Clin Exp Immunol 1993; 92:347-352

Inhibition by glucocorticoid and staurosporine of IL-4-dependent CD23 production in B lymphocytes is reversed on engaging CD40

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(Accepted for publication 19 January 1993)

SUMMARY

IL-4 synergizes with signals delivered through CD40 both for the induction of CD23/FcεRII expression and for IgE synthesis. Moreover, engagement of CD40 on the B cell surface by MoAb overcomes the ability of interferons, transforming growth factor-beta, or anti-CD19 to inhibit IL-4-dependent change. We now report that occupancy of CD40 relieves potent suppression of IL-4-induced CD23 production by glucocorticoid or the relatively broad-acting kinase inhibitor staurosporine. Interruption of the IL-4 signal was observed with concentrations of staurosporine considered to be selective for protein kinase C (PKC) inhibition ($IC_{50} = 10$ nM) but not with genistein or tyrphostins, effective inhibitors of tyrosine kinase activity. On ligation of CD40, staurosporine no longer inhibited the IL-4 signal: at concentrations of between 1 and 20 nM, staurosporine actually increased by as much as 100% the rate of CD23 production stimulated on simultaneous activation through CD40 and IL-4R. Such augmentation was not observed when the more specific PKC inhibitor RO-31-8220 was used; indeed, CD40 engagement was unable to overcome the ability of this inhibitor to block IL-4-promoted CD23 induction ($IC_{50} = 10$ μM). Occupancy of CD40 did, however, thwart completely the usual ability of prednisolone to inhibit the IL-4 signal leading to CD23 induction. Activation through CD40 left inhibition of phorbol ester-induced CD23 expression by staurosporine, RO-31-8220, or glucocorticoid unchecked. These findings further highlight the intimate level of cross-talk existing between CD40 and IL-4R on resting B lymphocytes to promote CD23 expression, a phenotypic change which preludes IgE synthesis.

Keywords IL-4 CD23 CD40 B lymphocytes inhibitors

INTRODUCTION

The Th2-derived cytokine IL-4 is a central and essential player in the series of events which drives uncommitted IgM/IgD$^+$ B cells toward IgE synthesis. A necessary second signal can be provided by Th membranes, antibody to CD40, or by lipopolysaccharide (LPS) in mouse and Epstein–Barr virus (EBV) in man (reviewed in [1]). Recent characterization of the CD40 counterstructure as an integral membrane glycoprotein of activated Th cells implicates it as a prime candidate for the previously undefined 'competence' factor of T cell membranes [2]. Thus, from the standpoint of the B cell, engagement of CD40 and IL-4R appears to provide the major physiological drive to IgE production.

Uncommitted B lymphocytes also provide the target population for IL-4-driven expression of CD23 [3]; moreover, IL-4-promoted CD23 production is similarly enhanced on signalling through CD40 with MoAb [4]. Membrane CD23 serves as the low affinity IgE receptor (FcεRII) of B cells and once shed—by active proteolysis—constitutes the bulk, if not all, of previously described IgE regulants [5]. Although definitive proof is still awaited, a large body of evidence implicates CD23 directly in the regulation of IgE synthesis [6]. At the very least, IL-4-dependent regulation of CD23 expression appears to be predictive of subsequent IgE production, suggesting that the mechanisms responsible for engendering the two phenotypes share some common pathways [7]. For example, the capacity of interferons (IFN), transforming growth factor-beta (TGF-β), or antibody to CD19, to turn off IL-4-driven CD23 expression is overridden on engaging surface CD40 [8]: occupancy of CD40 similarly counteracts inhibition by IFN of IL-4-promoted IgE production [9].

IL-4 turns on CD23 expression in human B cells through activating a second messenger cascade involving rapid and transient phosphoinositide (PI) hydrolysis followed by a sustained increase in intracellular cAMP [10,11]. Both the increase in cyclic adenosine monophosphate (cAMP) and the subsequent induction of CD23 are blocked by chelating intracellular Ca^{2+}

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using bis-(*o*-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid (BAPTA). The intracellular pathways triggered through IL-4R can be mimicked by pharmacological agonists to promote CD23 expression by a brief exposure of cells to phorbol ester and calcium ionophore followed by the addition of dBcAMP [10]. This precise sequence of change needs to be adhered to, inasmuch as preexposure of cells to dBcAMP down-regulates IL-4's ability to turn on CD23. The signal transduction pathway activated in resting B cells on engaging CD40—a member of the NGFR/TNFR/Ox40 family of receptors—has yet to be defined. Clearly though, the level of co-operativity existing between CD40 and IL-4R is considerable: this is underscored by the ability of IL-4 and MoAb to CD40 to promote the long term growth and survival of B cells [12].

The present study focuses on the functional cross-talk operating between CD40 and IL-4R by examining the actions of selective inhibitors of second messenger pathways on the induction of CD23. The influence of CD40 engagement on the previously reported ability of glucocorticoid (GC) to inhibit IL-4-driven change is also assessed [13]: GC are believed to modify cell behaviour at the transcriptional level through the binding of GC-receptor complexes to specific response elements [14]. The findings highlight the central role played by CD40 in determining the outcome of IL-4-driven B cell processes by demonstrating its ability to transduce signals which overcome the ability of otherwise potent pharmacological inhibitors to block CD23 induction, a prelude, if not a prerequisite, to IgE synthesis from previously uncommitted B cells.

MATERIALS AND METHODS

Reagents

Recombinant IL-4 was a kind gift from S. Gillis (Immunex Corporation, Seattle, WA); purified G28-5 antibody (CD40) was generously provided by J. Ledbetter (Bristol Myers-Squibb, Seattle, WA). Sheep antibody to human immunoglobulins and biotin-labelled BU38 (CD23) were from the Binding Site (Birmingham, UK). Prednisolone and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) were purchased from Sigma (Poole, UK). Staurosporine was from Mannheim Biochemica (Lewes, UK); BAPTA-AM, genistein, and verapamil from Calbiochem (Nottingham, UK). The protein kinase C (PKC)-selective inhibitor RO-31-8220 was a kind gift from G. Lawton (Roche Products Limited, Welwyn Garden City, UK); the tyrphostins AG34, AG490, and AG814, selective inhibitors of protein tyrosine kinases (PTK), were the gift of A. Levitzki (Department of Biochemistry, The Hebrew University, Jerusalem, Israel).

Cells

B cells were isolated from tonsils taken at routine tonsilectomy by negative selection protocols exactly as described previously [10]; resting B cells were separated as the high buoyant density fraction from a 60% Percoll (Pharmacia, Uppsala, Sweden) gradient as documented [10]. Cultures were performed in a humidified CO_2 atmosphere at 37°C in flat-bottomed tissue culture wells containing RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and antibiotics. For assessment of DNA synthesis, 10^5 cells were cultured in 200 μl of tissue culture medium; for CD23 induction, 10^6 cells were cultured in 1 ml of medium. In some experiments, sheep anti-human immunoglobulin at 100 μg/ml in PBS was coated onto tissue culture wells by overnight incubation at 37°C, followed by washing before addition of cells.

Table 1. Influence of inhibitors on IL-4-induced CD23 expression

	MFI surface CD23*			
	Exp. 1	Exp. 2	Exp. 3	Mean % inhibition†
Control	88	112	60	—
BAPTA‡	46	8	23	70
Staurosporine	17	12	4	87
Genistein	69	69	65	22
Verapamil	84	92	82	< 1

* B cells cultured at 10^6/ml for 2 days with or without IL-4 (1000 U/ml); CD23 expression assessed by staining washed cells with biotin-labelled BU38 followed by FITC-conjugated streptavidin and FACS analysis; results given as change in mean fluorescent intensity (MFI) due to presence of IL-4 with background (control) staining subtracted for each analysis.

† Mean per cent inhibition of CD23 induction calculated from all three experiments.

‡ Additions made 30 min before culture in IL-4: BAPTA-AM (5 μM); staurosporine (100 nM); genistein (40 μM); verapamil (10 μM).

Measurement of stimulation

DNA synthesis was measured at 56 h by pulsing triplicate cultures for a further 16 h with the addition of 0·5 μCi (18·5 kBq) contained in 50 μl medium. Incorporated radioactivity was determined and expressed as a mean value with replicates always within 15% of each other. Surface CD23 expression was measured by indirect immunofluorescence with BU38-biotin in the first layer and FITC-streptavidin in the second; 50 000 stained cells were analysed on a FACS IV (Becton Dickinson, Cowley, UK). CD23 levels in cell lysates and supernatants were determined using a sandwich ELISA assay on duplicate samples as described elsewhere [15]. For all determinations, mean values of three individual experiments are given: standard deviations were calculated and were always within 15% of the mean value obtained.

RESULTS

Staurosporine but not genestein inhibits IL-4-dependent CD23 expression

We have previously shown that the intracellular Ca^{2+}-chelating agent BAPTA substantially inhibits IL-4-driven CD23 expression in resting B lymphocytes [10]. This is confirmed in the present study and compared with the actions of other pharmacological inhibitors of different signal transduction pathways. As seen from results detailed in Table 1, staurosporine was somewhat more potent than the Ca^{2+}-chelator in its capacity to block IL-4-driven change, whereas the tyrosine kinase inhibitor genistein was relatively inactive: three tyrphostins (AG34, AG490, AG814)—specific inhibitors of tyrosine kinase activity—were completely without effect over a range of concentrations known to be active in systems dependent upon PTK

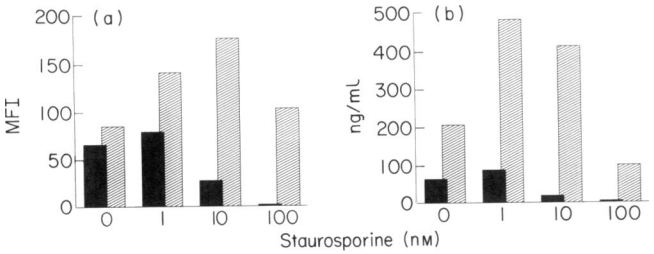

Fig. 1. Influence of staurosporine on CD23 induction. B cells were cultured as in Table 1 with IL-4 at 1000 U/ml and G28-5 (anti-CD40) at 1 μg/ml. (a) After 2 days, cells were removed, washed and analysed for surface expression of CD23, with results expressed as mean fluorescent intensity (MFI). (b) Cell supernatants from 3-day cultures were analysed for CD23 content by ELISA and results expressed as ng/ml of CD23. ■, IL-4; ▩, IL-4/G28-5.

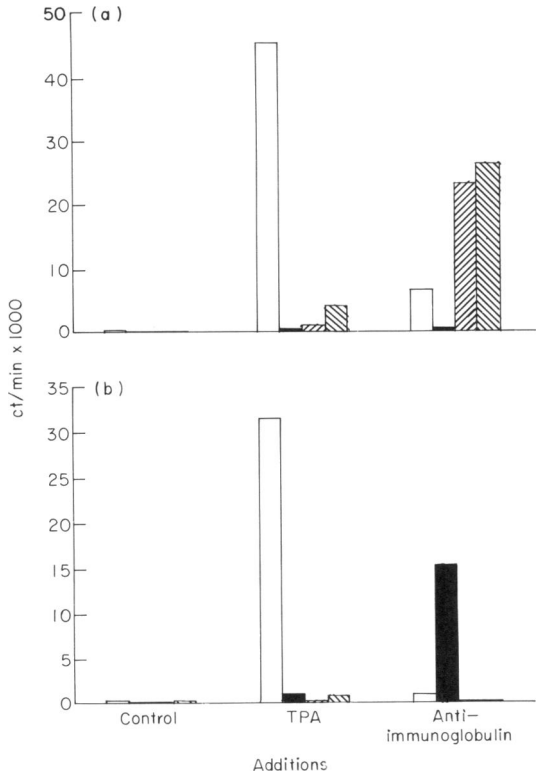

Fig. 2. Influence of inhibitors on B cell stimulations. Resting B cells were cultured at 10^5 per 200 μl in flat-bottomed microtitre wells for 3 days and DNA synthesis assessed over the last 16 h. TPA was added at 5 ng/ml, and anti-immunoglobulin coated onto plates at 100 μg/ml. Inhibitors staurosporine (Stau) and RO-31-8220 (RO) were added 30 min before culture at concentrations shown. Results given as mean ct/min radioactivity incorporated in triplicate cultures. (a) □, Control; ■, Stau 100 nM; ▩, Stau 10 nM; ▧, Stau 1 nM. (b) □, Control; ■, Stau 5 nM; ▩, RO 10 μM; ▧, RO 5 μM.

activity (data not given). No inhibition of IL-4-dependent CD23 induction was observed with the Ca^{2+}-channel blocker verapamil.

Genistein at 40 μM did inhibit by between 30% and 100% the appearance of soluble CD23 into cell culture supernatants (mean inhibition = 45% over four experiments; $P < 0{\cdot}01$, data not given). Thus the pathways leading to proteolysis of CD23 may depend upon activating an intrinsic tyrosine kinase. Studies performed in parallel on B cells and T cells activated with immobilized ligand to surface membrane (sm) immunoglobulin and to CD3 respectively, demonstrated that genistein was fully capable of inhibiting stimulations known to be tyrosine kinase-dependent: in both cases, inhibition due to the addition of 40 μM genistein was > 80% (data not given). Its lack of effect on IL-4-driven CD23 induction strongly suggests, therefore, that stimulation of tyrosine kinase activity is not required for signalling this phenotypic change through IL-4R; it also demonstrates that the inhibitory actions of staurosporine on IL-4 signalling are unlikely to be due to this compound suppressing a tyrosine kinase activity.

Staurosporine enhances CD40-augmentation of IL-4-driven CD23 production

Staurosporine has been found in other systems to be relatively selective for inhibition of PKC activity when used at concentrations of 10 nM or less [15]. Dose response studies revealed that 10 nM staurosporine provided an IC_{50} for inhibition of IL-4-driven CD23 induction whether assessed by measuring surface expression or the production of soluble CD23 in culture supernatants (Fig. 1). Inhibition was virtually complete at 100 nM, while at 1 nM a slight, but consistent enhancement on this change was observed ($P < 0{\cdot}05$ over four experiments).

As noted in previous studies, the addition of MoAb to CD40 somewhat augments the already high expression of CD23 induced by IL-4 at the B cell membrane and substantially increases the rate of soluble CD23 production (Fig. 1). Staurosporine at 1–10 nM enhanced CD40-augmented CD23 expression even further, with increases due to the addition of the 'inhibitor' exceeding 100%. Such change remained IL-4-dependent, inasmuch as no CD23 production was detected in the presence of MoAb to CD40 alone, either with or without the addition of staurosporine (data not detailed). Even at 100 nM, staurosporine failed to reduce the level of CD23 induced by IL-4 when CD40 was also being engaged (Fig. 1).

Comparison of the actions of staurosporine and its more PKC-selective analogue RO-31-8220 on B cell stimulations

Although the concentration range in which staurosporine exerted its dichotomous influence on IL-4-dependent change corresponded to that suggested as being selective for PKC activity, we nevertheless assessed the influence of its more PKC-specific analogue RO-31-8220 [16]. In order to validate the efficacy of the two compounds we initially examined their effect on B cell stimulations known to involve PKC activation: namely, the stimulation of resting B cells to DNA synthesis with either high dose phorbol or immobilized anti-immunoglobulin [17]. First, we found that over the dose range of 1–100 nM staurosporine blocked almost completely the phorbol ester-driven DNA synthesis; however, between 1 and 10 nM, staurosporine substantially enhanced the suboptimal stimulation of resting B cells provoked by immobilized anti-immunoglobulin, even though

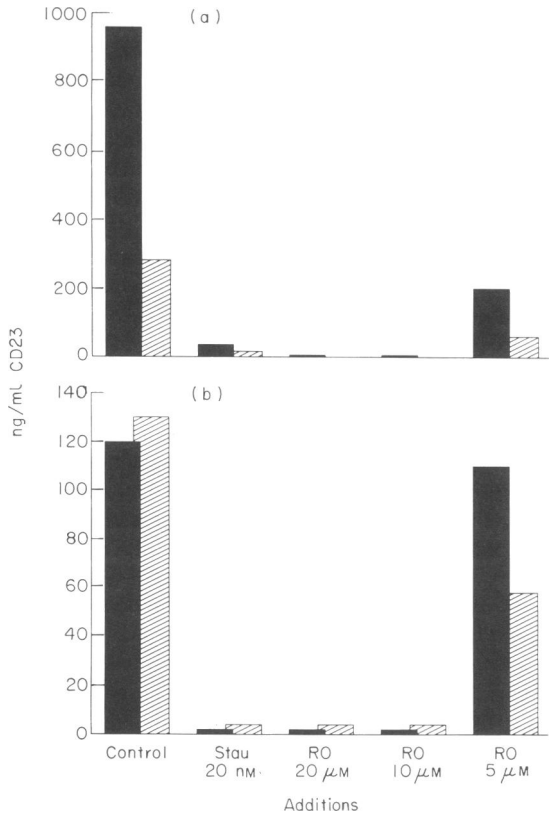

Fig. 3. Influence of inhibitors on CD23 induction. Cells cultured for 3 days as for Fig. 1 with inhibitors present as in Fig. 2, with CD23 content in lysates and supernatant being measured by ELISA. (a) Cultured with IL-4 (1000 U/ml), (b) with TPA (5 ng/ml). ■, Lysate; ▨, supernatant.

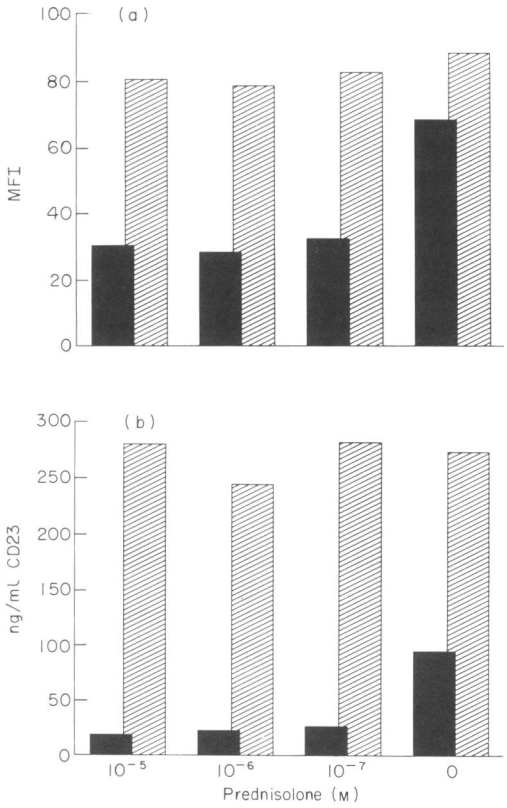

Fig. 5. Influence of prednisolone on CD23 induction. Cells were cultured as in Fig. 1: (a) surface CD23 on day 2; (b) supernatant CD23 on day 3. ■, IL-4; ▨, IL-4 + G28-5.

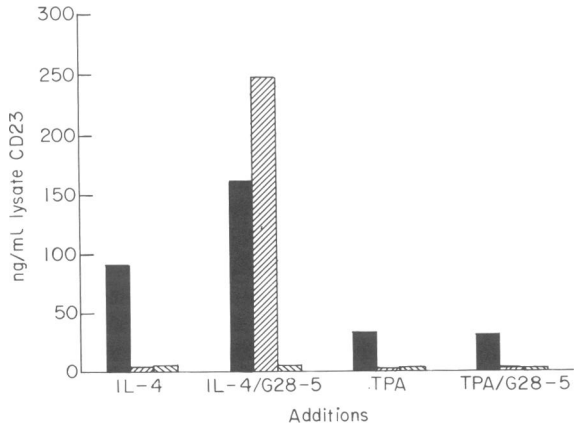

Fig. 4. Comparison of inhibitors on CD23 induction. Cells cultured for 3 days as for Fig. 3 in presence or absence of inhibitors as indicated, and CD23 content of lysates assessed by ELISA: IL-4, 1000 U/ml; G28-5, 1 μg/ml; TPA, 5 ng/ml. ■, Control; ▨, Stau 20 nM; ▧, RO 10 μm.

effective blocking of this response was observed at 100 nM of the inhibitor (Fig. 2a). When RO-31-8220 (which has a reported IC_{50} of inhibition on PKC-dependent T cell responses of 5–10 μM) was used, total blocking of both phorbol ester- and anti-immunoglobulin-driven DNA synthesis was observed (Fig. 2b).

At similar concentrations, and compared with the effect of 20 nM staurosporine for reference, RO-31-8220 also blocked IL-4-dependent induction of CD23 in these experiments, measured as total cell or supernatant content of CD23 (Fig. 3a). CD23 can also be induced on resting B cells through the chronic activation of PKC using high dose phorbol ester [3]. The influence of the two kinase inhibitors on this route of change can be judged from results presented in Fig. 3b. RO-31-8220 can be seen to act at an almost identical concentration range on both the pathways of CD23 induction.

CD40-occupancy does not counter inhibition of IL-4-driven CD23 production by RO-31-8220 nor inhibition of phorbol ester-driven production by staurosporine

Results presented in Fig. 4 reveal that engagement of CD40 is unable to relieve the inhibition of IL-4-dependent CD23 induction mediated by 10 μM RO-31-8220, whereas staurosporine again shows an enhancement on this phenotypic change. It can also be seen that MoAb to CD40 has no influence on the ability of either RO-31-8220 or staurosporine to block phorbol ester-promoted production of CD23.

Engagement of CD40 overrides the ability of glucocorticoid to inhibit IL-4-dependent CD23 production

Glucocorticoids have been shown to inhibit IL-4-dependent CD23 induction while enhancing IL-4-driven IgE synthesis; nevertheless, the latter change is still dependent upon the availability of the reduced levels of CD23 remaining [13]. Here we show that over a wide dose range of prednisolone, signalling through CD40 with MoAb is able to counter totally the ability of the glucocorticoid to inhibit IL-4-dependent induction of either membrane or soluble CD23 (Fig. 5). Prednisolone also suppressed phorbol ester-driven CD23 production in resting B cells by as much as 95% (mean = 87% over four experiments; s.d. = 8%): this inhibition was not relieved on engaging CD40 (data not given).

DISCUSSION

This study underscores the central role of CD40 in delivering signals that counteract inhibitors of IL-4-driven CD23 induction in resting human B cells, an observation previously made for inhibition by TGF-β, IFN, and anti-CD19 [8], and now extended to the potent pharmacological antagonists prednisolone and staurosporine. Importantly, glucocorticoids enhance IL-4-dependent IgE synthesis in mononuclear cell preparations, while suppressing CD23 production from purified B cells [13]. Given that activated T cells express CD40-ligand [2], the lack of inhibition on CD23 production by GC when CD40 is simultaneously engaged provides a rationale to its apparent discrepant behaviour on the two otherwise related IL-4-driven changes.

The present study indicates that CD23 induction: (i) requires an intact PKC signalling pathway; (ii) is independent of tyrosine kinase activity. Although results obtained using pharmacological inhibitors should be interpreted with caution, the lack of effect of genistein and tyrphostins together with the effective blocking by both staurosporine and its more PKC-specific analogue RO-31-8220 strongly favour such a conclusion. In addition, the two other agents capable of stimulating high-rate CD23 production in human B cells—namely, phorbol ester and EBV—each activate PKC chronically [18]. The similar efficacy of RO-31-8220 (and staurosporine) at inhibiting CD23 production provoked by IL-4 or phorbol ester provides further support for IL-4 utilizing a PKC-dependent pathway to engender this change.

The inhibition of both IL-4- and phorbol ester-driven CD23 production by glucocorticoid is also compatible with a central role for PKC in delivering the IL-4 signal. GC receptors appear to bind to the same gene response element as the phorbol ester-induced transcription factor AP-1 (composed of complexes of *jun* and *fos* gene products) [14]. Under some conditions, the binding of GC acts as a repressor of gene induction through the common response element: this would account for the capacity of GC to block almost totally phorbol ester-induced CD23 expression and support the requirement for PKC in the actions of IL-4.

An unexpected finding was that staurosporine actually enhanced—by as much as 100%—IL-4-driven CD23 production when CD40 was also being engaged. It was recently reported, however, and confirmed in the present study, that staurosporine at 1–100 nM is capable of augmenting stimulation of B cells by anti-immunoglobulin [19]. This change was shown to be dependent upon residual—albeit greatly reduced—PKC activity. One interpretation of these data is that staurosporine, a relatively broad-acting kinase inhibitor, blocks the activity of an enzyme that in resting B cells acts as a repressor of stimulation through a pathway shared by surface immunoglobulin and CD40. RO-31-8220, the more PKC-specific analogue of staurosporine, apparently fails to influence this putative suppressive pathway, but effectively knocks out PKC to block signalling through the routes described.

The pathway used by CD40 to transduce its signal in resting B cells is unknown. PI hydrolysis appears not to be involved [20]. Activation of tyrosine kinase has been shown to be stimulated through CD40 in pre-activated tonsillar B cells and in certain B cell lines, but the same authors failed to detect a similar response in small dense B cells [21]. We have found that tyrosine kinase inhibitors do not modify the ability of the CD40 signal to enhance IL-4-driven CD23 production in resting B cells (unpublished results). Whatever signal transduction system is used by CD40, it is likely to interact with a pathway stimulated through IL-4R which, although dependent upon, is distinct from PKC. One potential level of interaction between CD40 and IL-4R is via cAMP, a sustained elevation of which is generated in human B cells on binding of IL-4 [10,11].

This study indicates that when investigating factors regulating commitment of primary B cells to IgE synthesis—a phenotype heralded by CD23 expression—IL-4 cannot be considered in isolation. Any therapeutic modality aimed at disrupting the IL-4 signal should also take account of CD40 and its ligand.

ACKNOWLEDGMENTS

This work was supported with grants from the Medical Research Council (UK) and the Leukaemia Research Fund. M.F. is in receipt of a Royal Society Fellowship. Smith Kline-Beecham is also acknowledged for financial support.

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