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Immunoglobulin isotype production by cycling human B lymphocytes in response to recombinant cytokines and anti-IgM

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SUMMARY

Actively cycling populations of purified human tonsilar B lymphocytes were examined for their capacity to secrete IgM, IgA, IgE and IgG of all four subclasses in direct response to recombinant cytokines; in some experiments, monoclonal antibody to IgM (anti-μ) was included in order to explore the influence of antigen receptor ligation on immunoglobulin (Ig) production. Enhanced IgM release was seen on culture of the cycling cells with either interleukin-2 (IL-2), IL-4 or interferon-alpha (IFN-α). IL-2 and IFN-α also augmented IgA production, whereas IL-4 had no effect on this isotype. IL-4 did, however, encourage the production of the IgG subclasses IgG1, IgG2 and IgG3, while IL-2 augmented IgG1 and IgG3 release and IFN-α increased IgG1 levels. IgG4 production, and that of IgE, failed to be perturbed by any of the cytokines assayed. Neither IL-1α, IL-1β, IL-5 nor IFN-γ significantly altered the profile of Ig isotype release. When confronted with anti-μ, cycling B cells demonstrated a marked suppression in IgM production. Suppression could not be overcome by the addition to culture of the normally IgM-promoting IL-4. Concomitant with the reduction in IgM levels, an increase in IgG release was observed. This was comprised of elevations in IgG1 and IgG3. Although not influencing IgA release directly, anti-μ was found to promote increased IgA production in co-culture with either IL-2 or IFN-α. The findings are discussed in the context of recent findings on Ig isotype control in both human and murine systems.

INTRODUCTION

Although factors have been described that are seemingly capable of driving resting B cells directly to immunoglobulin (Ig) secretion (Karasuyama, Rolink & Melchers, 1988; Sidman & Marshall, 1984), it is likely that the major route to antibody production is via an actively cycling phase which would allow for the necessary expansion of infrequent antigen-responsive clones (Jelinek & Lipsky, 1983). In the mouse, the cytokines responsible for directing Ig isotype production from LPS-stimulated B cells have been extensively defined (Paul, 1987; Snapper, Finkelman & Paul, 1988; Miyajima *et al.*, 1988). Corresponding details in man have been slower to emerge, reflecting, in part, the lack of an equally efficient and appropriate stimulation strategy.

In the present study, a recently described activation protocol is exploited (Gordon *et al.*, 1987) that has the dual advantage not only of driving every B cell into active cell-cycle (Flores-Romo *et al.*, 1989) but also of mimicking the biochemical second messenger cascade initiated on the ligation of surface antigen receptors (Gordon *et al.*, 1987). Furthermore, the agonists used (phorbol dibutyrate and ionomycin) can be readily removed by simple washing, allowing the actions of agents introduced in reculture to be assessed in the absence of initial interfering stimuli. As it could be anticipated that activated B cells *in vivo* probably re-encounter antigen in combination with helper signals (MacLennan & Gray, 1986), Ig isotype regulation was examined not only in the presence of recombinant cytokines but also following the engagement of antigen receptors by using a recently described monoclonal antibody to IgM (Gordon *et al.*, 1989).

Abbreviations: ELISA, enzyme-linked immunosorbant assay; FCS, fetal calf serum; IFN, interferon; Ig, immunoglobulin; IL, interleukin; PBS, phosphate-buffered saline; PDB, phorbol dibutyrate.

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MATERIALS AND METHODS

Reagents

Phorbol dibutyrate (PDB) was purchased from Sigma (Poole, Dorset, U.K.) and ionomycin from Calbiochem (La Jolla, CA). Recombinant cytokines were obtained as follows: IL-1α, IL-1β and IL-2 from Genzyme (Boston, MA); IFN-α and IFN-γ from Janssen Biochimica (Beerse, Belgium); IL-5 was a kind gift of C. J. Sanderson (NIMR, Mill Hill, U.K.); IL-4 was isolated from culture medium of yeast cells transformed with an

expression vector containing full length cDNA for human IL-4 (Immunex Corporation, Seattle, WA). Cytokines were added to culture at concentrations determined in preliminary experiments as optimal for driving Ig production. Th BU1 murine monoclonal antibody to human IgM (Fcμ) was generated in the Department of Immunology, University of Birmingham, U.K. and was selected on its ability to stimulate DNA synthesis in resting B cells in co-culture with PDB, as reported elsewhere (Gordon *et al.*, 1989). The antibody is a mouse IgG2a, an isotype with negligible affinity for human B-cell Fc receptors (Gordon *et al.*, 1989), thereby avoiding potential immunomodulatory feedback by this route without the need for removal of the Fc portion.

B-cell culture

Highly purified resting tonsillar B cells were prepared by negative selections and Percoll (Pharmacia, Uppsala, Sweden) density gradients, exactly as described in detail elsewhere (Flores-Romo *et al.*, 1989). These cells were then cultured with PDB (1 ng/ml) and ionomycin (0·8 μg/ml) at 10^6/ml in RPMI-1640 supplemented with 10% fetal calf serum (FCS) and antibiotics, at 37° in 5% CO_2 humidified atmosphere. On Day 3, cells were taken from culture and washed (four times) in RPMI-1640 containing 1% FCS and re-cultured in either 1 ml tissue culture wells (10^6/ml) or in 200 μl flat-bottomed microwells (5×10^5/ml) for assessment of Ig production (see below) or DNA synthesis, respectively. DNA synthesis was determined as described previously using an 8-hr pulse of [^{3}H]thymidine ([^{3}H]TdR; Amersham, Amersham, Bucks, U.K.) (1μCi contained within 50 μl) prior to termination of culture. In some experiments, B cells were harvested after culture and examined for their expression of surface IgM using excess BU1 (50 μg/ml) as a first stage, followed by FITC-labelled sheep anti-mouse Ig (Dept. of Immunology, Birmingham) as a second stage for development. Fluorescent intensity was then quantified using a FACSIV flow cytometer (Becton-Dickinson, Mountain View, CA).

Enzyme-linked immunoassay (ELISA) for measurement of Ig

The typical sandwich approach for ELISA was used to quantify the isotypes in culture supernatants. The general procedures were as follows: primary anti-isotype antibodies were coated onto 96-well micro-ELISA plates (Dynatech Laboratories, Alexandria, VA) in 100 μl of PBS and left overnight at 4°. After washing with PBS plus Tween 20 (0·05%; Sigma), standard amounts of each Ig or culture supernatant in a final volume of 100 μl/well were added for 2 hr at 37°. The samples were then removed, the plates were washed with PBS-Tween 20 and the secondary anti-Ig peroxidase-conjugated antibodies were added at optimal dilutions and left incubating at 37° for 2 hr. After the final wash, the enzyme activity bound was detected using peroxidase substrate, and the absorbance at 492 nm was determined by an automated Multiskan ELISA reader (Titertek Multiskan MCC, Flow Labs, Rockville, MD). The Ig concentration in the samples was calculated by interpolation of the linear standard curve constructed with serial dilutions of known concentrations of pure Ig. The specificity of the assays was tested by including Ig of other classes at several hundred- or thousand-fold times higher than the lower concentration detected in the system, thus ruling out the possibility of cross-reactions in each case. For the IgG ELISA the lowest detectable concentration was 3·2 ng/ml and no cross-reaction was found with 100 μg/ml or IgM or 22 μg/ml of IgA. For the IgM assay the sensitivity was 4·2 ng/ml, whereas 44 μg/ml of IgG and 22 μg/ml of IgA were not detected. In the IgE assay, levels as low as 0·4 ng/ml could be detected, but not concentrations as high as 40 μg/ml of IgG, 22 μg/ml of IgA or 44 μg/ml of IgM. The limits of IgA detection was 3·9 ng/ml with no reaction from 44 μg/ml of IgG or 100 μg/ml of IgM in the IgA ELISA.

Sheep antibodies to Fcμ, Fcγ and Fcα, either unlabelled or peroxidase coupled, were prepared by Immuno-Diagnostic Research Laboratories (IDRL, Birmingham, U.K.). The anti-IgE antiserum was rendered monospecific by passing it through IgG and IgM columns. For the IgG ELISA, a mixture of three mouse monoclonal antibodies against Fabτ was used for coating and another monoclonal against Fcτ, peroxidase coupled, was used as secondary antibody. These anti-IgG reagents were a kind gift of M. Goodall (Dept. of Immunology, Birmingham), and the IgM, IgG standards and the IgM column were provided by R. A. Mageed (Dept. of Immunology, Birmingham). The standards for IgE were from Pharmacia AB (Uppsala, Sweden).

IgG subclasses were quantified by similar-based ELISA using kits developed by IDRL. Strips were presensitized with monoclonal antibodies specific for each human IgG subclass, which had been successfully evaluated in a IUIS/WHO collaborative study (Jefferis *et al.*, 1985). Binding of IgG isotypes from culture supernatants was evaluated using peroxidase-conjugated sheep anti-IgG at an optimal concentration for each subclass. The limits of sensitivity were as follows: IgG1, 1·2 ng/ml; IgG2, 1·8 ng/ml; IgG3, 2·0 ng/ml; IgG4, 2·8 ng/ml. No cross-reactivity was found between each isotype at levels up to 20 μg/ml.

RESULTS

Cytokine-directed Ig isotype production

High density tonsillar B lymphocytes were stimulated for 3 days with PDB and ionomycin, washed extensively and recultured with recombinant cytokines over a further 3–10 days before harvesting supernatants and determining the concentrations of the different Ig isotypes present. It was found that while the absolute and relative production of each isotype could vary among the different cell preparations studied, the direction of cytokine-promoted change on each individual Ig class or subclass was constant. Results given are therefore representative examples of such change. The degree of variability observed will be indicated where appropriate.

Recombinant IL-2 promoted significant enhancement of IgM and IgG release from cycling B cells and augmented IgA release to a more modest degree (Fig. 1; note that in some experiments, IL-2 promoted a five-fold enhancement of IgA release). Enhanced IgG production following exposure of cycling cells to IL-2 was found to reflect a dramatic increase in the release of the IgG1 subclass, although IgG3 levels were also elevated (up to seven-fold in some experiments); in no experiment did IL-2 promote any change in either IgG2 or IgG4 production (Fig. 1). IFN-α was found to encourage cycling B cells to release larger quantities of IgM, of IgA in particular, and also of IgG. With IFN-α, elevations in IgG levels were represented exclusively by IgG1, with no change in IgG2, IgG3 or IgG4 being noted in any experiment.

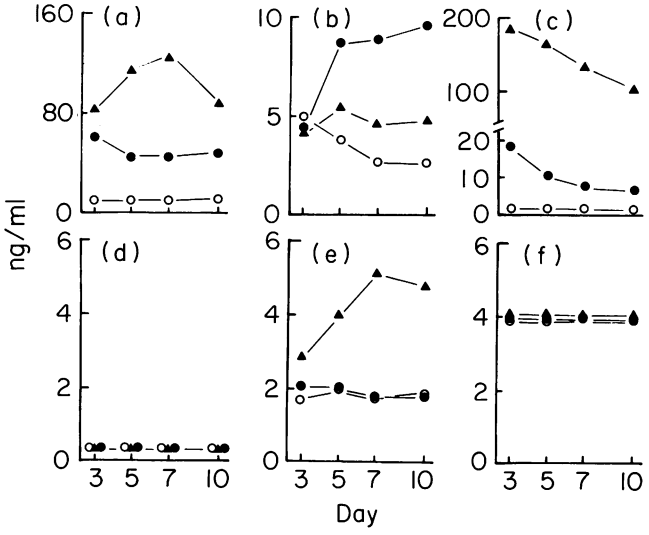

Figure 1. Influence of IL-2 and IFN-α on Ig isotype production. Resting B cells were stimulated for 3 days with PDB (1 ng/ml) and ionomycin (0·8 μg/ml), washed and recultured for the times indicated in: control medium (○); rIL-2 (200 U/ml) (▲); rIFN-α (1000 U/ml) (●). The concentrations of (a) IgM, (b) IgA, (c) IgG1, (d) IgG2, (e) IgG3 and (f) IgG4 present in culture supernatants were measured by ELISA.

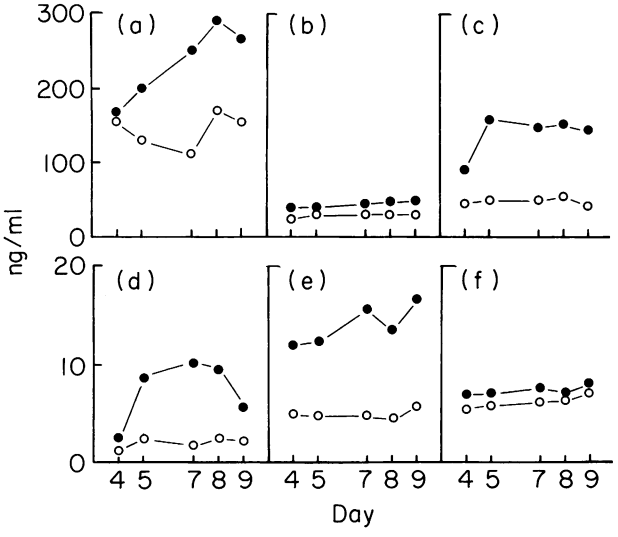

Figure 2. Influence of IL-4 on Ig isotype production. As for Fig. 1 but cells recultured in either control medium (○) or rIL-4 (1000 U/ml) (●).

In agreement with a recent study which used SAC-induced blasts as targets (Defrance *et al.*, 1988), IL-4 was found to promote, in the cycling B cells, increased production of both IgM and IgG but not significantly of IgA (Fig. 2). Augmented Ig production in the presence of IL-4 could be quite variable, ranging from two- to 13-fold for IgM and 1·5- to 6-fold for IgG. Again, increases in IgG1 constituted the bulk of the elevated IgG production, although IgG2 and IgG3 levels were also increased (Fig. 2). In no experiment was any significant change in IgG4 release noted as a consequence of exposing cycling cells to IL-4.

Recombinant cytokines IL-1 (α and β forms), IL-5 and IFN-γ were also assessed, over a range of concentrations, for their ability to modifiy Ig release from cycling B cells. IL-1 and IFN-γ were found to be fully active in the growth promotion of Epstein–Barr virus-transformed lymphoblastoid cells and in blocking IL-4-promoted CD23 induction, respectively (data not detailed); the IL-5 used was found to be potent in eosinophil differentiation assays (C. J. Sanderson, personal communication). Both IL-1 and IL-5 failed to promote change on any isotype in all experiments performed. Results with IFN-γ were less conclusive, with this cytokine often giving no change but occasionally showing a suppression of overall Ig production. None of the cytokines at any concentration tested was able to promote the release from cycling B cells of IgE to a level that was detectable in our assay (>400 pg/ml).

Influence of anti-μ on Ig isotype production

The reversible nature of the activation protocol adopted in this study afforded the opportunity to investigate the influence of antigen receptor engagement on the cytokine-directed Ig isotype production from actively cycling B cells, in the absence of potentially interfering initial stimuli. By confronting 3-day cycling cells with a monoclonal antibody to IgM (anti-μ) in the presence of IL-4, the aim was to approximate any potential re-encounter of cells with antigen.

Over a large number of experiments, anti-μ was found consistently to down-regulate the basal release of IgM from cycling B cells (Figs 3 and 4). The addition of increasing amounts of IL-4, which normally enhanced the release of IgM, failed to overcome the isotype suppression mediated by anti-μ (Fig. 3). Furthermore, the suppression was sustained. Even after 9 days of culture with anti-μ, IgM production remained low and, again, prolonged suppression failed to be relieved by IL-4 (Fig. 4). Coincident with the suppression of IgM production, anti-μ was seen to encourage a small but significant increase in IgG levels (Figs 3 and 4). This augmentation was observed whether or not IL-4 was also present in culture. IgA production, by contrast, remained essentially unchanged. The addition of IFN-γ, which can antagonize some actions of IL-4 on human B cells (Defrance *et al.*, 1988), failed to modify either basal, IL-4-promoted or anti-μ-modified Ig isotype production from the cycling population (Fig. 3).

A trivial explanation for the changes observed with anti-μ could be that the introduced antibody interfered with the sandwich ELISA used to detect Ig, yielding inappropriately low or high readings for IgM and IgG, respectively. This possibility was, however, discounted from experiments (not detailed) where inclusion into the IgM or IgG standard of the modulating antibody at the concentration used in functional assays failed to shift in either direction the titration curve of the two Ig.

The question of whether the anti-μ-promoted elevation of IgG production was isotype-selective or reflected a general up-regulation of all IgG subclasses was addressed next. Results presented in Table 1 indicate that the bulk of change resulted from elevations in IgG1 production, but with some contribution also from increases in IgG3. IgG2 and IgG4 production were essentially unaffected by the addition of anti-μ to cycling B-cell

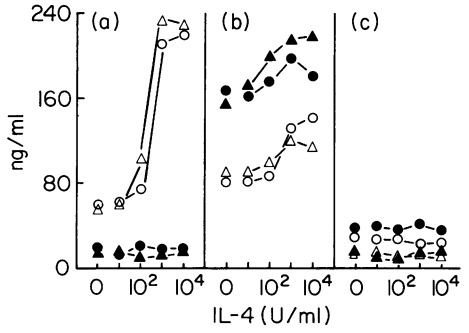

Figure 3. Monoclonal anti-μ promotes an IgM to IgG functional 'switch' in cycling B cells. Three-day stimulated B cells were recultured in the presence (●, ▲) or absence (○, △) of the monoclonal anti-μ chain antibody BU1 (25 μg/ml) with increasing concentrations of rIL-4, as indicated, either with (△, ▲) or without rIFN-γ (1000 U/ml) (○, ●). The concentrations of (a) IgM, (b) IgG or (c) IgA present in culture supernatants were measured by ELISA after 4 days of reculture.

Table 1. Influence of anti-μ on IgG subclass production

		ng/ml of IgG*							
		Exp. 1				Exp. 2			
Additions†	IFN-γ	G1	G2	G3	G4	G1	G2	G3	G4
Control	–	42	2	7	6	12	4	3	4
	+	45	2	5	6	14	6	5	5
BU1	–	73	3	12	5	21	9	9	5
	+	70	2	14	7	19	8	8	5
IL-4	–	166	11	29	7	48	15	19	4
	+	145	9	26	5	56	15	17	3
BU1+IL-4	–	270	12	56	6	93	18	37	5
	+	248	11	49	7	90	15	25	5

* Concentration detected in culture supernatants following 4-day reculture.

† Three-day-stimulated cells were washed and recultured for a further 4 days with additions as indicated: BU1 (anti-μ), 25 μg/ml; IL-4, 1000 U/ml; IFN-γ, 1000 U/ml.

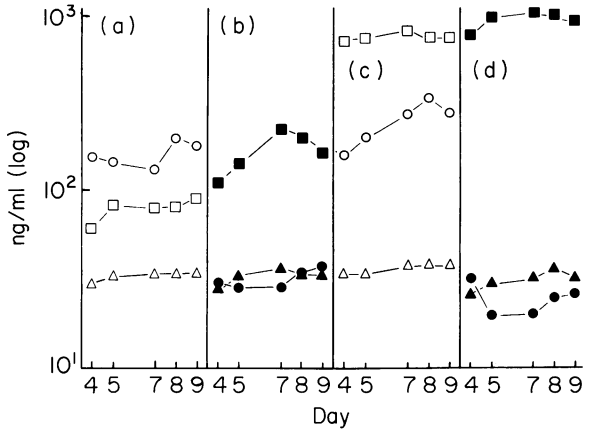

Figure 4. Kinetics of anti-μ-promoted functional 'switch'. As for Fig. 3, but culture supernatants harvested at times after reculture as indicated for measurement by ELISA of IgM (○, ●), IgG (□, ■) or IgA (△, ▲). Cells recultured in (a) control medium, (b) BU1 (25 μg/ml), (c) rIL-4 (1000 U/ml), and (d) BU1+rIL-4.

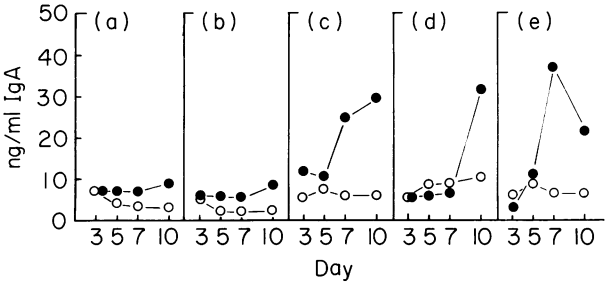

Figure 5. Anti-μ can promote a switch to IgA production. Three-day stimulated B cells were recultured for the times indicated, in the presence (●) or absence (○) of BU1 (25 μg/ml) and (a) control medium, (b) rIL-4 (1000 U/ml), (c) rIL-2 (200 U/ml), (d) rIFN-α (1000 U/ml), (d) rIL-2+rIFN-α. IgA concentration in culture supernatants was determined by ELISA.

cultures. Again, IFN-γ failed to modify IgG isotype release under any of the conditions explored.

IgA production can be enhanced by anti-μ

We noted previously that neither basal nor IL-4-directed IgA production from cycling B cells was significantly altered in the presence of anti-μ. However, as IL-4 left IgA levels unchanged anyway, the influence of anti-μ on IgA production was re-examined in the presence of IL-2 and IFN-α. Results presented in Fig. 5 reveal that on culture with either one or a combination of these two cytokines, IgA release from cycling cells was now augmented on the inclusion of anti-μ. It can be noted that this enhancement required a relatively long reculture with antibody and cytokine to be realized (Fig. 5).

DISCUSSION

The aim of this study was to define the influence of recombinant cytokines on Ig isotype production by purified populations of actively cycling human B cells. IL-2, IL-4 and IFN-α were each found to display a unique profile of Ig isotype enhancement from such populations. In agreement with an earlier study (Clutterbuck *et al.*, 1987), IL-5 was found to be ineffective on the purified B cells, possibly indicating a requirement for T cells and/or monocytes to drive the previously reported IgA production (Miyajima *et al.*, 1988). IL-1 and IFN-γ acting individually also failed to modify significantly Ig isotype production. These observations are again consistent with other studies which employed SAC-stimulated blasts as targets for cytokine action (Jelinek & Lipsky, 1987).

The Ig enhancing activity found for IL-2 is compatible with its known action as a T-cell replacing factor (TRF) (Callard *et al.*, 1986), while that of IFN-α is consistent with studies which

have indicated that its anti-proliferative effect on Daudi Burkitt lymphoma cells is accompanied by terminal differentiation (Exley *et al.*, 1987). It is interesting that while both IL-2 and IFN-α were capable of enhancing IgA production, IL-4 showed no appreciable effect on this isotype, a finding again in agreement with a recent study which utilized SAC blasts as targets (Defrance *et al.*, 1988). IL-4 did, however, show the broadest enhancement of the IgG subclasses, elevating the production of all isotypes, with the exception of IgG4, from the cycling B cells.

The control by recombinant cytokines of Ig isotype production from cycling human B cells as documented in this study appears to be quite different to that reported for LPS-stimulated murine B cells (Paul, 1987); for example, whereas in the human system IL-4 displayed a broad up-regulatory effect on Ig production and the actions of IFN-γ were limited, in mouse the former exhibits highly selective increases in IgE and IgG1 release, while the latter specifically and significantly augments IgG2a production. The most likely explanation for the apparent discrepancies in the two species probably relates to the activation strategies used. Studies on Ig isotype regulation in the human being reported at the completion of our study indicate that LPS in the murine system might be replacing signals that would normally be provided by T cells or monocytes. Thus, mutant EL4 thymoma cells have been shown to support IL-4-promoted IgE and IgG4 production from human B cells (Lundgren *et al.*, 1989); human IgG4 may represent the biological counterpart of murine IgG1. Epstein–Barr virus may be able to substitute for LPS in man, as it has been found to activate human B cells in a way that they can produce IgE in response to IL-4 (Thyphronitis *et al.*, 1989). Our findings in the present study on pure B-cell populations therefore do not appear to represent the full potential for Ig isotype regulation in man where other cells or stimuli are present.

An interesting finding to emerge from the present study was the small, but significant, apparent functional switch in Ig isotype production following the addition of anti-μ to culture. Suppression of isotype production by either a corresponding anti-isotype antibody or antigen is well documented (Symons, Clarkson & Hall, 1985; Abbas & Klaus, 1977) and appears to occur at the level of message production (Chen, 1988). The unexpected feature of this investigation was the concomitant increase in certain other Ig isotypes following IgM suppression by anti-μ. Thus, the inclusion of anti-μ increased modestly both basal and IL-4-promoted production of IgG1 and IgG3 and enhanced the IgA release occurring in the presence of either IL-2 or IFN-α. These observations apparently conflict with an earlier report where anti-μ was found to exert a general suppressive effect on overall Ig production (Maryama, Kubagawa & Cooper, 1985). In that study, however, antibody was present from initiation of culture, while in this study cells were already in active cycle before the antibody was introduced. It will be of interest to determine whether the increased production of non-suppressed Ig isotypes by anti-μ represents a direct molecular switching in IgM-positive cells or an indirect effect expanding on the few IgG- and IgA-bearing cells present in the culture.

In conclusion, this study has catalogued the direct effects of recombinant cytokines and anti-μ on actively cycling B cells with regard to Ig isotype production. The magnitude of change observed has been significant but relatively small by comparison with effects reported on LPS-stimulated murine B cells. Moreover, the cytokines analysed appeared more to be promoting enhanced Ig isotype production from pre-committed cells rather than invoking any real isotype switch. Thus, in terms of Ig production, soluble factors may be serving two quite distinct roles; one would reflect a direct effect on the B cells to up-regulate production from already-switched cells, the other, apparently requiring additional signals, would be on the switch mechanism itself. Clearly, additional studies are required to determine in detail what influence T-cell subsets, and also perhaps antigen-presenting cells, have in order to gain a full insight into Ig isotype control under conditions reflecting those prevailing *in vivo*.

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