Autocrine proliferating B cells are susceptible to terminal differentiation and apoptosis

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Abstract

Lymphocytes that proliferate autonomously are thought to be arrested at certain steps in differentiation. Here we demonstrate that autocrine proliferating B cells can be induced to terminal differentiation in the presence of ionomycin plus phorbol dibutyrate. The mature CD23^{high}/CD38^{low} B cell phenotype converts to the CD23^{low}/CD38^{high} plasma cell phenotype associated with increased immunoglobulin secretion and PC1 expression and a loss in surface immunoglobulin. Simultaneously, the cells arrest in proliferation and enter apoptosis at day 10. The cytokines IL-1 α , IL-6, TNF α and TNF β that are required to sustain continuous growth are secreted in substantially increased amounts mediating entry into apoptosis of the proliferating cells. Decrease in IL-10 secretion sustains this process. Our results draw the concept that once plasmacytoid differentiation is initiated, the growth sustaining network of autocrine cytokines is disturbed in a particular fashion depriving appropriate signals to suppress the differentiation associated apoptotic program.

Keywords: terminal differentiation; B cell maturation; autocrine growth control; apoptosis

Abbreviations: FACS, fluorescence activated cell sorter; Ig, immunoglobulin; mAb, monoclonal antibody; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide; PBU₂, phorbol dibutyrate; PKC, protein kinase C

Introduction

Normal lymphocytes are regulated by two precisely coordinated, but inversely related, genetic programs: one for proliferation and one for differentiation. The cellular development and homeostasis are maintained by a variety of mechanisms, particularly by positive and negative growth control, apoptosis, and immune selection. Increasing evidences suggest that certain cellular and molecular

events make the decision between activation, proliferation, tolerance, and apoptosis in B cells. Among these signals, the extent of cross-linking of the B cell antigen receptor plays a pivotal role providing a necessary but incomplete stimulus that requires additional signals, e.g., coreceptors, cytokines, and B cell-T cell contacts (Cohen, 1993; Krammer *et al*, 1994; Nemazee, 1993; Nossal, 1994). The ligand binding requirements for growth inhibition of immature B cells, however, are less stringent than those required for mature B cell activation (Udhayakumar *et al*, 1991) although extensive hypercross-linking of both surface IgM and IgD receptors is capable to induce apoptosis in mature resting B cells (Parry *et al*, 1994).

Defects in the control mechanisms of this process are likely to contribute to tumor development (Wyllie, 1993; Pardee, 1994; Hoffmann and Liebermann, 1994; Tlsty, 1995). On the other hand, induction of terminal differentiation resulting in programmed cell death may overcome the unlimited growth potential of the cell. This concept is sustained for leukemic/lymphoma cells by several examples: G-CSF and GM-CSF induced differentiation of established HL60 leukemic cells is associated with suppression of growth and clonogenicity (Begley *et al*, 1987); cytokine-induced differentiation of 4F10 lymphoma cells into immunoglobulin secreting cells is accompanied by growth inhibition (Eckmann *et al*, 1992); cross-linking of surface IgM inhibits proliferation of B lymphoma cells (Kim *et al*, 1992a,b) resulting in programmed cell death (Ishigami *et al*, 1992). B lymphoma cells, however, can either be inhibited or stimulated in proliferation, depending on whether the cells are stimulated with anti-Ig antibodies in soluble or immobilized form (van Endert and Moldenhauer, 1992).

In contrast to normal B cells dependent on B cell receptor-stimulation and to tumor derived cells with various defaults in growth control, autocrine dependent B cells autonomously sustain their proliferation by secretion of precisely defined amounts of certain cytokines. From the peripheral blood, we previously established autocrine B cells that continuously proliferate *in vitro* due to a subtle balanced network composed of the four synergistically acting cytokines IL-1 α , IL-6, TNF α and TNF β (Abken *et al*, 1992). Whereas autocrine proliferating lymphocytes are thought to be arrested at certain steps within their differentiation pathway, it is unresolved whether these cells can be induced to enter terminal differentiation and, finally, programmed cell death. Here we demonstrate that a substantial calcium influx plus a prolonged activation of protein kinase C does induce autocrine lymphocytes to convert from a mature B cell phenotype to a plasma cell like phenotype. This step in differentiation is accompanied by growth arrest and entry into programmed cell death due to certain alterations in the autocrine network of cytokines.

Results

Autocrine dependent B cells differentiate to plasmacytoid cells in the presence of ionomycin plus phorbol dibutyrate

The human B lymphocyte clone C-26tB7.3 was previously established from peripheral blood lymphocytes and shown to be autocrine growth dependent (Abken *et al*, 1992). The cells harbor rearranged immunoglobulin (Ig) gene segments, secrete IgA with monoclonally restricted H and L chain classes, and express a panel of surface markers indicative for mature B lymphocytes (Table 1). This phenotype is found stable for more than 2 years of continuous culture.

In the presence of ionomycin (0.8 µg/ml) plus phorbol dibutyrate (PBU₂) (3 ng/ml) the cells alter their morphology and attach to the culture dish within 24 h whereas the untreated cells grow in suspension (Figure 1). Cell staining revealed an increased basophilic cytoplasm and a small nucleus with dispersed, less dense chromatin and a prominent nucleolus compared to the untreated cells. Electron microscopic analysis additionally showed a well-developed Golgi apparatus and endoplasmic reticulum in contrast to the untreated cells.

In order to characterize the altered phenotype, we monitored certain surface marker antigens that are related to the differentiation program of B lymphocytes. CD19 and CD20 are expressed throughout pre-B and mature B cell ontogeny, whereas CD23 is preferentially found on mitogen-stimulated B cells but is absent on plasma cells (Uckun, 1990). Expression of CD54 is found on mature B cells and on plasma cells (Boyd *et al*, 1989) whereas CD38 is exclusively expressed on pre-B cells and on plasma cells but not on mature B cells (Uckun, 1990). The adhesion molecule CD11a (LFA-1) is continuously expressed during B cell differentiation although in decreased levels on plasma cells (Ahsmann *et al*, 1992). The plasma cell antigen PC1 is preferentially expressed on plasma cells (Anderson *et al*, 1985; Crawford and Ando, 1986). C-26tB7.3 cells were incubated with ionomycin, PBU₂, or ionomycin plus PBU₂ and were subjected to FACS analyses at day 5. We found

clear phenotypic differences between the parental and the treated cells (Table 1). The expression of CD20, CD23, CD11a (LFA-1), CD79, HLA DR, and surface immunoglobulin (slg) was decreased after treatment with ionomycin

Figure 1 C-26tB7.3 cells change their phenotype in the presence of ionomycin plus phorbol dibutyrate (PBU₂). C-26tB7.3 cells were cultured without supplements (a) or in the presence of ionomycin (0.8 µg/ml) plus PBU₂ (3 ng/ml) for 24 h (b). Whereas the predominant majority of the parental cells grow in suspension, nearly all treated cells adhere to the culture plate. (microscope magnification: 400 ×)

Table 1 Expression of surface markers of C-26tB7.3 cells in the presence of ionomycin and phorbol dibutyrate (PBU₂)

Surface marker	Mean fluorescence intensities (MFI ± S.E.M.) in the presence of			
	- - a	ionomycin	PBU ₂	ionomycin plus PBU ₂
Control	9 ± 7	14 ± 11	7 ± 5	18 ± 5
HLA class I	400 ± 55	510 ± 95	370 ± 40	620 ± 90
CD11a (LFA-1)	435 ± 105	220 ± 80	90 ± 70	140 ± 50
CD19	100 ± 14	150 ± 35	110 ± 25	40 ± 22
CD20	1200 ± 450	800 ± 100	250 ± 200	45 ± 32
CD21	280 ± 160	300 ± 120	120 ± 80	260 ± 140
CD23	1300 ± 380	980 ± 420	340 ± 275	30 ± 20
CD38	12 ± 8	40 ± 25	55 ± 30	140 ± 50
CD54 (ICAM-1)	220 ± 60	150 ± 90	230 ± 45	180 ± 80
CD74	53 ± 12	75 ± 21	64 ± 14	280 ± 30
CD79	340 ± 50	270 ± 60	310 ± 70	140 ± 50
PC1	250 ± 30	340 ± 110	280 ± 80	1250 ± 90
slg	760 ± 110	690 ± 80	580 ± 80	170 ± 40

C-26tB7.3 cells were cultured in the presence of ionomycin (0.8 µg/ml), phorbol dibutyrate (PBU₂) (3 ng/ml), or ionomycin (0.8 µg/ml) plus PBU₂ (3 ng/ml), respectively. At day 6, the cells were stained by immunofluorescence and mean fluorescence intensities (MFI) were determined by FACS analysis. The experiments were repeated six times and the data are expressed as the mean ± S.E.M. Control slg surface immunoglobulin. ^ano additive

plus PBU₂ compared to that of untreated cells (Figure 2). In contrast, CD38, CD74, plasma cell antigen PC1, and HLA class I expression increased after treatment whereas the expression of CD21 and CD54 (ICAM-1) was not altered. The pattern of CD23 and CD38 expression can be used to distinguish activated B cells from plasma cells (Terstappen *et al.*, 1990). Two-color FACS analysis revealed substantial differences between the treated and the untreated cell populations in respect to the coexpression of these antigens (Figure 3). Whereas the predominant majority (>80%) of the untreated cells expressed high levels of CD23 (CD23^{high}) and low levels of CD38 (CD38^{low}), the majority (>80%) of the treated cells expressed the reciprocal phenotype, i.e., CD23^{low}/CD38^{high}. The transition of the cells from the CD23^{high}/CD38^{low} to the CD23^{low}/CD38^{high} phenotype is indicative for plasma cell differentiation (Terstappen *et al.*, 1990). Accordingly, the amount of immunoglobulins (Ig) secreted by C-26tB7.3 cells into the culture supernatant was found increased after treatment with ionomycin plus PBU₂ (Figure 4). We furthermore found a twofold increase in the total protein content per cell and a threefold increase in the activity of mitochondrial dehydrogenases per cell (monitored by metabolism of MTT as substrate) indicating increased metabolic activities of the cells (data not shown). This set of analyses was repeated for two additional, autocrine dependent B cell lines (C-28t1/1 (IgG⁺) and B-10.15 (IgM⁺)) with similar results (data not shown) demonstrating that the observed phenotypic shift is a predominant event in this model.

Incubation of C-26tB7.3 cells with ionomycin (0.8 µg/ml) plus PBU₂ (3 ng/ml) increased threefold the activity of the

membrane bound protein kinase C (PKC) after 24 h compared to the untreated cells (Figure 5). PBU₂ alone only transiently raised the PKC activity to about 1.5-fold values after 30 min and ionomycin alone did not significantly alter PKC activities in the membrane fraction. Obviously, coincubation of C-26tB7.3 cells with ionomycin plus PBU₂ induced a prolonged increase of PKC activities compared to incubation with PBU₂ or ionomycin alone, respectively.

Specific inhibition of the PKC activity by chelerythrine (10 µg/ml) prevented differentiation of C-26tB7.3 cells in the presence of ionomycin plus PBU₂ as did chelating of extracellular Ca²⁺ by EGTA (1–10 mM). Incubation with PBU₂ alone did not induce plasmacytoid differentiation but altered growth kinetics of autocrine B cells dependent on the PBU₂ concentration: ≤1 ng PBU₂/ml increased whereas ≥3 ng PBU₂/ml suppressed proliferation of C-26tB7.3 cells. Activation of protein kinase A by incubation with dibutyl-*c*-AMP (25–500 µM), in contrast to PKC activation, increased cell proliferation but no plasma cell differentiation was recorded (data not shown). We conclude that both a substantial calcium influx and a prolonged increase in PKC activities are required to induce plasmacytoid differentiation of C-26tB7.3 cells.

The autocrine B cells loose autonomous growth stimulation and enter apoptosis after plasmacytoid differentiation

C-26tB7.3 cells proliferate autonomously for more than 2 years *in vitro* without detectable growth arrest or senescence. In the presence of ionomycin plus PBU₂,

Figure 2 The expression of certain B cell markers is altered after incubation of C-26tB7.3 cells with ionomycin plus phorbol dibutyrate (PBU₂). C-26tB7.3 cells were incubated in the presence of ionomycin (0.8 µg/ml) plus PBU₂ (3 ng/ml) and monitored for the expression of the B cell marker CD11a (LFA-1), CD19, CD20, CD21, CD23, CD38, CD54 (ICAM-1), and PC1. Control specifies immunofluorescence staining with a secondary antibody to indicate the background. The experiments were performed six times and the data are expressed by the mean ± S.E.M. MFI: mean fluorescence intensity

Figure 3 Correlated alteration in the expression of CD23 and CD38. C-26tB7.3 cells were cultured without additives or in the presence of ionomycin (0.8 μ g/ml) plus PBU₂ (3 ng/ml). At day 4 the cells were stained with fluorescein isothiocyanate(FITC)-conjugated anti-CD23 mAb and with phycoerythrin(PE)-conjugated anti-CD38 mAb and were subjected to FACS analysis. Plots representing approximately 5000 gated events are shown from a representative analysis. Controls illustrate the binding of a FITC- and PE-conjugated mAb with irrelevant specificity to monitor unspecific binding to the cells. FI: fluorescence intensity

however, the number of viable cells decreased to 50% within 10 days (Figure 6A). Simultaneously, the incorporation of BUdR into nuclei during a 10-h-incubation period decreased from 27% to less than 2% of the cells at day 5 indicating a substantial decrease in cycling cells. We moreover found oligonucleosomal fragmented DNA in increasing amounts reaching maximum on day 12–16. This observation is based (i) on electrophoretic separation of cellular DNA after *in vitro* labelling with α -³²P-dCTP by the Klenow DNA polymerase reaction (Figure 6B) and (ii) on a sandwich ELISA that detects mobilized nucleosomes in the cytoplasmic fraction of the cells (Figure 6C). The portion of cells with fragmented nuclear DNA rose from background levels at day 4 to 70–80% at day 12–16 (Figure 6D) as recorded by *in situ* labelling of DNA molecule ends by TUNEL techniques. We conclude that after arrest in proliferation in the presence of ionomycin plus PBU₂, the cells exhibit a median life span of 10 days and subsequently die by programmed cell death.

C-26tB7.3 cells secrete and re-internalize the cytokines IL-1 α , IL-6, TNF α , and TNF β that together are required in precisely defined concentrations to sustain continuous proliferation (Abken *et al*, 1992). In addition, the cells secrete constitutively IL-10 that, however, does not seem to be required for growth in limited dilution cultures. In the presence of ionomycin plus PBU₂, the C-26tB7.3 cells secrete substantially increased amounts of IL-1 α (sixfold), IL-6 (ninefold), TNF α (35-fold), and TNF β (3.5-fold) into the culture medium compared to the untreated cells. In contrast, secretion of IL-10 dropped to about 20% the level of the untreated cells (Figure 7).

In order to prove whether the altered concentrations of these cytokines contribute to growth arrest and apoptosis, we supplemented the medium of the autocrine proliferating C-26tB7.3 cells with the recombinant cytokines in those concentrations that we recorded after growth arrest. A series of substitution experiments revealed that addition of TNF α (1.7 ng/ml) induces growth arrest to certain degrees.

Figure 4 C-26tB7.3 cells secrete increased amounts of immunoglobulins in the presence of ionomycin plus PBU₂. C-26tB7.3 cells (2×10^6 cells/ml) were cultured in medium without additives (■) or in the presence of ionomycin (0.8 µg/ml) plus PBU₂ (3 ng/ml) (▨), respectively. At day 4, the cells were washed and cultured in fresh medium for 24 h. The amount of immunoglobulin (Ig) per ml secreted into the culture supernatant per 24 h was determined by ELISA and normalized to 2×10^6 viable cells

However, TNF α (1.7 ng/ml), TNF β (7 ng/ml), IL-1 α (150 pg/ml), plus IL-6 (100 pg/ml) together effectively induced growth arrest of C-26tB7.3 cells (Figure 8). Neutralization of IL-10 by an anti-IL-10 antibody in addition to supplementation with high amounts of IL-1 α , IL-6, TNF- α , plus TNF- β increased growth arrest whereas neutralization of IL-10 alone did not alter growth of C-26tB7.3 cells. Addition of IL-10 (0.2–2 ng/ml), however, did not reverse growth suppression by the four cytokines in high concentrations. No differentiation to a plasmacytoid phenotype was observed after addition of the altered amounts of the cytokines to continuously growing B cells. The substitution experiments demonstrate that the same cytokines that sustain continuous growth mediate growth arrest of autocrine B cells dependent on the cytokine concentration in the culture environment.

Discussion

The present analysis was performed to prove whether autocrine dependent B cells with unlimited proliferation potential can be induced to terminal differentiation and to apoptosis. Here we demonstrate that in the presence of ionomycin plus phorbol dibutyrate, the phenotype of the

Figure 5 Prolonged increase in the protein kinase C activity in the presence of ionomycin plus phorbol dibutyrate. C-26tB7.3 cells (10^6 cells/ml) were incubated in Iscove's medium without supplements or in the presence of ionomycin (0.8 µg/ml), phorbol dibutyrate (PBU₂) (3 ng/ml), or ionomycin plus PBU₂. The activity of the protein kinase C in the membrane fraction (closed columns) and in the cytoplasmic fraction (open columns), respectively, of the cells in presence of supplements (N) was normalized to the activity of the cells incubated without supplements (No)

mature B cells changes in a particular pattern: a shift from the CD23^{high}/CD38^{low} to the CD23^{low}/CD38^{high} expression profile, increase in Ig secretion, increased expression of PC1 and CD74, and a loss in surface Ig, CD11a(LFA-1), CD19, and CD20 expression. This particular change in phenotype is indicative for plasma cell differentiation (Terstappen *et al*, 1990; Anderson *et al*, 1985; Crawford and Ando, 1986). We recorded the same phenotypic shift in the three autocrine dependent B cell lines tested (C-26tB7.3, B-10.15, C-28t1/1) indicating that the plasma cell differentiation is a predominant event in this model.

Both a substantial calcium influx plus a prolonged stimulation of protein kinase C are required for induction of plasmacytoid differentiation because each ionomycin and PBU₂, respectively, did not initiate this process. Since

induction of differentiation by B cell receptor ligation generates sustained elevations of intracellular Ca²⁺ concentrations, it is tempting to speculate that the amplitude and/or duration of Ca²⁺ signalling may be an

Figure 6 In the presence of ionomycin plus porbolic dibutyrate, C-26tB7.3 cells arrest in autonomous proliferation and harbor oligonucleosomal fragmented chromosomal DNA. (A) C-26tB7.3 cells (10⁶ cells/ml) were incubated in Iscove's medium without supplements [●—●] or in presence of ionomycin (0.8 μg/ml) plus PBU₂ (3 ng/ml) [○—○]. The number of viable cells was determined by the trypan blue exclusion assay. (B) C-26tB7.3 cells (10⁶ cells/ml) were cultured without additive (lane 1), or in presence of ionomycin (0.8 μg/ml) plus PBU₂ (3 ng/ml) (lanes 2–5). At day 3 (lane 2), 7 (lane 3), 12 (lane 4), and 16 (lane 5), respectively, cells were lysed and high molecular weight DNA was sedimented by centrifugation. Fragmented DNA in the supernatant was labelled with α-³²P-dCTP by Klenow polymerase reaction, separated by agarose gel electrophoresis, and visualized by autoradiography. (C) Oligonucleosomal DNA-histone complexes that accumulate during apoptotic degradation of chromosomal DNA were monitored by a sandwich ELISA as described in Materials and Methods. Briefly, the DNA-histone complexes within the supernatant of a cellular lysate were captured by an anti-DNA antibody anchored onto a microwell plate and were quantitatively monitored by a peroxidase-coupled anti-histone antibody. Recordings of cells incubated with ionomycin (0.8 μg/ml) plus PBU₂ (3 ng/ml) [N] were normalized to those of an untreated control culture [N₀]. (D) C-26tB7.3 cells were incubated in the presence of ionomycin (0.8 μg/ml) plus PBU₂ (3 ng/ml). By means of the terminal transferase reaction in presence of FITC-dUTP (TUNEL technique) and fluorescence microscopy, cells in apoptosis were visualized. The portion of TUNEL positive cells from total number of cells (%) is recorded

Figure 7 C-26tB7.3 cells secrete altered amounts of cytokines into the culture medium after induction of the plasmacytoid phenotype. C-26tB7.3 cells (10^6 cells/ml) were cultured in Iscove's DME medium without additives (■), or in presence of ionomycin ($0.8 \mu\text{g/ml}$) plus PBU $_2$ (3 ng/ml) (▨), respectively. At day 3, the cells were washed, plated in fresh culture medium (10^6 cells per ml) for 24 h, and the amount of the specified cytokines in the culture supernatant were determined by ELISA. The experiment was repeated five times and the mean $\pm$ S.E.M. is given. The medium background was below detection limit of the assay

important parameter in the biological response and in the induction of apoptosis of B cells. Although this proposal seems reasonable in view of the known susceptibility of lymphocytes to Ca^{2+} ionophore-induced apoptosis, an additional prolonged PKC stimulation is here shown to be required to contribute to the induction of differentiation and apoptosis of autocrine B cells. In B lymphoma, B-CLL cells, and Burkitt's lymphoma cell lines, an imbalance in slg-dependent signalling pathways, which could favour sustained intracellular Ca^{2+} increase, has been proposed to be causal for the apoptotic death (Benhamou *et al*, 1994; McConkey *et al*, 1991; Knox *et al*, 1992).

Whereas elevation of the cytosolic Ca^{2+} level represents a common trigger for apoptosis in various cells (McConkey and Orrenius, 1995), in some model systems, however, elevation of the cytosolic Ca^{2+} level has been shown to inhibit apoptosis. In IL-3 dependent hematopoietic progenitor cells, the calcium ionophore A23187 suppresses apoptosis (Rodriguez-Tarduchy *et al*, 1992, 1994) whereas phorbol esters protect the IL-3 dependent cells from apoptosis (Rajotte *et al*, 1992). The effect of the ionophore in that system is due to secretion of IL-4 which in turn promotes survival (Rodriguez-Tarduchy *et al*, 1994).

Autonomous proliferation of the C-26tB7.3 cells depends on the secretion of IL-1 α , IL-6, TNF α , and TNF β in subtle balanced amounts (Abken *et al*, 1992). After induction of plasmacytoid differentiation, the cells arrest in proliferation and secrete these cytokines in substantially increased amounts. When added in those altered concentrations to the untreated proliferating C-26tB7.3 cells, the same cytokines that are required for autocrine growth stimulation mediate growth arrest indicating that the potential of the

cytokines to sustain continuous proliferation is strictly dose dependent. We speculate that the implementation of an underlying cell death program is inhibited as long as the cytokine network of autocrine stimulation is available to suppress the apoptotic process. In the induction of growth arrest of autocrine B cells, decrease in IL-10 secretion acts synergistically with increase in IL-1 α , IL-6, TNF α , and TNF β secretion because neutralization of IL-10 enhanced growth arrest and cell death. Addition of IL-10, however, did not prevent apoptosis of the cells in presence of ionomycin plus PBU $_2$. This is in accordance to the report that in lymphoma cells IL-10 expression does not protect from calcium-mediated apoptosis (Levy and Brouet, 1994; Liu *et al*, 1991). In B-CLL cells, IL-10 exhibits proapoptotic activities (Fluckiger *et al*, 1994) whereas the apoptosis of germinal center B cells is prevented by IL-10 (Levy and Brouet, 1994).

Several contradictory observations imply that the situation of re-entry into the differentiation and apoptosis pathway seems to be different in the autocrine non-tumorigenic B cells compared to tumorigenic B lymphoma cells. In lymphoma cells, TNF α ($1-10 \text{ ng/ml}$) protects against calcium dependent apoptosis but does not potentiate the protective effect of phorbol esters on ionomycin-induced apoptosis (Genestier *et al*, 1995). Treatment of lymphoma cells with ionomycin results in apoptosis that can be prevented by phorbol ester mediated PKC activation (Ishigami *et al*, 1992; Knox *et al*, 1992; Bonnefoy-Berard *et al*, 1994; Kim *et al*, 1992a,b; Vazquez *et al*, 1991). In B lymphoma cells insufficient PKC activation is suggested to be related to slg-induced growth inhibition and apoptotic death (Benhamou *et al*, 1990; Sarthou *et al*,

Figure 8 Continuous growth of C-26tB7.3 cells is inhibited in the presence of altered concentrations of the cell secreted cytokines. The autonomously proliferating C-26tB7.3 cells (10^6 cells/10 ml) were incubated in Iscove's medium without additives [●—●] or supplemented with the recombinant human cytokines in those concentrations that were recorded after incubation with ionomycin (0.8 μ g/ml) plus PBU₂ (3 ng/ml) (cf. Figure 7) [○—○]: TNF α (1.7 ng/ml), TNF β (7 ng/ml), IL-1 α (150 pg/ml), and IL-6 (100 pg/ml). Alternatively or additionally, an anti IL-10 antibody (10 μ g/ml) was added. The number of viable cells was determined by trypan blue assay

1989). This is in agreement with the observation that PKC activation rescued several lymphoma cells from anti-Ig-induced cell death: Peyer's patch B-cells (Motyka *et al*, 1993), Burkitt's lymphoma cells (Knox *et al*, 1992), Ramos cells (Valentine and Licciardi, 1992), and the immature BKS-2 lymphoma cells (Muthukkumar *et al*, 1993). Moreover, PKC activation prevents germinal center B cells (Knox *et al*, 1992), ilial Peyer's patch B cells (Motyka *et al*, 1993), and resting splenic B cells (Illera *et al*, 1993) from spontaneous apoptosis. These studies suggest that insufficient PKC activities may be at least partially responsible for both antigen-induced apoptosis in immature B cells and for apoptotic death-by-default of mature B

cells deprived of antigenic stimulation. Since sustained PKC activation blocks the ability of anti-Ig antibodies to induce Ca²⁺ mobilization in B cells and in lymphomas (Bijsterbosch and Klaus, 1987; Gold and deFranco, 1987), low level PKC activation might result in sustained intracellular Ca²⁺ elevation which in turn would trigger the apoptosis program.

Some discrepancies in the effect of calcium influx plus PKC stimulation to B cells at various stages of differentiation and neoplastic transformation may moreover reflect differential susceptibilities of the cells to these signals. This may be due to multiple PKC isoforms, which may differentially respond to antigenic stimulation and vary in

substrate specificity and activation requirements (Marquez *et al*, 1992). Regarding susceptibility of B cells to differentiation and apoptosis signals, it is unresolved, however, whether or not PKC isoform expression is developmentally regulated.

Materials and Methods

Cell lines

The B lymphocyte lines C-26tB7.3, C-28t1/1 and B10.15 were independently established from peripheral blood lymphocytes as described (Abken *et al*, 1993). Cells were cultured in Iscove's DME medium supplemented with insulin (0.1 U/ml), transferrin (1 µg/ml), pyruvate (1 mM), oxaloacetic acid (1 mM), HEPES (20 mM), and 10% fetal calf serum (FCS) ('Myoclonal' plus serum, Gibco BRL, Paisley, Scotland). To monitor the cell viability, metabolic degradation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) was colorimetrically determined (Mosmann, 1983).

Assays for apoptotic cells

Cells (10^7) were lysed in 500 µl of lysis buffer (5 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% Triton X-100) for 15 min at 4°C and high molecular weight DNA was sedimented by centrifugation at $10\,000 \times g$ for 20 min. The DNA fragments within the supernatant were radioactively labelled with α - 32 P-dCTP by terminal transferase reaction and analyzed by agarose gel electrophoresis. Alternatively, the nucleosomal DNA fragments were captured by an anti-DNA antibody anchored onto a microwell plate and quantitatively monitored by a POD-conjugated anti-histone antibody ('cell death detection assay', Boehringer Mannheim, Mannheim, Germany). For *in situ* detection of apoptotic cells, cells on cytospin slides were fixed, permeabilized, and incubated in the presence of terminal deoxynucleotidyl transferase and FITC-labelled dUTP (TUNEL technique) (Gavrieli *et al*, 1992). After washing, the cells with accumulation of chromosomal DNA breaks are detected by fluorescence microscopy.

FACS analyses

Cells were labelled with mouse monoclonal antibodies specific for the human CD11a, CD19, CD20, CD21, CD23, CD38, CD54, CD74, CD79 antigen, respectively, conjugated with FITC or PE. Fluorescence analyses were performed on a FACScan (Becton-Dickinson, Mountain View, Cal.).

Cytokine detection

Cell-free culture supernatant was obtained from a 24 h culture of cells seeded at 10^6 cells per ml. The concentration of the particular cytokine was monitored by ELISA tests (TNF α and IL-6 ELISA from Boehringer Mannheim, Mannheim, Germany; TNF β , IL-1 α , and IL-1 β ELISA test from BB British Biotechnology, Abingdon, U.K.; IL-10 ELISA test from Biosource, Camarillo, Cal.).

Analysis of the PKC activity (Casnellie, 1991)

Cells (5×10^6) were washed in PBS containing 1 mM sucrose and resuspended in 500 µl of lysis buffer (Tris HCl (20 mM), pH 7.5, sucrose (330 mM), EDTA (2 mM), EGTA (0.5 mM), PMSF (2 mM)). The cells were lysed by ultra sonication at 4°C and the lysate centrifuged at $10\,000 \times g$ for 30 min. The supernatant contains the

cytoplasmic fraction, the sediment the membrane fraction. The sediment was stirred for 1 h in lysis buffer, Triton X-100 (1%) at 4°C and subsequently centrifuged at $10\,000 \times g$ for 30 min to obtain the cleared membrane fraction. To monitor the PKC activity, cytoplasmic or membrane fraction (5 µl), respectively, was incubated in 100 µl of reaction buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl $_2$, 0.5 mM CaCl $_2$, 0.25 µg/ml bovine serum albumin) in presence of 1,2-dioleoyl-sn-glycerol (2 µg), phosphatidylserine (10 µg), the synthetic PKC substrate Ac-MBP(4-14) (25 µM) (Boehringer Mannheim, Mannheim, Germany), and of γ - 32 P-dATP (0.5 µCi) for 12 min at 30°C. The reaction mixture was fixed onto P80 paper and subsequently washed in phosphoric acid (0.1 M) and in ethanol (96%). Radioactivity bound to the paper was determined by scintillation spectrometry.

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