

Effect of recombinant human erythropoietin on human IgE production *in vitro*

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SUMMARY

Recombinant human erythropoietin enhanced spontaneous IgE production (200–300% enhancement) in cultures of peripheral blood mononuclear cells (MNC) from atopic patients. In contrast, IgG and IgA production were only slightly enhanced (30–50% enhancement), and IgM production was not affected by erythropoietin. The enhancement of IgE production by erythropoietin was indirect since it required T cells and monocytes. However, erythropoietin effect was specific since enhancement was blocked by anti-erythropoietin antibody but not by control antibody. Interleukin-4 (IL-4) also enhanced spontaneous IgE production from atopic MNC. However, the enhancing effect by erythropoietin is different from that by IL-4, since the erythropoietin effect was not blocked by anti-IL-4 antibody, and conversely IL-4 effect was not blocked by anti-erythropoietin antibody. In contrast to the enhancing effect on atopic MNC, erythropoietin failed to induce IgE production in cultures of MNC from normal donors while IL-4 induced IgE production from normal MNC. However, when normal MNC were pre-incubated with IL-4, erythropoietin enhanced IgE production from IL-4-pre-incubated MNC. Moreover, B cells separated from IL-4-pre-incubated MNC produced IgE which was enhanced by erythropoietin. However, this effect required T cells and monocytes. These results indicate that erythropoietin could regulate ongoing IgE production *in vitro* by T cell- and monocyte-dependent mechanisms.

Keywords erythropoietin IgE production interleukin-4

INTRODUCTION

In vitro human IgE production is regulated by many factors. We have previously reported that ongoing IgE production by B cells could be inhibited by natural killer (NK) cells or anti-CD23 antibody (Kimata *et al.*, 1987; Sherr *et al.*, 1989). However, supernatants from NK cells or those from T cells from atopic patients enhanced IgE production by purified B cells in the absence of T cells (Sherr *et al.*, 1987; Kimata, Sherr & Saxon, 1988). In contrast, in the presence of T cells, interleukin-4 (IL-4) enhanced IgE production by B cells both from atopic patients and normal donors (del Prete *et al.*, 1988; Sarfati & Delespesse, 1988; Pène *et al.*, 1988a; Yang, de Weck & Stadler, 1988; Vercelli *et al.*, 1989). Since IL-4 failed to induce IgE production from B cells or B cell lines (Kimata & Saxon, 1988; Sherr *et al.*, 1989), the above results suggest that other cytokine(s) may play roles in IgE production *in vitro*. While we were studying the IgE regulation *in vitro*, we noticed that recombinant human erythro-

poietin enhanced spontaneous IgE production. The relationship of erythropoietin and IL-4 is discussed.

MATERIALS AND METHODS

Reagents

The following recombinant human cytokines and antibodies were kindly provided by each company or institute: erythropoietin (specific activity of 2×10^5 U/mg determined by radioimmunoassay; Miyazaki *et al.*, 1988) and rabbit anti-erythropoietin antibody (Kirin Brewery Co. Ltd., Gunma, Japan), interleukin-1 β (IL-1 β) (Otsuka Pharmaceutical Co., Tokushima, Japan), IL-2, interferon-alpha (IFN- α) and IFN- γ (Takeda Chemical Industries, Osaka, Japan), IL-4 and rabbit anti-IL-4 antibody (Ono Pharmaceutical Company, Osaka, Japan), IL-5 (Suntory Research Center, Osaka, Japan), IL-6 (kind gifts from Drs T. Hirano and T. Kishimoto, Institute for Molecular and Cellular Biology, Osaka, Japan), granulocyte-macrophage colony-stimulating factor (GM-CSF, Sumitomo Pharmaceuticals, Osaka, Japan). The culture medium was complete RPMI which consisted of RPMI 1640 (M.A. Bioproducts, Walkersville, MD) containing 10% fetal calf serum (FCS) (Irvine Scientific), 2 mM glutamine, 50 U/ml penicillin, and 50 μ g/ml streptomycin.

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Cell preparation

Donors were either atopic patients (serum IgE levels > 300 U/ml) or normal healthy donors (serum IgE levels < 50 U/ml). Heparinized peripheral blood was drawn and mononuclear cells (MNC) were prepared by Ficoll-Hypaque centrifugation, and T cells were separated by rosetting with sheep erythrocytes twice and monocytes were obtained by plastic adherence as previously described (Kimata *et al.*, 1987). This monocyte fraction contained <1% CD3⁺ T cells, <1% CD20⁺ B cells and >98% CD11b⁺ monocytes. To obtain highly purified B cells, B cell fractions were depleted of monocytes and NK cells by L-leucine methyl ester pre-incubation methods (Thiele, Kurosaka & Lipsky, 1983). Briefly, B cells were suspended at a concentration of 5×10^6 cells/ml. They were incubated with L-leucine methyl ester (Sigma Chemical Co., St Louis, MO) in concentrations of 5 mM at 22°C for 40 min. This B cell fraction contained <1% CD3⁺ T cells, <1% CD11b⁺ monocytes, <1% CD16⁺ NK cells and >98% CD20⁺ B cells. Contamination of monocytes and NK cells in T cells were also removed by L-leucine methyl ester preincubation. This T cell fraction contained <1% CD11b⁺ monocytes, <1% CD16⁺ NK cells, <1% CD20⁺ B cells and >98% CD3⁺ T cells. IL-4-pre-incubated MNC or B cells were prepared as follows: normal MNC were cultured (2×10^6 /ml) with IL-4 (200 U/ml) for 7 days. They were washed thoroughly and resuspended in fresh complete RPMI. Purified B cells were separated from IL-4-pre-incubated MNC by depletion of T cells by sheep erythrocyte rosetting twice, followed by L-leucine methyl ester incubation methods for depletion of monocytes and NK cells. This B cell fraction contained >98% CD20⁺ B cells. At the same time, T cells and monocytes were freshly separated from the same donors as above. As controls, MNC were pre-incubated with medium alone and B cells were separated as above.

Culture system

MNC (2×10^5 cells/200 μ l/well) from either atopic patients or normal donors were cultured in 96-well U-bottomed microtitre plates (Costar, Cambridge, MA). Purified B cells from atopic patients (1×10^5 cells/200 μ l/well) were cultured with or without various numbers of T cells or monocytes either separately or together. Cells were stimulated with various concentrations of erythropoietin with or without other factors. After 10 days of incubation, immunoglobulins in the supernatants were measured by ELISA (Kimata *et al.*, 1988). IL-4-pre-incubated MNC or control-medium-pre-incubated MNC (2×10^5) were cultured with erythropoietin for 7 days. IL-4-pre-incubated B cells or control-medium-pre-incubated B cells (1×10^5) were cultured with various concentrations of erythropoietin in the presence or absence of freshly separated autologous T cells (1×10^5) and monocytes (2×10^4). After 7 more days of culture, IgE was measured. Net IgE production was calculated by subtracting the values of IgE obtained in parallel cultures containing cycloheximide (50 μ g/ml).

RESULTS

Enhancement of spontaneous IgE production from atopic MNC by erythropoietin

As shown in Fig. 1, erythropoietin enhanced spontaneous IgE production from atopic MNC in a dose-dependent manner. As little as 0.01 U/ml of erythropoietin enhanced IgE production

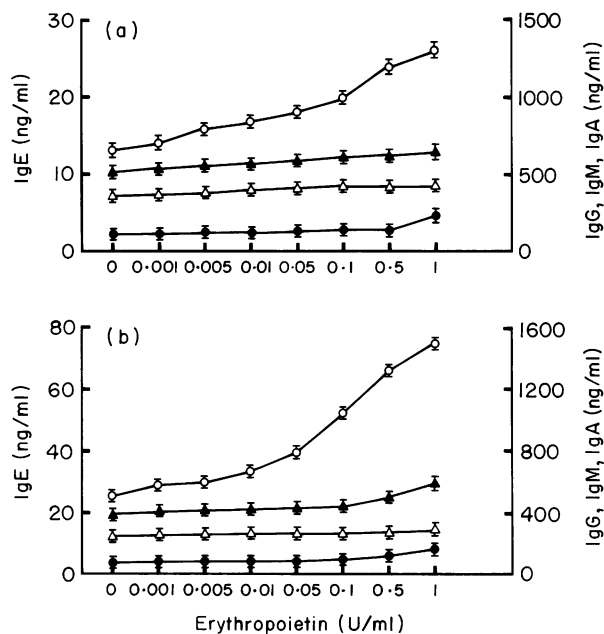


Fig. 1. Effects of erythropoietin on immunoglobulin production by atopic MNC. MNC from two atopic patients (a, b) were cultured with various concentrations of erythropoietin. Immunoglobulins were measured on day 10 of cultures. Values are the mean $\pm$ 1 s.d. from triplicate cultures. ○, IgE; ▲, IgG; △, IgM; ●, IgA.

significantly. In contrast, erythropoietin enhanced IgA (Fig. 1 a, b) and IgG (Fig. 1b) production only slightly at high concentrations, and IgM production was not enhanced by any of the concentrations of erythropoietin tested.

Requirement of T cells and monocytes for erythropoietin-induced enhancement of IgE production from atopic B cells

It has been reported that induction of IgE production by IL-4 required T cells and monocytes (Pène *et al.*, 1988a; del Prete *et al.*, 1988). Therefore, we studied whether enhancement of IgE production by erythropoietin requires T cells and monocytes. To address this question, highly purified B cells, T cells and monocytes from atopic patients were separated. As shown in Fig. 2a, erythropoietin-induced enhancement of IgE production required T cells. Maximal enhancement was observed at a 1:1 T:B cell ratio in the presence of monocytes. However, slight but significant enhancement of IgE production was also observed in the absence of monocytes. The role of monocytes was then studied. As shown in Fig. 2b, the addition of monocytes potentiated erythropoietin-induced enhancement of IgE production in a dose-dependent fashion in co-cultures of purified B cells and autologous T cells.

Erythropoietin enhances spontaneous IgE production by different mechanisms by IL-4

IL-4 is known for enhancement of IgE production from atopic MNC (Yang *et al.*, 1988). Because erythropoietin effects require T cells, it is possible that erythropoietin may stimulate T cells to produce IL-4 which in turn acts on B cells for IgE production. Thus we studied whether erythropoietin-induced IgE enhancement was mediated by IL-4. As shown in Fig. 3, erythropoietin-induced enhancement of IgE production was blocked by anti-erythropoietin antibody but not by control antibody or by anti-

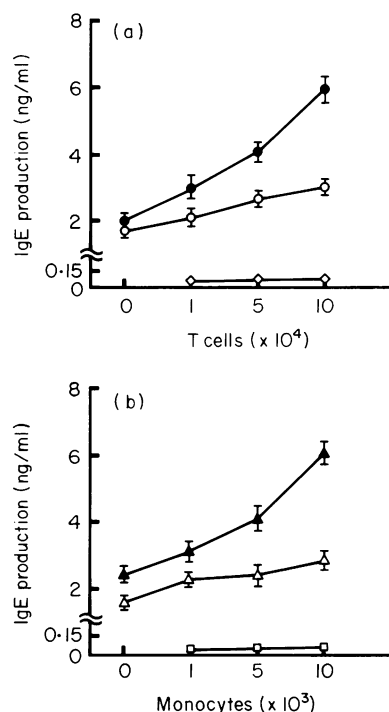


Fig. 2. Requirement of T cells and monocytes for enhancement of IgE production by erythropoietin. (a) Purified atopic B cells (1×10^5) were cultured with various numbers of autologous T cells in the absence (○) or presence (●) of monocytes (1×10^4). T cells alone (◇) were also cultured; (b) purified atopic B cells (1×10^5) were cultured with various numbers of autologous monocytes in the absence (△) or presence (▲) of T cells (1×10^5). Monocytes alone were also cultured (□). They were stimulated with erythropoietin (1 U/ml) for 10 days, and IgE was measured. Values are the mean $\pm$ 1 s.d. from triplicate cultures.

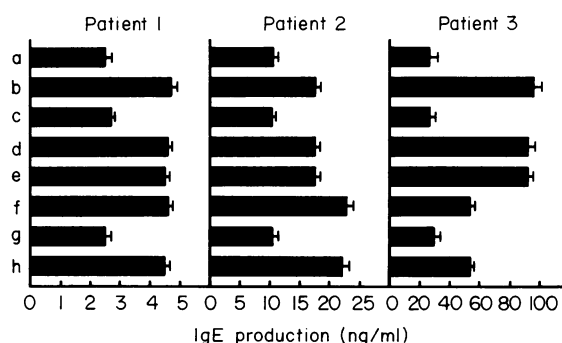


Fig. 3. Specificity of the erythropoietin effect. MNC from three atopic patients were cultured with indicated factors for 10 days, and IgE was measured. a, Medium; b, erythropoietin (1 U/ml); c, erythropoietin (1 U/ml) + anti-erythropoietin antibody (1/500); d, erythropoietin (1 U/ml) + control antibody (1/500); e, erythropoietin (1 U/ml) + anti-IL-4 (10 µg/ml); f, IL-4 (200 U/ml); g, IL-4 (200 U/ml) + anti-IL-4 antibody (10 µg/ml); h, IL-4 (200 U/ml) + anti-erythropoietin (1/500). Values are the mean $\pm$ 1 s.d. from triplicate cultures.

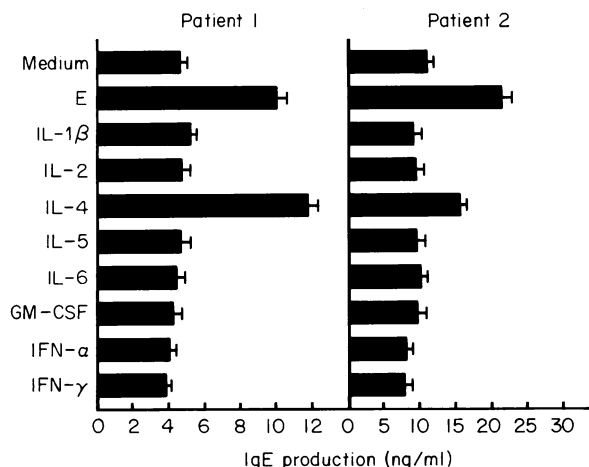


Fig. 4. Effects of various cytokines on IgE production from atopic MNC. MNC from two atopic patients were cultured with various cytokines. Erythropoietin (E) was used at 1 U/ml, IL-1β at 100 U/ml, IL-2 at 100 U/ml, IL-4 at 200 U/ml, IL-5 at 100 ng/ml, IL-6 at 100 U/ml, GM-CSF at 10 000 U/ml, IFN-α at 1000 U/ml and IFN-γ at 1000 U/ml. IgE was measured on day 10 of cultures. Values are the mean $\pm$ 1 s.d. from triplicate cultures.

IL-4 antibody. Conversely, IL-4-induced enhancement of IgE production was blocked by anti-IL-4 antibody but not by anti-erythropoietin antibody. Various cytokines were also tested in their effect on atopic MNC. While erythropoietin and IL-4 enhanced IgE production, no other cytokines, including IL-1β, IL-2, IL-5, IL-6, GM-CSF, IFN-α and IFN-γ, enhanced IgE production. For dose range of these cytokines, see legend of Fig. 4. We did test cytokine concentrations 10–1000-fold lower and these also failed to enhance IgE production (data not shown). These results indicate that the enhancement of IgE production by erythropoietin was specific and not mediated by IL-4.

Effect of erythropoietin on freshly separated MNC, IL-4-pre-incubated MNC or B cells from normal donors

We also studied the effect of erythropoietin on MNC from normal donors. Erythropoietin (0.01–1.0 U/ml) failed to induce IgE production (< 0.15 ng/ml) by freshly separated MNC from normal donors while IL-4 (200 U/ml) induced IgE production (0.7–3.0 ng/ml in three experiments). Since erythropoietin enhanced ongoing IgE production from atopic MNC, we suspected that erythropoietin might enhance IL-4-induced ongoing IgE production from normal MNC. Therefore, effect of erythropoietin on IL-4-pre-incubated MNC or B cells from normal donors was tested. As shown in Fig. 5, IL-4-pre-incubated normal MNC produced IgE, and this IgE production was enhanced by the addition of erythropoietin in a dose-dependent fashion. Similarly, purified B cells separated from IL-4-pre-incubated MNC produced small but significant IgE. Erythropoietin enhanced this IgE production in the presence but not absence of freshly separated autologous T cells and monocytes. In contrast, erythropoietin had no effect on control-medium-pre-incubated MNC or B cells even in the presence of T cells and monocytes.

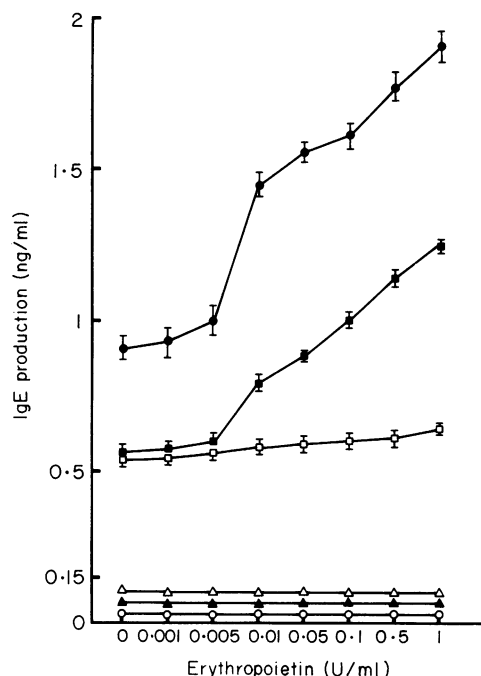


Fig. 5. Effect of erythropoietin on IL-4-preincubated MNC or B cells from normal donors. MNC (2×10^5) pre-incubated with IL-4 (●) or with medium (○) were cultured with various concentrations of erythropoietin. B cells (1×10^5) from IL-4-pre-incubated MNC were cultured with various concentrations of Epo in the absence (□) or presence (■) of freshly separated autologous T cells (1×10^5) and monocytes (2×10^4). As control, B cells from medium-pre-incubated MNC were also cultured with various concentrations of erythropoietin in the absence (Δ) or presence (▲) of T cells and monocytes as above. On day 7 of incubation, IgE was measured. Values are the mean $\pm$ 1 s.d. from triplicate cultures.

DISCUSSION

We have demonstrated that erythropoietin enhances spontaneous IgE production from atopic MNC and purified atopic B cells in the presence of T cells and monocytes. Therefore, the enhancing effect of erythropoietin on IgE production is indirect; however, the effect is specific since enhancement of IgE production was completely blocked by anti-erythropoietin antibody but not by control antibody. IL-4 has been shown to enhance IgE production from atopic MNC (Yang *et al.*, 1988). It is possible that erythropoietin may stimulate T cells to produce IL-4, and produced IL-4 acts on B cells for IgE production. However, this is unlikely, since enhancement of IgE production by erythropoietin was blocked by anti-erythropoietin antibody but not by anti-IL-4 antibody, and conversely enhancement of IgE production by IL-4 was blocked by anti-IL-4 antibody but not by anti-erythropoietin antibody. In addition to that, supernatants from erythropoietin-stimulated MNC or T cells failed to induce CD23 on purified B cells (data not shown) which was known for IL-4 assay (Defrance *et al.*, 1987). Moreover, no other cytokines including IL-1 β , IL-2, IL-5, IL-6, GM-CSF, INF- α and IFN- β enhanced IgE production. Thus erythropoietin is unique for its enhancing effect on IgE production.

In contrast to atopic MNC, erythropoietin failed to induce IgE production from normal MNC which did not produce detectable IgE. Thus mechanisms by erythropoietin and IL-4 on

IgE production by MNC from normal donors were also different. However, IL-4-pre-incubated MNC produced IgE which was enhanced by erythropoietin (Fig. 5). Similarly, B cells purified from IL-4-pre-incubated MNC produced IgE in cultures with medium alone. Erythropoietin enhanced this IgE production in the presence of freshly separated T cells and monocytes (Fig. 5). Thus, erythropoietin could enhance ongoing IgE production from B cells activated *in vivo* and *in vitro*.

Enhancement of IgE production by erythropoietin was dose dependent. As little as 0.005–0.01 U/ml of erythropoietin enhanced IgE production. Because serum erythropoietin levels were 0.01–0.03 U/ml (Goto *et al.*, 1989), this dose was physiological. However, the *in vivo* role of erythropoietin on IgE production remains to be elucidated. Although maximal enhancement of IgE production requires 0.5–1 U/ml of erythropoietin, this is not surprising. It has been recently reported that erythropoietin at relatively high concentrations acted on various target cells. For example, erythropoietin (1 U/ml) promoted growth of clonogenic myeloblastic leukaemic cells in the presence of IL-3 or GM-CSF (Asano *et al.*, 1988). Similarly, erythropoietin (1 U/ml) enhanced granulocyte/macrophage and multi-lineage colonies in the presence of IL-3 and IL-6 (Rennick *et al.*, 1989). Moreover, *in vivo* administration of erythropoietin in humans caused increase in myeloid and megakaryocytic, as well as bone marrow erythroid, progenitor cells (Geissler *et al.*, 1989). The erythropoietin gene can be detected in mouse bone-marrow-derived macrophages (Vogt, Pentz & Rich, 1989), and the receptors for erythropoietin were detected on murine placenta and megakaryocytes (Sawyer, Kranz & Sawada, 1989; Fraser *et al.*, 1989). These results indicate that erythropoietin may play many roles in immune and hematologic responses. Our reports were the first that erythropoietin enhanced ongoing IgE production. Moreover, erythropoietin also enhanced ongoing IgE production from a human IgE-producing plasma cell line. AF-10 (manuscript submitted).

It has recently been reported that IL-4-induced human IgE production was enhanced by IL-5 or IL-6 while IFN- α or IFN- γ suppressed it (Pène *et al.*, 1988a, 1988b; Vercelli *et al.*, 1989, 1990). Such cytokine interactions may exist in erythropoietin on IgE regulation. This possibility is currently under investigation.

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REFERENCES

- ASANO, Y., OKAMURA, S., SHIBUYA, T., HARADA, M. & NIHO, Y. (1988) Growth of clonogenic myeloblastic leukemic cells in the presence of human recombinant erythropoietin in addition to various human recombinant hematopoietic growth factors. *Blood*, **72**, 1682.
- DEFRANCE, T., AUBRY, J.P., ROUSET, F., VANBERVLIET, B., BONNEFOY, J.Y., ARAI, N., TAKEBE, Y., YOKOTA, Y., LEE, F., AKAI, K., DE VRIES, J. & BANCHEREAU, J. (1987) Human recombinant interleukin 4 induced Fc ϵ receptors (CD23) on normal human B lymphocytes. *J. exp. Med.* **165**, 1459.
- DEL PRETE, G., MAGGI, E., PARRONCHI, P., CHRETIEN, I., TIRI, A., MACCHIA, D., RICCI, M., BANCHEREAU, B., DE VRIES, J.E. &

- ROMAGNANI, S. (1988) IL-4 is an essential factor for the IgE synthesis induced *in vitro* by human T cell clones and their supernatants. *J. Immunol.* **140**, 4193.
- FRASER, J.K., TAN, A.S., LIN, F.-K. & BERRIDGE, M.V. (1989) Expression of specific high-affinity binding sites for erythropoietin on rat and mouse megakaryocytes. *Exp. Hematol.* **17**, 10.
- GEISSLER, K., STOCKENHUBER, F., KABRNA, E., HINTERBERGER, W., BALCKE, P. & LECHNER, K. (1989) Recombinant human erythropoietin and hematopoietic progenitor cells *in vivo*. *Blood*, **89**, 2229.
- GOTO, M., MURAKAMI, A., AKAI, K., KAWANISHI, G., UEDA, M., CHIBA, H. & SASAKI, R. (1989) Characterization of monoclonal antibodies directed against human erythropoietin that recognize different determinants. *Blood*, **74**, 1415.
- KIMATA, H., SHANAHAN, F., BROGAN, M., TARGAN, S. & SAXON, A. (1987) Modulation of ongoing human immunoglobulin synthesis by natural killer cells. *Cell. Immunol.* **107**, 74.
- KIMATA, H. & SAXON, A. (1988) Subset of natural killer cells is induced by immune complexes to display Fc receptors for IgE and IgA and demonstrates isotype regulatory function. *J. clin. Invest.* **82**, 160.
- KIMATA, H., SHERR, E.H. & SAXON, A. (1988) Human natural killer (NK) cells produce a late-acting B-cell differentiation activity. *J. clin. Immunol.* **8**, 381.
- MIYAZAKI, H., KOZUTSUMI, H., KATO, T., HOSHI, S., TAMURA, S., KUBOTA, M. & SUZUKI, T. (1988) Production of monoclonal antibodies against human erythropoietin and their use in the purification of human urinary erythropoietin. *J. Immunol. Methods*, **113**, 261.
- PÈNE, J., ROUSSET, F., BRIERE, F., CHRETIEN, I., BONNEFOY, J.-Y., SPITS, H., YOKOTA, T., ARAI, N., ARAI, K., BANCHEREAU, J. & DE VRIES, J.E. (1988a) IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons γ and α and prostaglandin E_2 . *Proc. natl Acad. Sci. USA*, **85**, 6880.
- PÈNE, J., ROUSSET, F., BRIERE, F., CHRETIEN, I., WIDEMAN, J., BONNEFOY, J.-Y. & DE VRIES, J.E. (1988b) Interleukin 5 enhances interleukin 4-induced IgE production by normal human B cells. The role of soluble CD23 antigen. *Eur. J. Immunol.* **18**, 929.
- RENNICK, D., JACKSON, J., YANG, G., WIDEMAN, J., LEE, F. & HUDAK, S. (1989) Interleukin-6 interacts with interleukin-4 and other hematopoietic growth factors to selectively enhance the growth of megakaryocytic, erythroid, myeloid, and multipotential progenitor cells. *Blood*, **73**, 1828.
- SARFATI, M. & DELESPESE, G. (1988) Possible role of human lymphocyte receptor for IgE (CD23) or its soluble fragments in the *in vitro* synthesis of human IgE. *J. Immunol.* **141**, 2195.
- SAWYER, S.T., KRANZ, S.B. & SAWADA, K. (1989) Receptors for erythropoietin in mouse and human erythroid cells and placenta. *Blood*, **74**, 103.
- SHERR, E.H., MACY, E., KIMATA, H., GILLY, M.A. & SAXON, A. (1989) Binding the low affinity Fc ϵ R on B cells suppresses ongoing human IgE synthesis. *J. Immunol.* **142**, 481.
- SHERR, E.H., STEIN, L.D., DOSCH, H.M. & SAXON, A. (1987) IgE-enhancing activity directly and selectively affects activated B cells: Evidence for a human IgE differentiating factor. *J. Immunol.* **138**, 3836.
- THIELE, D.L., KUROSAKA, M. & LIPSKY, P.E. (1983) Phenotype of the accessory cell necessary for mitogen-stimulated T and B cell responses in human peripheral blood: delineation by its sensitivity to the lysosomotropic agent, L-leucine methyl ester. *J. Immunol.* **131**, 2282.
- VERCELLI, D., JABARA, H.H., ARAI, K., YOKOTA, T. & GEHA, R.S. (1989) Endogenous interleukin 6 plays an obligatory role in interleukin 4-dependent human IgE synthesis. *Eur. J. Immunol.* **19**, 1419.
- VERCELLI, D., JABARA, H.H., LAUENER, R.P. & GEHA, R.S. (1990) IL-4 inhibits the synthesis of IFN- γ and induces the synthesis of IgE in human mixed lymphocyte cultures. *J. Immunol.* **144**, 570.
- VOGT, C., PENTZ, S. & RICH, I.N. (1989) A role for the macrophage in normal hemopoiesis. III. *In vitro* and *in vivo* erythropoietin gene expression in macrophages detected by *in situ* hybridization. *Exp. Hematol.* **17**, 391.
- YANG, X.-D., DE WECK, A.L. & STADLER, B.M. (1988) Effect of recombinant human interleukin 4 on spontaneous *in vitro* human IgE synthesis. *Eur. J. Immunol.* **18**, 1699.