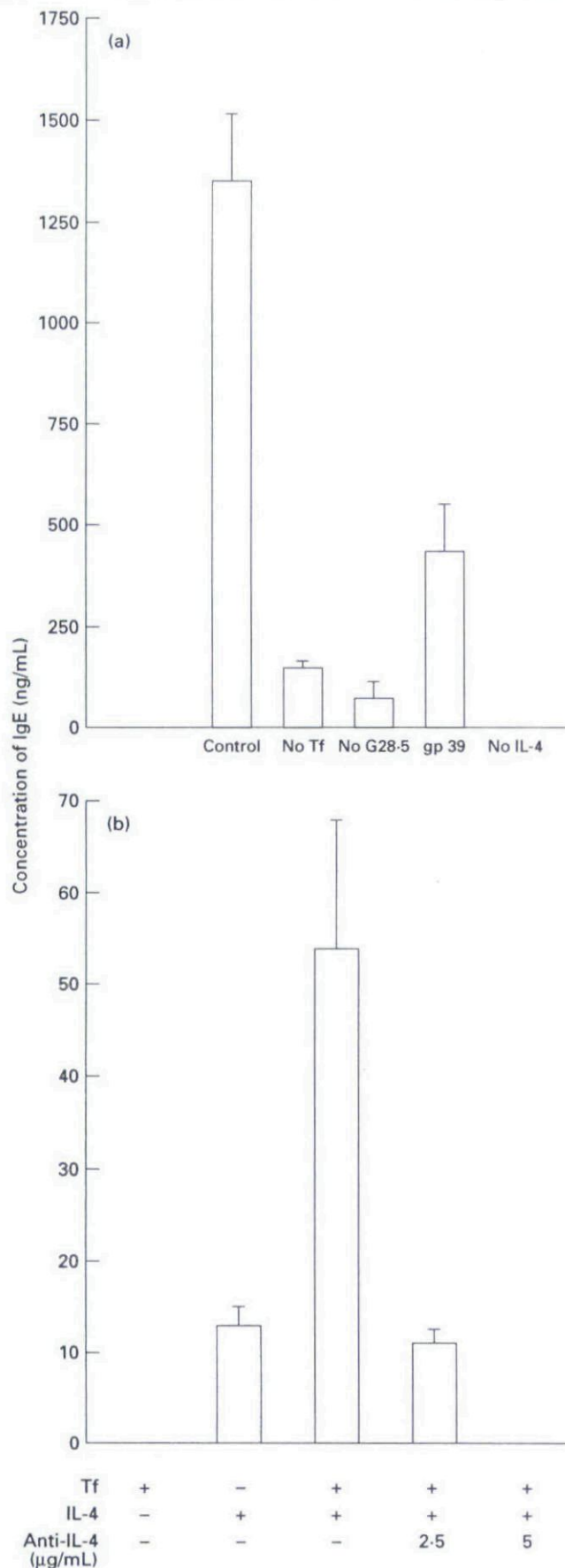
demonstrating the requirement for IL-4 (as shown when not added or blocked by anti-IL-4 antibody) and Tf (Fig. 2b). High levels of IgE could also be detected in cultures stimulated with recombinant CD40 ligand (gp39 or CD40L) instead of anti-CD40 antibody.

Role of T cells in in vitro IgE synthesis

The IL-4 stimulated T cell-containing IgE culture system described above is not dependent on activating the T cells with anti-CD3 antibody [11,12]. However, a proportion (6–8%) of the T cell population in this culture system appeared to be endogenously activated, as determined by FACS analysis using anti-CD25 or anti-HLA-DR MoAbs. As T cell-depleted cultures released approximately 20 times more IgE than did T cell-containing cultures, we investigated how T cells influenced the latter system. Reconstitution experiments were performed, adding T cells back to T cell-depleted cultures. This has resulted in a dose-related inhibition of IgE production (Fig. 3). Further experiments were performed, adding back control or irradiated (4500 rads) T cells to the T cell-depleted cultures (Figs 4a and b, respectively). These experiments demonstrated that a low T cell/non-T cell ratio (1:1) in the absence of anti-CD40 favoured enhanced IgE production in comparison to cultures containing less than 0.1% T cells. However, at higher T cell/non-T cell ratios (4:1) IgE production was inhibited (Fig. 4). It is interesting to note that cultures that had received irradiated T cells produced on average 20-fold more IgE (compare Figs 4a and 4b); with the difference between the mean values at 1:1 T cell/non-T cell ratio being statistically significant ($P = 0.019$).

Table 1. Production of IgE by IL-4 stimulated T cell-containing and IL-4/anti-CD40 stimulated T cell-depleted cultures, using PBMC from three donors. Results are expressed at mean $\pm$ SEM of quadruplicate wells: a–f denotes paired data, i.e. T cell-containing and T cell-depleted cultures were performed on the same day using the same PBMCs

IgE concentration (ng/mL)					
T cell-containing cultures			T cell-depleted cultures		
SJS	FS	PT	SJS	FS	PT
54 $\pm$ 11.2 ^a	41 $\pm$ 4.8 ^d	23 $\pm$ 3.2 ^e	512 $\pm$ 28 ^a	1840 $\pm$ 134 ^d	410 $\pm$ 48 ^c
105 $\pm$ 3.4 ^b	84 $\pm$ 14	44 $\pm$ 4 ^f	1373 $\pm$ 140 ^b		1268 $\pm$ 156 ^f
70 $\pm$ 14 ^c	71 $\pm$ 7.5	47 $\pm$ 5.5	2217 $\pm$ 163 ^c		
88 $\pm$ 6.4		29 $\pm$ 5.3			
33 $\pm$ 4.8		28 $\pm$ 3.6			
69 $\pm$ 12					
81 $\pm$ 9					



Discussion

In this paper we describe simplified culture conditions for *in vitro* synthesis of human IgE from peripheral blood lymphocytes in the presence and absence of T cells. As expected, in the presence of T cells IgE synthesis was IL-4 dependent. Transferrin, as an iron transporting protein, was also required for optimum IgE production. The conditions for our T cell-containing culture were based on the method described by Claassen *et al.* [10] and was not dependent on activating the T cells with immobilised anti-CD3 antibody [11,12]. Indeed, under our culture conditions anti-CD3 antibody tended to suppress, rather than stimulate, IgE synthesis (unpublished data). In the system described here, IgE was produced without the addition of anti-CD3 antibody; presumably levels of CD40 ligand (gp39) expressed on endogenously activated T cells were sufficient to provide this non-cognate B cell signal. The presence of such endogenously activated T cells was confirmed by FACS analysis, using anti-CD25 or anti-HLA-DR MoAbs.

Production of IgE by PBMCs that have been depleted of T cells was dependent on both IL-4 and anti-CD40 antibody. Omission of anti-CD40 antibody from the culture resulted in severely diminished IgE production, that could be partially restored by the addition of recombinant gp39 (CD40L). Anti-CD40 antibody was more efficient than recombinant gp39 in stimulating IgE synthesis, presumably because anti-CD40, being bivalent (compared with soluble monovalent gp39), would deliver a stronger signal by cross-linking CD40 on the B cell membrane. A comparatively small amount of IgE was still being produced (0–60 ng/mL) when T cells and anti-CD40 were absent from the culture system, thereby suggesting that B cells may have been endogenously activated. Alternatively, CD40L (gp39) transcripts have

Fig. 2. (a) In order to determine the importance of IL-4, Tf and anti-CD40 for IgE synthesis in the T cell-depleted system, cultures with or without these reagents were established. The results demonstrate that IL-4, anti-CD40 (G28.5) and Tf are necessary for optimal *in vitro* synthesis of IgE from PBMCs depleted of T cells. The gp39 (CD40L) molecule can partially replace the need for anti-CD40 in this system, and IgE production is fully dependent on IL-4. (b) Likewise, T cell-containing cultures were established with or without IL-4 or Tf. The results demonstrate that IL-4 and Tf are necessary for optimal *in vitro* synthesis of IgE from PBMCs. IgE production is again fully dependent on IL-4 as IgE is undetectable in the absence of this cytokine. Furthermore, IL-4 activity and IgE production can be inhibited by the addition of an anti-IL-4 antibody. In both figures, results are expressed as means $\pm$ SEM of quadruplicate culture wells, using blood donated by SJS, a non-atopic female.

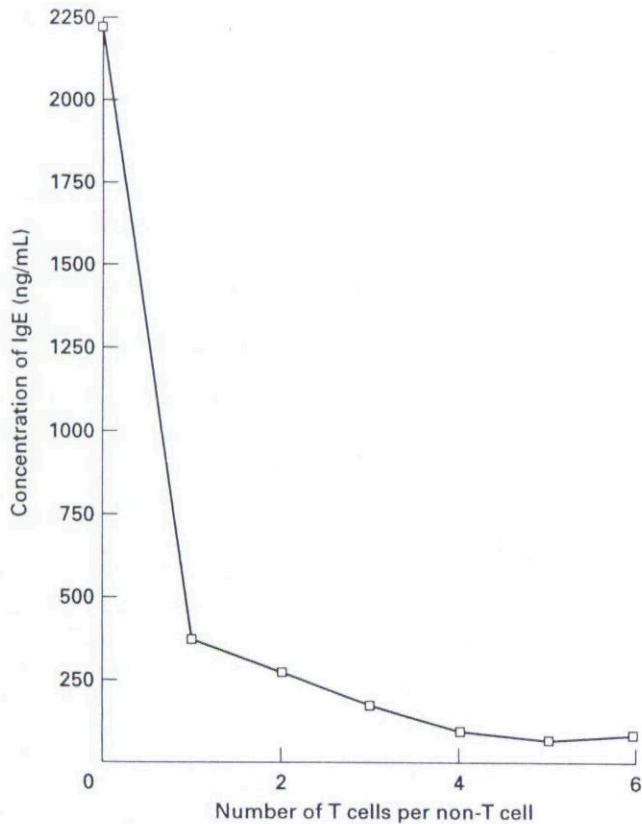


Fig. 3. Addition of T cells to T cell-depleted cultures results in a dose-dependent inhibition of IgE synthesis. Results are based on measuring IgE concentration in five pooled culture wells using blood donated by SJS, a non-atopic female.

been detected in human blood basophils, natural killer cells (NK) and monocytes [13] and it was possible that these cells provide the cultured B cells with low levels of non-cognate help. Indeed, freshly isolated human blood basophils have been shown to express CD40L and to induce IgE production [14].

As T cell-depleted cultures released on average 20 times more IgE (range 410–2220 ng/mL; mean 1270 ng/mL) than did T cell-containing cultures (range 23–105 ng/mL; mean 58 ng/mL), this suggested that T cells were down-regulating IgE production. We used reconstitution experiments to investigate how T cells influenced

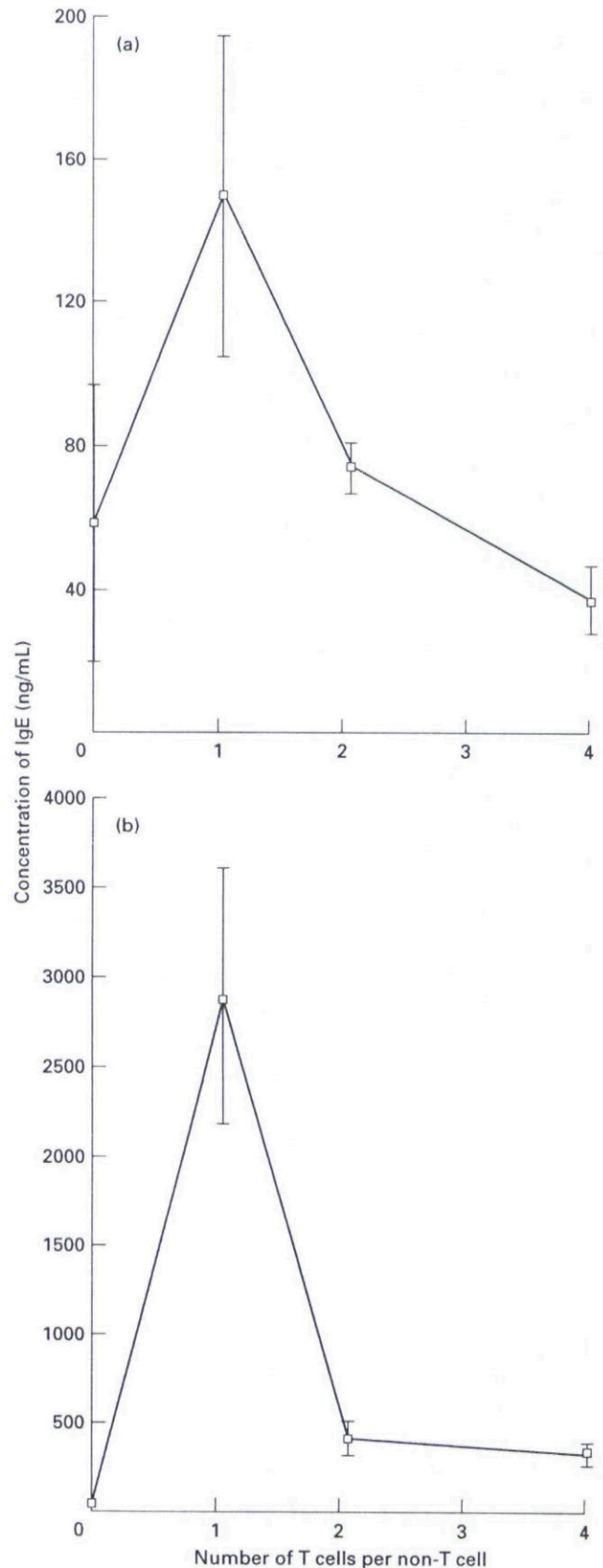


Fig. 4. Effects of adding T cells (a) or irradiated (4500 rads) T cells (b) to T cell-depleted cultures that were not stimulated with anti-CD40. Low T cell/non-T cell ratio (1:1) favoured enhanced IgE production in comparison to cultures containing no T cells. Higher T cell/non-T cell ratios inhibited IgE production. Irradiated T cells stimulated a much stronger IgE response than non-irradiated T cells. Results are expressed as mean $\pm$ SEM of quadruplicate culture wells using blood donated by SJS, a non-atopic female.

the latter system. Addition of T cells to the IL-4/anti-CD40 stimulated T cell-depleted cultures resulted in a dose-dependent inhibition of IgE production. An explanation for this inhibition may be that PBMC-derived T cells are predominantly TH₁, a T helper cell subset known to suppress IgE synthesis by secreting IFN γ [4]. This would also explain why stimulating T cells with immobilized anti-CD3 antibody tended to suppress IgE synthesis.

In the absence of anti-CD40 a biphasic response was observed, with low numbers of T cells increasing, and high numbers of T cells decreasing, IgE production. The optimal ratio of T cells (untreated or irradiated) to non-T cells was found to be 1:1; increasing this ratio to 2:1 or more there was a marked inhibitory effect on IgE synthesis. Our experiments also show that at the optimal T cell/non-T cell ratio of 1:1 irradiated (non-replicating) T cells supported higher IgE synthesis than did non-irradiated T cells, which again can be explained in terms of PBMC-derived T cells being predominantly TH₁. These results are consistent with a previous report demonstrating that irradiated T cells enhanced, whilst non-irradiated T cells suppressed, IgE synthesis [15].

Use of the culture conditions described here will facilitate studies of *in vitro* IgE synthesis in a number of ways. Firstly, they will alleviate the problems of low and inconsistent quantities of IgE produced in other previously described culture systems [16,17]. Secondly, as conditions for IgE synthesis in the presence or absence of T cells are demonstrated using peripheral blood (rather than tonsillar cells used in other T-cell depleted systems), it is now possible to have directly comparable T cell-containing and T cell-depleted cultures (i.e. same cell source and reagent concentration). Furthermore, as peripheral blood is more readily obtainable than tonsils (which are often removed due to infection) donor status (i.e. atopic or non-atopic) can be more easily selected. The availability of these culture systems will therefore be of use for determining whether potential modulators of IgE synthesis act on T cells or on other cell types, as we have shown recently with Cyclosporin A [18].

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