Immunology 1989 **66** 228–232

Phorbol ester and calcium ionophore are sufficient to promote cell replication in cultures of quiescent human B lymphocytes

L. FLORES-ROMO, D. FOSTER, G. R. GUY* & J. GORDON *Departments of Immunology and *Biochemistry, University of Birmingham, Birmingham*

Accepted for publication 3 October 1988

SUMMARY

Highly purified, resting B cells could be induced to grow for up to 10 days by culturing in the presence of a synergistic combination of a tumour-promoting phorbol ester and the calcium ionophore ionomycin. In spite of evident cell death occurring, four to five times as many viable B lymphocytes could be harvested at the end of culture than were initially plated. Soluble factors derived from T cells (interleukin-2, commercial B-cell growth factor) or monocytes (interleukin-1) failed to augment further the growth-promotion observed. Evidence is presented to suggest that an autocrine component might be necessary for maintenance of the cell-cycle and growth initiated by the phorbol ester and calcium ionophore combination. The significance of these findings to B-cell physiology are discussed.

INTRODUCTION

Much attention is being focused on the control of growth and differentiation among the various cellular components of the immune system. Availability of recombinant growth factors and monoclonal antibodies to functional cell surface moieties has allowed a dissection of the processes involved by examining purified cell populations in defined tissue culture conditions (Gordon & Guy, 1987). However, in the majority of studies reported, potential growth-modulating agents have been assessed by their ability to promote metabolic change such as seen on the incorporation of radionucleotide precursors into cellular RNA or DNA. Using this approach, it has been shown for both B and T lymphocytes that phorbol esters and calcium ionophores, agonists of the phosphoinositide 'dual pathway' of signalling, when combined, by-pass the requirement for antigen receptor triggering in the initiation of 'mitogenesis' (Truneh *et al.*, 1985; Guy *et al.*, 1985). Indeed, studies from our own group have shown this combination to be the most efficient signal available for driving resting human B lymphocytes into DNA synthesis (Walker *et al.*, 1986). While we have confirmed that stimulation under these conditions, as monitored by thymidine incorporation, is reflected in cell cycle progression (Walker *et al.*, 1986), it has not been determined whether such a synergistic combination of phorbol ester and ionomycin is capable of driving quiescent B lymphocytes to active cell division, either in the absence or presence of presumed growth-promoting soluble factors. This question is addressed directly in the present study.

Correspondence: Dr J. Gordon, Dept. of Immunology, the Medical School, Vincent Drive, Edgbaston, Birmingham B15 2TJ, U.K.

MATERIALS AND METHODS

Reagents

Phorbol myristic acetate (PMA) and phorbol dibutyrate (PDB) were purchased from Sigma (Poole, Dorset) and the calcium ionophore ionomycin from Calbiochem (La Jolla, CA). Commercial B-cell growth factor (BCGF) was obtained from Cellular Products Inc. (Buffalo, NY) and represented a cocktail of growth-promoting activities derived from lectin-stimulated peripheral blood T lymphocytes. Interleukins (IL) 1 and 2 were from Genzyme (Boston, MA) and supplied, respectively, as natural monocyte-derived material and recombinant product.

B-cell cultures

Resting B lymphocytes were purified from human tonsillar tissue by negative selections and Percoll (Pharmacia, Uppsala, Sweden) density gradients exactly as described elsewhere (Walker *et al.*, 1986). Cells prepared in this way were cultured at concentrations for times and with additions indicated in the text in RPMI-1640 containing 10% fetal calf serum, 5×10^{-5} 2-mercaptoethanol and antibiotics at 37° in a humidified 5% CO_2 atmosphere.

Cell counts and viabilities were performed in duplicate, the latter by vital dye exclusion. Cell cycle analysis was performed on a fluorescent-activated cell sorter (FACS IV), analysing for each sample 50,000 fixed cells stained with propidium iodide as described in detail elsewhere (Guy *et al.*, 1985). For the measure of DNA synthesis, cells plated in 200 μl of culture medium recieved a 1 μCi pulse of [^{3}H]thymidine contained in 50 μl of medium for the final 16 hr of culture. All determinations were

performed in quadruplicate and results given are mean values. Replicates were never found to differ by more than 10% and were normally within 5% of each other.

RESULTS

B-cell growth in the presence of PMA and ionomycin

It has been shown previously that resting B lymphocytes respond optimally by DNA synthesis to synergistic combinations of PMA and ionomycin and, to a more limited extent, to PMA alone at high dose (> 2 ng/ml) (Walker *et al.*, 1986). In the present study, the effect of these agents on B-cell growth was measured. Results expressed in Fig. 1a are typical of several such experiments performed. It can be seen that the best growth response was obtained using a combination of ionomycin at 0·8 μg/ml and PMA at either 0·1 or 0·2 ng/ml. Increasing the PMA concentration further (to 0·4 ng/ml) essentially negated its ability to synergise with the calcium ionophore for promoting growth. In the absence of PMA, ionomycin was found to be toxic for the B cells. No dose of PMA by itself over the range of 0·1–2 ng/ml was growth stimulatory (results not detailed). The cell yields obtained from cultures containing a 'mitogenic' dose of PMA (2 ng/ml) were identical to those from control cultures (Fig. 1a). Over the range of experiments, and as typified in Fig. 1a, optimal synergistic combinations of the two agents led to a doubling in cell number by Days 5–6 of culture, while by Days 8–10 four times as many cells were present than at initiaion of the experiment. No further increase was noted beyond Day 10, and by Days 12–15 the number of viable cells present fell sharply (not detailed). It was confirmed that, at termination of culture, only B cells ($CD19^+$) and no T cells ($CD3^+$) or monocytes ($OKM1^+$) were present.

Influence of soluble factors

As the B-cell cultures were essentially devoid of T cells or monocytes, representative 'growth factors' from each cell type were assessed in order to determine whether the growth-promoting effect noted above could be improved upon. It is clear from inspection of Fig. 1b–d and by comparison with Fig. 1a that neither IL-1, IL-2 or commercial T-cell-derived BCGF was capable of extending the growth-promoting properties of PMA and ionomycin. Indeed, in most instances, the soluble factors were providing negative influences to the effects of the pharmacological agonists. A detailed comparison of the contribution from the different soluble factors to both cell number and cell viability in these cultures is shown for Day 6 in Table 1. Again, the results confirm that the 'growth factors' used provided no improvement for either of these parameters over optimal PMA and ionomycin combinations alone.

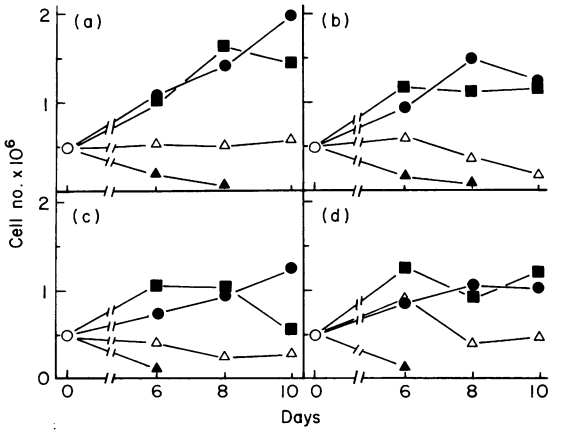

Figure 1. Growth of purified B cells. Resting B cells were stimulated with either PMA alone at 2 ng/ml (▲) or with ionomycin at 0·8 μg/ml plus PMA at 0·1 ng/ml (●), 0·2 ng/ml (■) or 0·4 ng/ml (△). On Day 3 of culture, the following additions were made: (a) none; (b) IL-1 (2·5 U/ml); (c) commercial BCGF (10% v:v); (d) IL-2 (200 U/ml). The concentrations used had previously been shown as optimal in co-stimulation assays with tonsillar B cells. Numbers of viable cells on days indicated are shown.

B-cell stimulation in the presence of PDB and ionomycin: relationship to cell density

In the initial experiments using PMA and ionomycin to promote B-cell growth, it became clear that a major limiting factor was the accumulation of metabolites and other toxic compounds arising from both active cell replication and a relatively high rate of cell death. Although cultures could be split and refed with the stimulating agents in order to minimize this problem, it was difficult to assess the continuing concentrations of PMA that would be present due to its potential degradation and irreversible intracellular binding. It has been shown recently that PDB can replace PMA in synergistic combination with ionomycin for B-cell stimulation (Gordon *et al.*, 1987). This reagent has the advantage of binding reversibly so that the levels of phorbol ester maintained in cultures could be more carefully controlled. For this reason, we next explored the effects of PDB and ionomycin on B-cell growth. In preliminary experiments, PDB was titred into cultures of primary B cells, together with an optimal concentration of ionomycin for different starting concentrations of B cells in order to optimize the system. For this initial screen, DNA synthesis at Day 3 was used as a measure of the response. It can be seen from Fig. 2 that for PDB optimal stimulation with ionomycin over a range of cell concentrations was obtained at 1 ng/ml. Also, the magnitude of the response can be seen to be highly dependent upon the initial plating density of B cells.

Cell cycle analysis of B cells restimulated with PDB and ionomycin

The rationale for replacing PMA by PDB as a co-stimulant with ionomycin was the ability to remove from and control more precisely the concentrations of phorbol ester in the culture system. To assess whether PDB was both effectively removed and could also maintain the cell cycle when replaced in culture, cells stimulated for 3 days with PDB plus ionomycin were washed and then replated in either control medium, medium containing either PDB or ionomycin alone, or with the two agents together. A further 3 days later, cell cycle status was assessed by FACS analysis (Fig. 3). It can be seen that stimulated cells replated in medium alone accumulated in the G0/G1 phase of cycle, with insignificant numbers cycling through S and G2/M stages. Thus the stimulants had been effectively removed. Replenishing cultures with PDB alone left this profile unchanged, whereas the additional replacement of ionomycin resulted in a cell cycle profile essentially indis-

Table 1. Growth and viability of stimulated B cells at Day 6 of culture

Additions*		Control		IL-1		IL-2		BCGF	
TPA (ng/ml)	CaI	Cell no.†	Viability‡	Cell no.	Viability	Cell no.	Viability	Cell no.	Viability
2·0	–	0·19	35	0·19	34	0·12	24	0·12	26
0·1	+	1·12	63	1·0	66	0·74	66	0·74	59
0·2	+	1·08	62	1·26	65	1·34	66	1·15	57
0·4	+	0·54	57	0·66	53	0·84	65	0·4	47
0	–	<0·15	<20	–	–	–	–	–	–

* Resting B cells stimulated for 6 days, as in Fig. 1 with TPA, at concentrations indicated, and ionomycin (CaI) present at 0·8 μg/ml where shown. IL-1, IL-2 or BCGF were added at Day 3 as in Fig. 1.

† Cell number ($\times 10^6$) is of viable cells and means of duplicate cultures.

‡ Viability of total populations given as percentages.

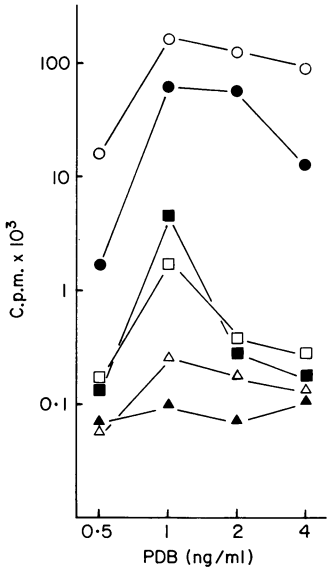

Figure 2. Response of B cells to PDB plus ionomycin. Resting B cells were plated in the presence of PDB at concentrations indicated with ionomycin at 0·8 μg/ml for 72 hr receiving a pulse of [^{3}H]thymidine for the final 16 hr. Cells were plated at the following cell densities (10^5/ml): 10 (○); 5 (●); 2·5 (■); 1·25 (□); 0·625 (△); 0·312 (▲). Cells cultured without PDB or with ionomycin alone failed to incorporate [^{3}H]thymidine significantly above background levels.

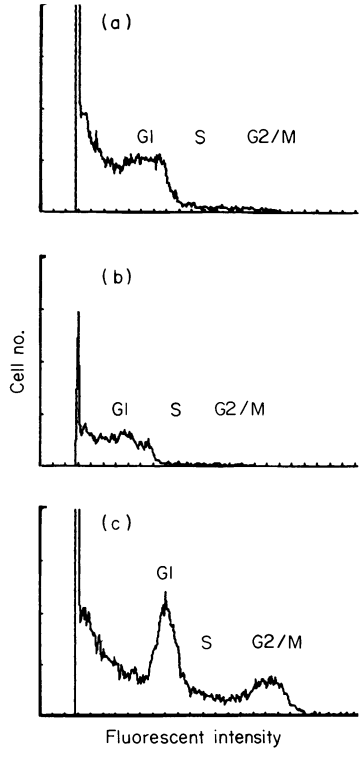

Figure 3. Maintenance of cell cycle in stimulated cells. Resting B cells were cutured for 3 days in the presence of PDB (1 ng/ml) and ionomycin (0·8 μg/ml) at a concentration of 5×10^5/ml, washed and replated at the same starting concentration for a further 3 days in the presence of: (a) control medium; (b) PDB; (c) PDB plus ionomycin. Cell cycle status at Day 6 is shown.

tinguishable from that which would be obtained if analysing a continually growing B-cell line. The addition of ionomycin by itself resulted in massive cell death (not shown).

B-cell growth response to PDB and ionomycin

Using conditions established as optimal for short-term DNA synthesis, B-cell cultures were intitiated with PDB and ionomycin as indicated in Fig. 4. By Day 5 of culture, viable cell number had increased approximately four-fold whether cultures were initiated at 5 or $2{\cdot}5 \times 10^5$ cells per ml. As when using PMA as co-stimulant, it was noted that such cultures, if undisturbed, turned acidic, resulting later in overwhelming cell death. Therefore in some experiments, cells were taken from culture at Day 5, washed and then replated with PDB plus ionomycin at the initial lower cell concentrations. Three days later (Day 8 after initial primary culture) a modest further increase in cell number was noted for cells which had been replated at 5×10^5/ml but not for those recultured at $2{\cdot}5 \times 10^5$/ml. When such recultures were analysed for cell cycle status, it was seen that only those re-initiated at the higher cell density were maintaining the cycle, whereas those at lower density were accumulating in the G0/G1

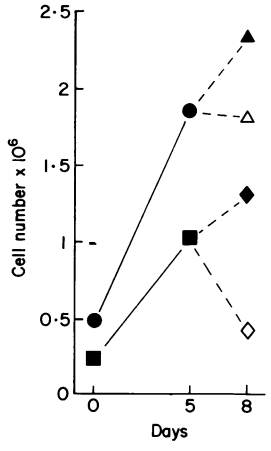

Figure 4. Growth of B cells cultured with PDB and ionomycin. Cultures were initiated with B cells at either 5×10^5 (●) or $2{\cdot}5 \times 10^5$ (■) per ml in the presence of PDB plus ionomycin, as in Fig. 3. On Day 5, cells were counted and washed, then replated at new seeding concentrations of 5×10^5 (◆,▲) or $2{\cdot}5 \times 10^5$ (◇,△) per ml with the PDB/ionomycin combination. The effective cell growth by Day 8 is indicated by hatched lines.

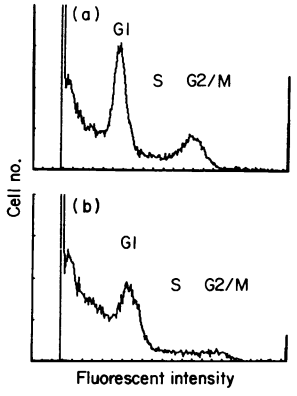

Figure 5. Maintenance of cell cycle in replated cells. Cells from experiments initiated in Fig. 4 at 5×10^5/ml, washed at Day 5 and replated at 5×10^5 (a) or $2{\cdot}5 \times 10^5$ (b) were analysed for cell cycle status on Day 8. For both Figs 4 and 5 experiments were performed in flat-bottom microwells (growth area = 0·32 cm^2).

phase (Fig. 5). Thus, even where the second messenger agonists were presented optimally in reculture, a critical B-cell density was required to sustain an active growth cycle.

DISCUSSION

The results presented herein show, for the first time, that agents which mimic the second messenger pathways generated in B lymphocytes on cross-linking their antigen receptors are capable of promoting real growth in these cells over a period of several days in tissue culture. Although the duration (8–10 days) and magnitude (four- to five-fold increase) of the growth response observed may not appear over impressive, reports on the replication of human B cells *in vitro*, even at these modest levels, are most notable by their absence. There have been only very occasional, infrequent successes described previously and the long-term, often indefinite, nature of such responses, when reported, must question whether the B cells in those studies did, indeed, remain normal (e.g. Maizel *et al.*, 1982).

For murine B cells, however, elegant studies have been presented from Melchers & Lernhardt (1985) to indicate that, albeit limited, growth can be driven and controlled in cultures that have been initiated with the mitogen lipopolysaccharide (LPS) and subsequently sustained with monocyte- and T-cell-derived soluble factors. In these studies, cell viabilities initially remained high and replication proceeded in an orderly manner dependent upon the precise timing and nature of the signals supplied. A major limitation to the success of our own attempts to promote growth of human B cells has been the appreciable concommitant cell death ensuing on stimulation. This not only reduced the apparent numbers of cells in culture but also interfered with the replication of the remaining viable population. The cell death observed cannot be attributed to the stimulating agents used, as comparable effects have been noted with stimuli considered to be more physiological (unpublished observations) or to the fetal calf serum batch, as several have been tested. Indeed, given the success of Melchers & Lernhardt (1985) who used defined serum-free medium for their murine B-cell cultures, we also examined the stimulation of human B cells under their identical conditions and actually found cell death to be increased. While we are unable to account for the high level of cell death in our cultures, part of the explanation might be that at least a proportion of cells are undergoing terminal differentiation on stimulation. This notion is upheld by the finding of large amounts of secreted IgM (but not IgG or IgA) in cultures of human B cells which have been stimulated with phorbol esters and ionomycin (our own unpublished observations).

Surprisingly, the 'growth factors' tested in this study failed to increase the number of viable cells being generated on stimulation with phorbol ester and ionophore. Indeed, a reduced rate of division was often obtained. We believe that this diminution of replication reflects the dual properties of the cytokines used in as much as these 'growth factors' also possess differentiating capacity (Gordon & Guy, 1987). Also, under the conditions of assay, the signals being continually delivered by the second messenger agonists might well overide any potential modulating influence from the soluble factors added.

While the T-cell- and monocyte-derived factors failed to contribute to the cell replication observed, there was evidence that an autocrine factor might have been necessary to sustain the growth cycle in B-cell cultures initiated with the phorbol ester and calcium ionophore combination. Thus, when stimulated cells were plated below a critical number, they stopped cycling and were found to accumulate in the G0/G1 stages of cycle. This occurred even where cultures had been restimulated with optimal concentrations of the second messenger agonists. The nature of this apparent autocrine requirement, previously observed in other T-cell-independent B-cell stimulations, is currently under investigation (Gordon, Guy & Walker, 1985).

We believe this to be the first report describing reliable, short-term growth of primary human B lymphocytes *in vitro*. It is anticipated that these preliminary observations will form the basis of further study where long-term cultures might be obtained in the absence of any transforming agent, thus allowing for a detailed analysis of normal growth regulation at successive cell division. It may be, however, that the inevitable

fate of the continually stimulated B cell is terminal differentiation leading quickly to senescence of the culture (Melamed *et al.*, 1985). Again, the system described herein should also allow for the examination of that possibility and of the mechanisms involved.

ACKNOWLEDGMENTS

The Medical Research Council (U.K.) provided financial support for this study. We would like to thank Michelle Millsum for preparing the B cells.

Dr L. Flores-Romo was in receipt of a Fellowship from Fundacion Mexicana para la Salud.

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