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TPA (12-O-tetradecanoyl-phorbol-13-acetate) activation and differentiation of human peripheral B lymphocytes

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Summary. The effect of the tumour-promoting phorbol ester TPA (12-O-tetradecanoyl-phorbol-13-acetate) on normal human peripheral blood and tonsil B lymphocytes was investigated. A strong DNA-synthesis response with the maximum at day 4 was detected. This response was, however, inhibited by increasing concentrations of serum in the medium. The membrane Ig expression was changed with a rapid decrease in IgD expression and a slower decrease in IgM and IgG expression. TPA-induced Ig secretion was detected in 12 out of 22 tested donors and the response was found to be independent of T cells and macrophages.

The expression of four monoclonal antibody-detected B cell activation and differentiation markers, B1, B2, LB1 and BB1, was followed. The results indicate activation and differentiation of the B cells.

INTRODUCTION

Phorbol ester tumour promoters induce a multitude of effects in lymphoid cells. They can act synergistically with concanavalin A or with phytohaemagglutinin to elicit mitogenic responses in mouse, human and guinea-pig lymphocytes (Baily *et al.*, 1982; Rosenstreich & Mizel, 1979; Wang, McLain & Edelman, 1975) and are mitogenic themselves for some of these cells (Baily *et al.*, 1982; Wang *et al.*, 1975). Several human lymphoblastoid lines exhibit phorbol ester responses, including interferon production (Klein & Vilcek, 1980) and transient growth inhibition (Castagna & Rochette Egly, 1979) in B cell lines, and enhanced differentiation in T cell lines (Nagasawa & Mak, 1980). Recently TPA has been shown to induce the production of T cell-growth factors (interleukin 2) by mouse cells (Farrar *et al.*, 1980), as well as by human peripheral blood lymphocytes (Sando *et al.*, 1981). Blood lymphocytes from patients with chronic lymphocytic leukaemia (CLL) have recently been shown to differentiate following TPA stimulation (Nadler *et al.*, 1981a; Tötterman *et al.*, 1981; Tötterman, Nilsson & Sundström, 1980). The induction of insulin receptors and Ig secretion, which are markers of activated lymphocytes and terminal B lymphocyte differentiation, respectively, could not be detected in these studies.

Since many studies with TPA are made on lymphocyte cell lines or freshly explanted cells from leukaemia or lymphoma patients, it is of increasing importance to understand how TPA affects normal lymphocytes. The aim of the present work was to study in some detail the TPA response of normal human B lymphocytes with respect to markers of activation and differentiation. For the former, the DNA-synthseis response was investigated as the major marker although insulin receptor expression was also studied. The ability of TPA to induce terminal differentiation

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of B cells was measured by Ig secretion and changes in the expression of surface membrane immunoglobulins. The effect of TPA treatment on the expression of four different monoclonal antibody-detected B cell-associated markers was also followed. One of these, the B1 marker, is expressed on all B lymphocytes except plasma cells (Nadler *et al.*, 1981a; Stashenko *et al.*, 1981). The B2 marker, in contrast, is restricted to certain subpopulations of B cells (Nadler *et al.*, 1981b; Stashenko *et al.*, 1981). It is expressed weakly on half of the B cells in blood and its appearance may be associated with that of IgD expression. LB1 and BB1 both detect activation markers. The LB1 antibody reacts with both T and B cells, whereas BB1 reacts only with activated B cells (Yokoshi, Holly & Clark, 1982). The changes occurring are discussed in relation to current knowledge of B cell differentiation.

MATERIALS AND METHODS

Monoclonal cells obtained from the buffy coat band by Ficoll–Isopaque centrifugation or by mincing tonsils through a fine metal mesh and clearing the suspension from debris by Ficoll–Isopaque centrifugation (Böyum, 1968) were washed and incubated with carbonyl iron for 1 hr at 37°. Iron phagocytising cells were removed with a magnet. To separate T and B lymphocytes, the cells were allowed to form rosettes with sheep red blood cells (SRBC) in 20% serum and 3% dextran T 70 (Pharmacia, Uppsala, Sweden). The rosetting cells were separated from non-rosetting cells by Ficoll–Isopaque centrifugation (Jondal, Holm & Wigzell, 1972). The non-rosetting fraction was again incubated with SRBC and rosettes were separated on a Ficoll–Isopaque gradient to remove residual T cells.

Immunofluorescence

Five hundred thousand cells were incubated with 50 μl fluorescein conjugated rabbit anti-human IgM, IgG, IgD, or $\kappa + \lambda$ chains (Dako Immunoglobulins, Copenhagen, Denmark) diluted 1:10 in BSS (1·2 mM $CaCl_2$ 138 mM NaCl, 5·4 mM KCl, 8·1 mM $MgSO_4$, 0·5 mM KH_2PO_4, 0·3 mM Na_2HPO_4 pH 6·9). After 45 min on ice, the cells were washed and resuspended in BSS: glycerol 1:1.

Staining with the monoclonal antibodies was performed with an indirect method. The cells were suspended in the monoclonal antibody at appropriate dilutions for 30 min at 0°, washed twice and resuspended in fluorescein-conjugated rabbit anti-mouse Ig (Dako) diluted 1:10. After a second 30 min incubation the non-bound antibodies were removed by washing. The cells were resuspended in BSS:glycerol 1:1 and observed in a fluorescence microscope.

Cell culture

Cells were cultured in microtitre plates (Flow Lab, Ayrshire, U.K.) at a concentration of 10^5 cells in 200 μl RPMI-1640 supplemented with 100 μg/ml streptomycin and 100 U/ml penicillin and 5% foetal bovine serum unless otherwise indicated. The cultures were incubated in a 5% CO_2, 100% humidified atmosphere at 37°. TPA (12-O-tetradecanoyl-phorbol-13-acetate) was purchased from Sigma Chemicals, U.S.A., and used at a concentration of 5 ng/ml. The Ig concentration in the supernatants was assayed by an ELISA method described elsewhere (Engvall & Perlman, 1972). The sensitivity was 5 ng/ml for IgG, 15 ng/ml for IgM, and 10 ng/ml for IgA.

DNA synthesis was assayed by incorporation of [^{3}H]-thymidine; 0·5 μCi was added to each well and the cultures were incubated for 15 hr before harvesting and counting bound radioactivity.

Insulin receptors were assayed as previously described (Åman *et al.*, 1982).

RESULTS

TPA-induced DNA synthesis was detected first after 3 days incubation (Fig. 1), with the peak on day 4, in agreement with others (Wang *et al.*, 1975). The serum concentration of the medium was shown to influence the magnitude and the duration of the response. High serum concentrations (10%) reduced and delayed the peak of the response (Fig. 1). Similar results were obtained with two tested lots of fetal bovine serum and one tested lot of newborn calf serum. We found the optimal serum concentration to be 2% for two donors and 5% for two other donors.

To investigate whether the serum inhibition of the response could be reduced by increasing the amount of TPA, we incubated cells with TPA concentrations ranging from 1 to 100 ng/ml in 2% or 20% serum (Fig. 2). High TPA concentrations failed to restore the response.

The effect of TPA treatment on the membrane Ig expression of purified B lymphocytes was studied by immunofluorescence (Table 1). These results showed a rapid decrease in the percentage of IgD positive cells after 24 hr incubation with subsequent disappearance

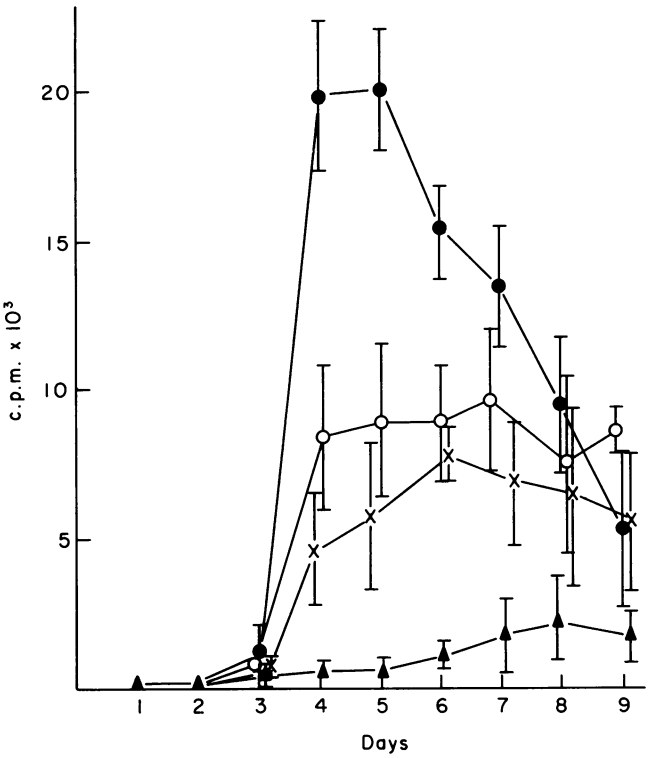

Figure 1. [^{3}H]-thymidine incorporation in human B lymphocytes incubated for 1–9 days with 5 ng TPA/ml in different concentrations of foetal bovine serum: (●) 2% serum; (○) 10% serum; (×) 20% serum. (▲) Control without TPA in 10% serum. Results are expressed as the mean c.p.m. of six donors. *Vertical bars* indicate SD.

of this isotype. In contrast, IgM expression was relatively unchanged at day 3, although by day 5 only a minority of cells were expressing this isotype. With regard to IgG expression, while there was a trend towards diminished expression of this isotype by day 5, the change was variable between donors with no consistent pattern emerging. The total number of surface Ig-positive cells, as judged by staining for light chains, did not alter dramatically until day 5 when on average only about one-third of the cells were positive.

The cell culture supernatant of TPA-treated and control cells were tested for secreted immunoglobulins of IgM, IgG, and IgA classes. In 12 out of the 22 donor populations tested, significant immunoglobulin secretion was induced due to the presence of TPA. The other 10 gave low levels of secretion equivalent to the control samples. One of the non-inducible donors was tested three times with consistent negative results.

The Ig concentration in the supernatants of TPA-treated cells at day 4 varied from <20 to 950 ng/ml IgG and from 3 to 250 ng/ml IgM. Small amounts of IgA were detected in the supernatants from two donors (<50 ng/ml).

Three tonsil B lymphocyte samples were tested, and all of them responded with a strong induced Ig synthesis of between 800 and 1400 ng/0·5 $\times$ 10^6 cells for IgM, between 2000 and 2500 ng for IgG, and between 200 and 790 ng for IgA (results from two samples are shown in Table 2).

The Ig concentration in the supernatants was tested after incubation for 4 days with different TPA concentrations. TPA concentrations between 1 and 100 ng/ml were found to induce Ig secretion and the optimal concentration was 10 ng/ml (Fig. 3).

Figure 4 shows the amount of extracellular Ig produced from six responding donors during 7 days in culture with and without TPA. The main TPA-induced IgG production was detected between days 2 and 4, while IgM production was observed mainly between days 2 and 3.

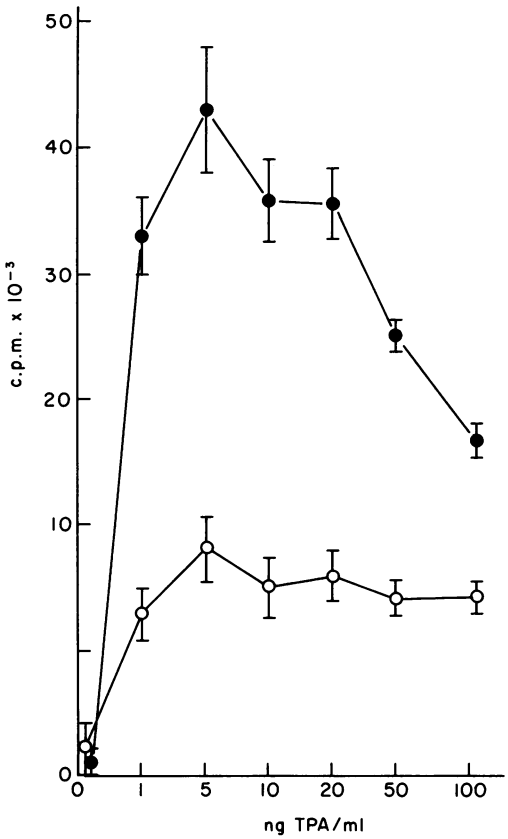

Figure 2. [^{3}H]-thymidine incorporation in human B lymphocytes incubated for 4 days with different TPA concentrations in 2% (●) and 20% (○) foetal bovine serum. Vertical bars indicate SD on results of triplicate determinations.

The T cell dependence of the Ig secretion was investigated by mixing T lymphocytes at different ratios to a highly purified B lymphocyte preparation. The B cell populations prepared from blood contained at least 85% Ig-positive cells, less than 1% SRBC-rosetting cells. Ninety per cent or more reacted for the B1 marker, and 5% reacted with the OKT3 and OKM1 antibodies. B cell preparations from tonsils contained at least 90% Ig-positive cells, 98% or more of the cells expressed the B1 marker, and less than 1% expressed the OKT3 marker. No cells reacted with the OKM1 antibodies.

From the results given in Table 2, it is clear that under the conditions of the experiments, T cells did not augment the TPA-induced secretion of either IgG or IgM that was observed when using B cells alone.

The expression of the monoclonal antibody detected antigens was tested after 1, 3 and 5 days of incubation with or without TPA present (Table 3). The B1 expression decreased slightly from a mean of 93% of the cells to a mean of 84% after TPA exposure for 24 hr. At day 3 82% of the cells were positive, but by day 5 the number of positives had decreased to 40% weakly positive cells. The control samples showed no significant change in expression.

The B2 antigen was found on a mean of 55% of the untreated cells and the expression showed a reproducible increase following TPA exposure for 1 day to 77% positive cells. By day 3, however, a decrease to 17% positive cells were noted. After this the number of B2-positive cells remained low. The control tests showed that the expression remained stable over 1 day but a decrease was noted at day 3.

Nine per cent of the cells originally expressed the BB1 marker. Following the TPA exposure, the number of positive cells increased to 14% at day 1, 42% at day 3, and to 48% at day 5. The number of positive cells in the control culture rapidly increased to 57% at day 1, but decreased again to 14% by day 3. LB1 was found on a mean of 11% of the cells, with a

Table 1. Membrane immunoglobulin expression on TPA-treated human peripheral blood B lymphocytes

Incubation time (days)	Percentage positive cells				Control			
	IgM	IgD	IgG	κ+λ	IgM	IgD	IgG	κ+λ
0	58±7	56±4	12±6	89±4				
1	59±3	5±3	13±6	88±2	62±4	41±9	14±7	84±5
3	53±6	1±0·5	8±4	80±6				
5	18±6	0	7±3	36±5				

The Ig expression was studied in immunofluorescence microscope before starting the TPA treatment and after 1, 3 and 5 days of incubation. The mean percentage positive cells from four donors is shown with standard deviation.

Table 2. TPA-induced Ig secretion of blood or tonsil B lymphocytes with or without T cells in different ratios

		B/T	B only	2	1	0·5	T only	Control (B only)
Blood	Donor 1	IgM	185	100	190	70	5	45
		IgG	300	325	290	275	10	80
	Donor 2	IgM	190	150	200	250	5	25
		IgG	400	380	380	380	10	110
Tonsils	Donor 3	IgM	880	800	810	890	60	130
		IgG	2110	2200	2190	1900	150	200
	Donor 4	IgM	1100	990	1350	1290	30	210
		IgG	2500	2200	2600	2200	220	380

Purified B and T cell preparations were mixed at different B cell/T cell ratios in medium with 5 ng/ml TPA. The total cell concentration was in all $0{\cdot}5 \times 10^6$/ml. The Ig concentration in the supernatant was assayed after five days incubation and is expressed as ng Ig/$0{\cdot}5 \times 10^6$ B cells. Controls without TPA.

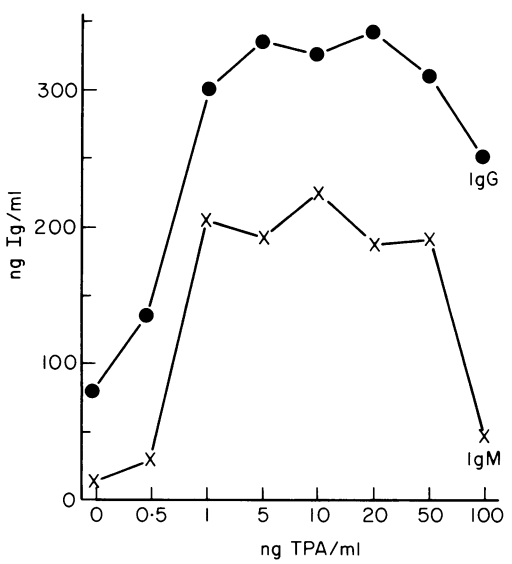

Figure 3. Ig concentration in supernatants of B+T lymphocyte cultures incubated for 5 days with different concentrations TPA in 5% serum. Results from one typical experiment are shown.

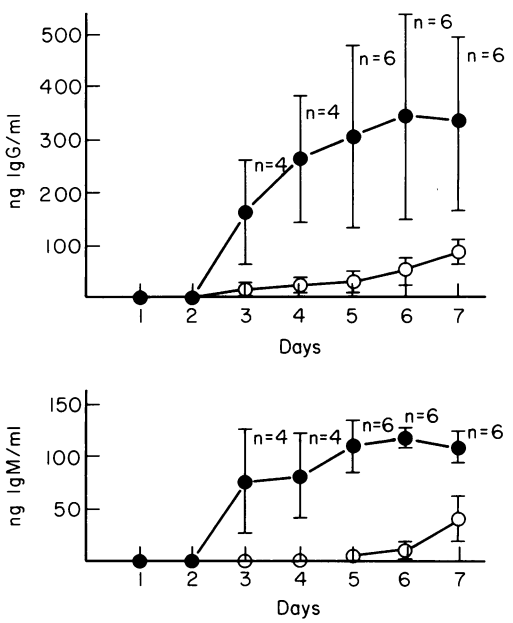

Figure 4. Immunoglobulin concentrations in B lymphocyte cultures during 7 days of incubation with 10 ng TPA/ml. (■) TPA-stimulated cultures. (□) Control. Vertical bars indicate SD.

modest increase to 17% following 1 day of exposure to TPA; at day 3 51% were staining strongly. A small decrease to 34% was noted at day 5. The control culture showed a similar pattern as seen with the BB1 expression with a strong increase to 43% positive cells at day 1 and a decrease to 11% at day 3.

Insulin receptors have been reported as a specific marker of activated lymphocytes (Krug & Krug, 1972). We have however been unable to detect any elevated insulin binding to normal B cells after different incubation periods with TPA.

Table 3. Expression of surface markers detected by the monoclonal antibodies B1, B2, BB1 and LB1 on TPA-stimulated blood B lymphocytes

Incubation time (days)	Percentage positive cells							
	B1		B2		BB1		LB1	
	C	TPA	C	TPA	C	TPA	C	TPA
0		93±3		48±3		9±3		11±3
1	93±3	84±3	49±6	76±3	35±8	16±4	43±10	17±5
3	94±6	82±5	17±4	17±6	14±2	41±16	10±4	51±11
5	ND	40±8	ND	24±9	ND	47±12	ND	40±19

The marker expression was studied by immunofluorescence microscopy before starting the TPA treatment and after 1, 3 and 5 days incubation. The mean percentage positive cells is shown with standard deviation.

DISCUSSION

In this study we have shown that serum modulates the capacity of blood lymphocytes to respond to TPA by DNA synthesis. Our results, at first inspection, appear to fit the data of Horowitz, Greenebaum & Weinstein (1981), who showed that the specific binding of phorbol esters to the cell membrane of embryonic rat fibroblasts was inhibited by serum or other body fluids. These authors showed that serum contains one or two factors that competes with the binding of phorbol esters. To investigate if the inhibition of DNA synthesis in our system was due to binding competition we tried to eliminate the serum inhibition by increasing the TPA concentrations. We also incubated the cells with TPA in serum-free medium for 1 hr before adding the serum to 20%. In both experiments however, the inhibition was not decreased. These results indicate that serum inhibition of TPA-generated DNA synthesis in lymphocytes is not due to binding competition. Full elucidation of this phenomenon will require further experimentation.

We have been unable to detect the activation specific marker of insulin receptors on TPA-stimulated B lymphocytes. This suggests that DNA synthesis and the appearance of insulin receptors may reflect different levels of activation, and TPA induces the former of these but not the latter in normal B cells. The inability to detect elevated insulin binding is consistent with earlier findings on TPA-treated CLL-cells (Tötterman *et al.*, 1981).

The immunofluorescence studies of the Ig expression showed a rapid decrease of IgD expression after TPA stimulation. These results support the hypothesis that the B cells were activated and/or induced to differentiation. IgM and IgG decreased both more slowly and to a lesser extent during the incubation period. No overt switch from expression of IgM to IgG class was seen however.

The capacity of cells to secrete Ig is the most reliable marker of progression towards terminal differentiation in B lymphocyte populations. In our study we observed a modest but significant increase in the production of extracellular Ig following exposure of cells to very low amounts of TPA in over half of the populations studied. The inability to detect an increased Ig secretion from some donors was shown to be a reproducible phenomenon and this deserves further investigation. *In vitro* pokeweed mitogen (PWM)-stimulated lymphocytes initiate Ig secretion after the peak of DNA synthesis and both DNA synthesis and Ig secretion are inhibited by irradiation of the cells (Choi & Good, 1977).

In our study, however, TPA-induced Ig secretion could be detected clearly before DNA synthesis. Surprisingly then, irradiation of the cells did completely inhibit the Ig secretion (data not shown). It may well be that the TPA-induced secretion came from a few B cells already activated *in vivo* and in more advanced stages of differentiation. This possibility is further supported by the strong response from tonsil B cells. Tonsils contain more activated cells than blood as evidenced from their reactivity with monoclonal antibodies recognizing activation markers (unpublished observations). The fact that the Ig production preceded the DNA synthesis of the bulk population

and that mainly IgG was produced is consistent with this suggestion. Further experiments may show whether already activated B cells are selectively triggered to terminal B cell differentiation and Ig secretion by TPA stimulation. B cell preparations which were depleted of phagocytic cells and contained less than 1% of SRBC-rosetting cells, responded by Ig secretion. When T cells were added, no increase of Ig production was seen. These results indicate that under the conditions of the experimental system the Ig secretion was independent of T cell help. This apparently contradicts other authors who have reported both a strong T cell and macrophage dependence of the Ig secretion response after 5 or 6 days TPA exposure (Ralph & Kishimoto, 1982; Sugavara, 1982). Their experiments, however, were performed with concentrations of TPA between 1 and 5 μg per ml which is 100 to 500 times the concentration used by us. In addition, the authors of this studies measured Ig secretion by a plaque assay which detects the number of secreting cells while we used an ELISA which assayed the total amount of secreted Ig. It may be that their very high concentration of TPA induced T cells to produce factors that amplified the B cell response.

The monoclonal antibody-detected markers showed an equally dramatic change on the B lymphocytes after TPA stimulation. The pan-B cell marker B1 decreased slightly during the first 3 days, but by day 5 only 40% of the cells remained weakly positive. These results are in good agreement with data from other experiments on peripheral blood cells where B1 expression decreased at days 4 and 5 as a result of PWM-induced differentiation (Stashenko *et al.*, 1981). In our system the initial decrease in B1 expression is associated with Ig secretion. The rapid loss of B1 at day 5 in TPA-exposed cells was, however, not associated with Ig secretion.

The B2 expression showed a transient increase on the TPA-exposed cells but was clearly lost on a majority of the cells by day 3. The B2 antibody probably detects limited subpopulations of B cells and was found on only half of the purified B cells in our preparations. Again our results are in agreement with the report of Stashenko *et al.* (1981) who worked with PWM stimulation of normal B cells. While it has been suggested that the B2 antigen is expressed together with IgD it is clear in our study that while IgD disappears after 1 day of TPA exposure, the number of B2-expressing cells actually increases. It is of course possible that TPA and PWM may push the B cells along different compartments of B cell differentiation.

The BB1 and LB1 antibodies react with activated B cells and activated B+T cells, respectively. The number of cells expressing these markers increased dramatically on TPA stimulation, indicating that these cells were indeed activated. In contrast to the BB1 expression which remained high, the LB1 marker decreased again by day 5. It is probable that cells at this stage left the differentiation step during which the LB1 marker is expressed.

The number of cells positive for the activation markers in the control cultures showed a rapid but transient increase. These changes may reflect some non-specific activation due to the manipulations of the cells during the purification or alternatively may reflect some real physiologic modulation when B cells in circulation are released from control factors. The TPA-stimulated samples showed no rapid BB1–LB1 increase however.

In conclusion, the markers detected by monoclonal antibodies suggest that TPA both activates and induces the differentiation of peripheral B cells *in vitro*. This can occur independently of T cells and macrophages. The rapid loss of IgD and induction of DNA synthesis further indicate induction of differentiation and activation respectively. However, the lack of insulin receptor expression and late (i.e. after DNA synthesis) Ig secretion differs from the pattern seen with other mitogens. TPA-induced changes in normal B cells thus appears to be a useful system for probing events associated with B lymphocyte differentiation.

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