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Regulation of resting and cycling human B lymphocytes via surface IgM and the accessory molecules interleukin-4, CD23 and CD40

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SUMMARY

Experiments were designed in order to compare directly the ability of a new and potent monoclonal anti-μ chain antibody to initiate or maintain stimulation in resting and cycling B lymphocytes, respectively. Resting B cells could be stimulated by soluble anti-μ only in the presence of additional signals; these could be supplied by a high dose of phorbol ester or a combination of interleukin-4 (IL-4) and the CD40 antibody, G28-5. Immobilization of anti-μ not only increased the magnitude of the resting B-cell response but also diminished the co-factor requirements. The 'background' stimulation obtained when using a high concentration of immobilized anti-μ was unexpectedly reduced in the presence of IL-4 alone. The duration, but not the magnitude, of the IL-4 signal required for promoting optimal responses varied with the co-stimulation applied. Importantly, the threshold concentrations of soluble anti-μ needed to trigger the resting B cells were reduced upon the addition of each co-stimulant. With actively cycling B cells, both soluble and immobilized anti-μ were now capable of sustaining stimulation which could be prolonged on the addition of IL-4 and/or G28-5. In both resting and cycling populations, a strong correlation was noted between the magnitude of stimulation elicited when IL-4 was present and the release of the soluble CD23 molecule. Moreover, IL-4-promoted, but not other, stimulations could be augmented up to 10-fold by the inclusion of the CD23 antibody MHM6. Both the resting and cycling B-cell populations were found to secrete IgM in direct response to IL-4 and G28-5; this factor-driven production of IgM was differentially modulated by soluble and immobilized anti-μ in the two populations.

INTRODUCTION

The probable site for initiating a B-cell response to T-dependent (TD) antigen is within the paracortex of secondary lymphoid organs (MacLennan & Gray, 1986). Here, migrating B cells that have bound antigen congregate with helper T lymphocytes (*Th*) on interdigitating cells (IDC). Cognate interaction between the B cell and Th cells at this stage would presumably be amplified by enhanced expression of MHC class II antigen on the B cell as a result of IL-4 release from the Th cell (Paul & Ohara, 1987). It would be anticipated that the expression of B-cell-associated CD23 would also be increased at these sites—indeed, cognate T–B interactions have been shown to lead to a particularly potent induction of the CD23 surface molecule (Crow, Jover & Friedman, 1986). In addition to the signals delivered through antigen receptor, IL-4 receptor and possibly CD23, a role for the CD40 antigen could also be envisaged at this early stage of B-cell activation. The 50,000 MW CD40 glycoprotein is not only expressed strongly on IDC but, again, is up-regulated on the B-cell surface by IL-4 (Gordon *et al.*, 1988b); although the natural ligand for CD40 is presently undefined, antibodies to CD40 are capable of providing potent co-stimulation signals to both resting and cycling B cells (Clark & Ledbetter, 1986; Gordon *et al.*, 1987).

The sequelae to the initial TD response represent a bifurcation generating: (i) extrafollicular proliferation with corresponding IgM antibody production; and (ii) the follicular blast reaction which, through the formation of germinal centres, provides the possibility of affinity maturation by somatic mutation, and of isotype switching (MacLennan & Gray, 1986). B cells cycling within germinal centres will gain the opportunity to re-encounter antigen, this time presented as immune-complexes on CD23-rich follicular dendritic cells (FDC) (Gordon *et al.*, 1989). These B cells still express the CD40 antigen and may again encounter IL-4-delivered signals via T cells within the light zone of the germinal centre.

Most studies that have analysed *in vitro* the role of cytokines or functional surface antigens in the process of B-cell activation have employed experimental protocols which have best developed the activities sought, for example pre- or co-stimulation of B cells with phorbol esters, *Staphylococcal aureus* Cowan Strain I (SAC) or immobilized anti-immunoglobulin. Such approaches

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have revealed the individual importance of IL-4, CD23 and CD40 in B-cell regulation (reviewed by Gordon & Guy, 1987). In the present study, we have attempted to define the function of these major regulatory molecules under a variety of conditions, focusing on their interaction with signals delivered via antigen receptor. This approach has been facilitated by generating a new and particularly potent monoclonal antibody to IgM, the functional characteristics of which were compared directly for defined resting and cycling B-cell populations under differing modes of ligand presentation.

MATERIALS AND METHODS

Reagents

Phorbol dibutyrate (PDB) and phorbol myristic acetate (PMA) were purchased from Sigma (Poole, Dorset) and ionomycin from Calbiochem (La Jolla, CA). Recombinant IL-4 was isolated from the culture medium of yeast cells transformed with an expression vector containing full length cDNA for human IL-4. Neutralizing rabbit antiserum to IL-4 was used at a dilution of 1:10,000, which abolished completely the activity of 1000 U/ml of IL-4 in B-cell co-stimulation assays. The CD40 antibody G28-5 is a murine IgG1 mouse monoclonal antibody and was a generous gift from J. A. Ledbetter (Oncogen, Seattle, WA). MHM6 is also a murine IgG1 antibody belonging to the CD23 cluster. Monoclonal antibodies to IgM were all generated in the Department of Immunology, Birmingham, with the exception of 4B8, which was supplied by R. Armitage, ICRF Tumour Immunology Unit, London and HB57, Mu18, MuB3 and P24, which were the kind gift of P. Mongini, Mount Sinai School of Medicine, NY.

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 (Cairns *et al.*, 1988). For generating actively cycling populations, these cells were then cultured at 10^6 ml with PDB (1 ng/ml) and ionomycin (0·8 μg/ml) in RPMI-1640 supplemented with 10% fetal calf serum (FCS) and antibiotics at 37° in a 5% CO_2 humidified atmosphere. On Day 3, cells were taken from culture and washed four times in RPMI-1640 containing 1% FCS. Resting or cycling B cells were cultured or recultured in either 1 ml tissue culture wells (10^6/ml) or in 200 μl flat-bottomed microwells (5×10^5/ml) for assessment of IgM and soluble CD23 production or DNA synthesis, respectively, exactly as described before (Cairns *et al.*, 1988). Antibodies were coupled to tissue culture wells where indicated by overnight incubation at 37° in carbonate buffer, pH 9·6, as described in Cairns *et al.* (1988). Plates were washed extensively in phosphate-buffered saline, pH 7·2, before cell culture. Concentrations given in the text are nominal referring to coating levels only; for most experiments, a pre-determined optimal coating concentration was used. In the preliminary screen, antibodies were purified by affinity-chromatography against Sepharose 4B coupled with IgM. Subsequent studies with BU1 used the IgG2a fraction of the ascites fluid purified by ion-exchange chromatography.

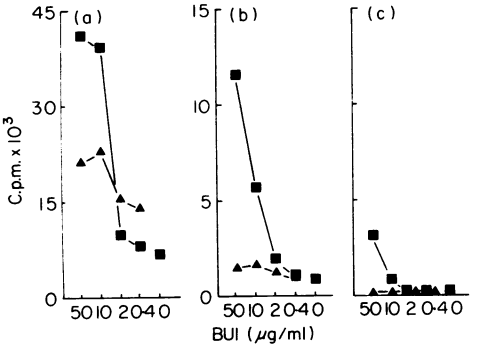

Figure 1. Co-stimulation of resting B cells with BU1 and PMA. B cells at 10^5 per well were cultured for 64 hr in the presence of soluble (▲) or immobilized (■) BU1 as indicated and DNA synthesis was determined by the incorporation of [^{3}H]TdR for the final 16 hr. PMA was added at: (a) 2 ng/ml; (b) 0·2 ng/ml; (c) 0 ng/ml. Results are represented as mean c.p.m. of triplicate determinations which never varied by more than 10% of each other. Note that concentrations for immobilized BU1 are nominal as the efficiency of antibody coating to wells is unknown (actual amounts used for coating tissue culture wells were equivalent to 10-fold those indicated for soluble antibody).

RESULTS

Functional characteristics of BU1, a novel monoclonal anti-μ

It has previously been shown that monoclonal antibodies directed against surface IgM differ widely in their ability to stimulate B cells (Rudich, Winchester & Mongini, 1985). In the present study we screened 20 monoclonal anti-μ (see the Materials and Methods), initially in a co-stimulation assay with PMA, and found similar variability in their stimulation capacity (data not shown). Only a few of the antibodies gave consistently high stimulation indices, and amongst these BU1 was seen to be particularly potent. This IgG2a antibody was subsequently selected for inclusion into the detailed study.

The relative efficiencies of soluble and immobilized BU1 were compared. Results detailed in Fig. 1 show that, by itself, soluble BU1 was incapable of stimulating resting B cells over the range of concentrations studied, while at high coating levels, immobilized BU1 could provide a modest and variable degree of stimulation (Table 1). When presented together with a low dose of PMA (0·2 ng/ml), again only the immobilized antibody was stimulatory. With a higher dose of PMA (2ng/ml), both soluble and immobilized BU1 were now able to augment the basal level of DNA synthesis occurring, with optimal stimulation for soluble antibody being obtained at concentrations between 10 and 50 μg/ml (Fig. 1). At a defined optimal coating concentration of BU1 it was found that the dose-requirements for TPA in co-stimulation with immobilized antibody were identical to those observed for obtaining a synergistic response with the calcium ionophone ionomycin (Guy *et al.*, 1985); for soluble BU1, PMA concentrations which were by themselves partially mitogenic were needed to obtain augmented responses (Fig. 2). These observations on human tonsillar B cells are in total contrast to those which have been reported for murine splenic B cells where phorbol esters were found to antagonize stimulations mediated through surface IgM (Mizuguchi *et al.*, 1986).

It was of interest to determine at what point in the activation sequence the signal transduced through surface IgM was

Table 1. Stimulation of resting B cells via surface IgM and accessory molecules

Additions*	[^{3}H]TdR incorporation in response to†		
	Control	Soluble BU1	Immobilized BU1
None	142±36	247±57	14,438±4067
IL-4	196±40	3104±469	1246±320
G28-5	1884±248	14,659±3890	46,339±12,339
IL-4+G28-5	7878±2105	33,914±6977	102,753±30,503

*IL-4, 1000 U/ml; G28-5, 1 μg/ml; soluble BU1, 25 μg/ml; immobilized BU1, 250 μg/ml coating concentration.

†Results expressed as c.p.m. incorporated within a 16-hr pulse on Day 3 of culture and represent the means of three different experiments ±SD.

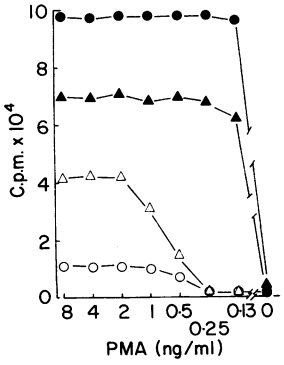

Figure 2. Dose-response of PMA co-stimulation with BU1. As for Fig. 1. (○) No antibody; (△) soluble BU1, 25 μg/ml; (▲) immobilized BU1, 250 μg/ml (nominal); (●) ionomycin, 0·8 μg/ml.

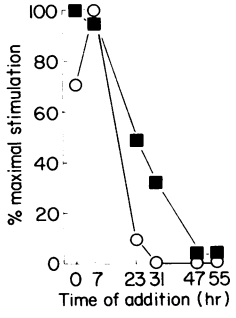

Figure 3. Temporal requirements for BU1 in PMA co-stimulation assay. Cells were cultured as in Fig. 1 with PMA at 2 ng/ml and either BU1 (○) at 25 μg/ml or G28-5 (■) at 1 μg/ml added at time indicated. Results expressed as percentage maximal stimulation which for BU1 was 47,342±1306 c.p.m. and for G28-5 was 84,463±2987 c.p.m.; PMA only gave 5636±470 c.p.m.

required to interact optimally with that delivered by the phorbol ester. Results from delayed addition experiments revealed a very early requirement for the antibody in the activation process to obtain significant co-stimulation with PMA (Fig. 3). These requirements were compared with those for signalling B cells

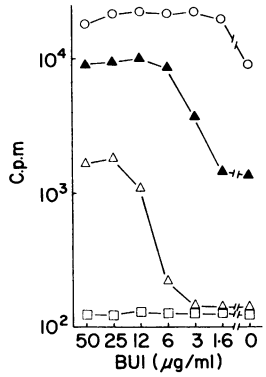

Figure 4. Stimulation of resting B cells with BU1, IL-4 and G28-5. B cells were cultured as in Fig. 1 with varying concentrations of soluble BU1 as indicated with the following additions: (□) none; (△) IL-4, 1000 U/ml; (▲) G28-5, 1 μg/ml; (○) IL-4+G28-5.

through the CD40 antigen using the G28-5 antibody. As noted previously, this signal was also required early on in the co-stimulation of resting B cells to achieve an optimal response (Clark & Ledbetter, 1986). The small increase in the co-stimulatory activity of BU1 noted on delaying its addition by a few hours prior to the sudden fall off observed at 24 hr was reproducible.

Contribution of IL-4 and CD40 to the stimulation of resting B cells through surface IgM

By itself, IL4 was found to co-stimulate modestly with soluble BU1 (Table 1). The CD40 antibody G28-5, which, as seen before, is weakly stimulatory for resting B cells (Gordon *et al.*, 1988b) also promoted enhanced DNA synthesis with soluble BU1. The highest degree of stimulation was obtained when soluble BU1 was presented to resting B cells in the presence of both IL-4 and G28-5. With immobilized BU1, augmented stimulation was obtained with G28-5 alone, although the response could again be improved upon with the addition of IL-4. The level of stimulation reached under these conditions was similar to that obtained when applying an optimal combination of phorbol ester and calcium ionophore. Unexpectedly, IL-4 added in the absence of G28-5 decreased the direct mitogenicity observed when presenting cells with a high coating density of immobilized BU1. This observation was reproducible over several experiments when resting tonsillar B cells were used as targets and is reminiscent of the down-regulation of the TPA response by IL-4 noted in an earlier study (Gordon *et al.*, 1988a).

It was possible that the co-stimulants being added not only increased the magnitude of stimulation through surface IgM but also diminished the threshold requirements for the ligand. This was, indeed, seen to be the case: with only IL-4 present, 25 μg/ml of soluble BU1 were required for optimal co-stimulation; with G28-5, 6 μg/ml were required, while with a combined IL-4/G28-5 co-stimulus optimal responses were obtained with 1·6 μg/ml or less of soluble BU1 (Fig. 4).

We next asked whether the mode of presentation of antibody or the additional signals received might alter the threshold concentration of IL-4 required to promote an optimal response in the resting B cells. Results obtained (not detailed) indicated

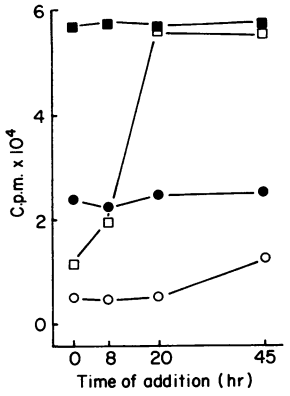

Figure 5. Temporal requirement for IL-4. B cells were cultured as in Fig. 4 in the presence of IL-4/G28-5 either with (■, □) or without (●, ○) soluble BU1 at 25 μg/ml. Rabbit anti-IL-4 (1:10,000) (□, ○) or medium alone (■, ●) was added at times indicated.

this not to be the case, with optimal effects being found with 1000 U/ml of IL-4 under all experimental conditions. It remained possible, however, that the duration of the IL-4 signal required might be different under the varying conditions of stimulation. This was explored by intervening in the stimulation at various times with a neutralizing anti-IL-4 antibody. Now a difference was seen, in that with soluble anti-μ the IL-4 signal was needed for a shorter time in order to obtain maximal stimulation through CD40 (Fig. 5).

Maintenance of the B-cell cycle through surface IgM, IL-4 and CD40

Actively cycling populations were generated by stimulating high density tonsillar B cells for 3 days with phorbol dibutyrate and ionomycin and then washing extensively to remove the mitogenic signals (Flores-Romo *et al.*, 1989). It was found that these populations could then be re-stimulated through their surface IgM using either soluble or immobilized BU1; with the latter, the stimulations were somewhat more sustained (Fig. 6). Either mode of presenting antibody could also enhance further the DNA synthesis promoted on replating cycling cells with PMA in the absence of ionomycin (data not shown). Similarly, BU1 in either form was capable of synergizing with either IL-4 or G28-5, alone or in combination, for maintaining the stimulation of the cycling B-cell population (Fig. 6). Again, the immobilized antibody was seen to sustain the cycle somewhat longer than did the soluble form (Fig. 6).

Release of soluble CD23 and induction of IgM secretion from resting and cycling B cells

There is mounting evidence that soluble fragments of the CD23 antigen contribute to IL-4-initiated growth-promotion in B cells (reviewed by Gordon *et al.*, 1989). Measurement of soluble CD23 (sCD23) by ELISA in culture supernatants indicated a strong correlation between sCD23 release and the level of stimulation obtained where IL-4 was present (Table 2). IL-4 by itself, while efficient at inducing surface CD23 expression, failed to promote significant release of the soluble molecule. However, when added jointly with G28-5, even resting B cells could now

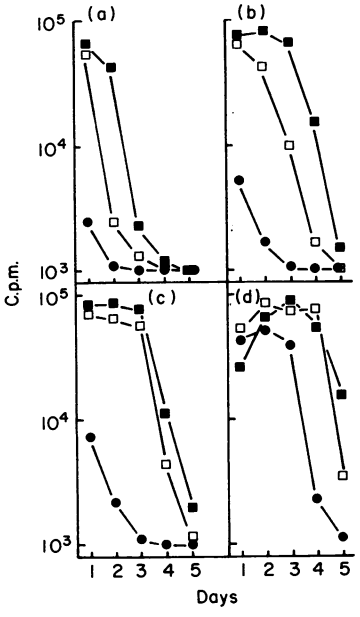

Figure 6. Maintenance of stimulation in cycling B cells. B cells stimulated for 3 days with PDB and ionomycin and then washed to remove these stimuli were recultured at 10^5/well for times indicated before receiving an 8 hr pulse of [^{3}H]TdR and DNA synthesis determined. Additions on replating are: (●) none; (□) soluble BU1 at 25 μg/ml; (■) immobilized BU1 at 250 μg/ml (nominal); (a) none; (b) IL-4, 1000 U/ml.; (c) G28-5, 1 μg/ml; (d) IL-4+G28-5.

Table 2. Release of IgM and sCD23 in response to BU1 and accessory molecules

		Release of IgM and soluble CD23 (ng/ml)†			
		Resting B cells		Cycling B cells	
BU1	Additions*	IgM	sCD23	IgM	sCD23
None	None	<2	<5	10±4	<5
	IL-4	<2	12±4	39±12	27±6
	G28-5	<2	<5	<2	<5
	IL-4/G28-5	47±16	104±28	51±20	128±31
Soluble	None	<2	<5	<2	<5
	IL-4	<2	17±6	11±5	95±18
	G28-5	<2	<5	<2	<5
	IL-4/G28-5	13±6	238±65	14±4	467±90
Immobilized	None	<2	<5	13±5	<5
	IL-4	<2	15±3	43±17	98±15
	G28-5	<2	<5	<2	<5
	IL-4/G28-5	107±23	465±102	34±13	583±86

*Additions made as for Fig. 6.

†Resting B cells cultured for 7 days, 3 day-cycling cells for a further 4 days before harvesting supernatants for measurements. Results given as means of three different experiments ± SD.

Table 3. Enhancement of IL-4-promoted stimulations by MHM6

	[^{3}H]TdR incorporation into†			
	Resting B cells		Cycling B cells	
Additions*	Control	MHM6	Control	MHM6
None	170	206	448	869
BU1	196	244	803	1052
IL-4	243	355	9517	21,516
G28-5	2669	3663	7247	9585
IL-4+G28-5	4387	48,070	46,418	93,102
IL-4+BU1	732	3931	33,052	38,319
BU1+G28-5	8441	10,218	12,491	18,406
IL-4+BU1+G28-5	29,453	83,284	63,795	64,933

*Additions made as for Fig. 6; MHM6 added at 25 μg/ml.

†[^{3}H]TdR incorporation assessed by a 16-hr pulse on Day 3 of plating or re-plating resting and cycling B cells, respectively, and represent means of triplicate determinations which never varied by more than 10% of each other.

be encouraged to high rate sCD23 release. This was augmented by the addition of soluble BU1 and even further in the presence of immobilized BU1. A very similar pattern of sCD23 release was seen with the cycling population (Table 2). Interestingly, although a combination of BU1 and G28-5 was efficient at sustaining the cycle of stimulated B cells, this was not accompanied by sCD23 release in the absence of IL-4.

Clearly, entry into and maintenance of the cycle are not the only sequelae to B-cell activation in a physiological context. As an indicator of any differentiation that might be occurring, the level of IgM production was quantified, again by ELISA. Neither IL-4 nor G28-5 alone promoted IgM production from resting B cells, but when added jointly they were capable of inducing significant IgM release. Interestingly, the IgM secretion evoked by IL-4 and G28-5 was substantially down-regulated by soluble BU1 but significantly up-regulated by immobilized BU1. In the already-cycling cells, IgM secretion was induced by IL-4 alone and this production was not modified further by the addition of G28-5. As with resting B cells, soluble BU1 suppressed the IL-4-promoted IgM production from the cycling population, while immobilized BU1 showed little effect on IgM release in either a positive or negative direction (Table 2).

Contribution from stimulations through the CD23 antigen

We have previously shown that the CD23 antibody MHM6 is capable of augmenting the stimulation of resting B cells initiated with a high dose of phorbol ester (Gordon *et al.*, 1986). Experiments detailed in Table 3 illustrate that MHM6 is also able to enhance stimulations promoted with the more physiological stimuli employed in the present study and does so for both resting and cycling B cells. With resting B cells, the most potent augmentation obtained with MHM6 (approximately 10-fold) was observed for cells that had been stimulated with IL-4 and G28-5; where BU1 was also included, MHM6 enhanced the stimulation >two-fold. With cycling B cells, the greatest enhancement elicited by MHM6 was obtained on joint addition with IL-4, either alone or together with G28-5 (Table 3). Stimulations which were independent of IL-4 were only minimally affected by MHM6.

DISCUSSION

Clearly, the prime consideration in the development and regulation of a B-cell response is the antigen receptor. We have attempted to mimic the influence of antigen *in vitro* by probing B-cell function with a new, highly potent antibody to IgM. This antibody was capable of delivering, via antigen receptors, information to the B cell which enabled it to respond vigorously to signals transmitted via CD40 and IL-4 receptors. In agreement with earlier reports, optimal stimulation was obtained with resting B cells only when anti-μ was presented on a solid support (Parker, 1975). For B cells already in cycle, both soluble and immobilized antibody were effective at delivering the stimulatory signal. In this respect, cycling B cells were more sensitive than resting B cells to signalling through surface IgM. It was of interest that while IL-4 not unexpectedly augmented the stimulation of cycling B cells to either soluble or immobilized anti-μ, it was found to inhibit the direct response of resting B cells to immobilized anti-μ. This down-regulation of an anti-immunoglobulin signal by IL-4 has probably not been observed previously as most other studies have tended to use pre-stimulation protocol rather than adding the two agents simultaneously. Our observations in this study are, however, remarkably similar to the down-regulation by IL-4 of TPA-driven B-cell stimulation noted previously (Gordon *et al.*, 1988a). Both types of stimulation (i.e. TPA and anti-μ) will involve translocation of PKC from the cytosol to the membrane and it may be this step that the IL-4 generated signal interferes with (Guy, Gordon & Michell, 1989). It is worthwhile noting that IL-4 can also antagonize the actions of IL-2 (Jelinek & Lipsky, 1988) and, at least in T cells, IL-2 has been shown to trigger PKC translocation (Bonvini *et al.*, 1987). IL-4-mediated inhibition of resting B-cell stimulation by immobilized anti-μ did not reflect redirection into a differentiation mode as neither production of IgM nor of other Ig classes (data not detailed) could be detected. It is of interest that ligation of CD40 was able to override the inhibitory influence of IL-4 in this system.

The above findings, *in toto*, indicate that the detailed outcome of a B-cell response to antigen receptor stimulation will depend not only on what accessory signals are received but also on when they are received and in what combination. This presumably reflects, in turn, events *in vivo* where a B cell receiving a TD antigen stimulus will migrate into appropriate areas of the secondary lymphoid tissue at appropriate times to receive the appropriate instructions. Our findings from the *in vitro* model also indicate that the nature and combinations of accessory signals received will alter the antigen threshold required to elicit an optimal stimulus in the responding B cell.

The present study confirms and extends the notion of an important role for CD23 in IL-4-promoted B-cell stimulations (Gordon *et al.*, 1989). Thus, under the conditions of activation employed here, a close correlation was observed between the magnitude of IL-4-promoted B-cell responses and the release of soluble CD23 fragments, as had been noted previously for phorbol ester-initiated cultures (Gordon *et al.*, 1988a). The role for soluble CD23 in B-cell regulation was recently reviewed in detail (Gordon *et al.*, 1989). Furthermore, some new observa-

tions indicate that certain antibodies to CD23 can effectively neutralize IL-4-stimulated B-cell responses (Delespesse, Sarfati & Hofstetter, 1989). In the present study, the CD23 antibody MHM6 was able to augment stimulations of both resting and cycling B cells initiated with IL-4, demonstrating that the functional properties of this antibody are not limited to phorbol ester-promoted stimulations. Somewhat paradoxically, a relatively high rate of CD23 production was required to register a stimulatory effect with the MHM6 antibody. This may relate to stabilization of autostimulatory forms of soluble CD23 by this antibody, as has been mooted recently (Gordon *et al.*, 1988a) and as has been shown for other antibodies in several hormonal systems (Aston, Cowden & Ada, 1989).

The ability of IL-4 and G28-5 to promote IgM secretion in resting B cells in the absence of antigen receptor ligation was unexpected. No detectable production of any other Ig class was noted with this stimulation strategy (L. Flores-Romo and J. Gordon, unpublished observations). Down-regulation of the induced IgM production in both resting and cycling B cells by soluble anti-μ is consistent with previous observations on suppression of isotype production either by anti-isotype or by antigen (Symons, Clarkson & Hall, 1985; Abbas & Klaus, 1977). In murine B cells anti-μ has been shown to inhibit selectively mRNA for secretory μ chains (Chen, 1988). Interestingly, immobilizing the anti-μ negated this inhibitory action and, for resting cells, significantly enhanced the IgM secreted in response to IL-4 and G28-5. These observations indicate that the mechanism involved in anti-isotype suppression requires internalization of the ligand and/or antigen receptor. It could thus be envisaged that depending upon the nature of antigen presentation, quite different outcomes could ensue on ligating antigen receptors at different stages of the B-cell response *in vivo*.

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