SYNERGISTIC ACTION OF A23187 AND PHORBOL ESTER ON HUMAN B CELL ACTIVATION\textsuperscript{1}

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We have investigated the existence of a synergy occurring between the calcium ionophore A23187 and phorbol myristate acetate (PMA) with respect to human B cell proliferation and differentiation. The combination of A23187 (250 to 500 nM) with nonmitogenic concentrations of PMA (1 to 3 ng/ml) resulted in a strong proliferative response in human tonsillar, spleen, and peripheral blood B cells. This proliferation could not be blocked by anti-Tac antibody at concentrations that effectively inhibited T cell proliferation under similar culture conditions, suggesting that IL 2 and its receptor are not involved in B cell proliferation in this system.

During a 3-day culture period, A23187 (500 nM) did not activate B cells in terms of changes in cell size or in the expression of transferrin receptor, HLA-DR, and Tac antigen. PMA at a nonmitogenic concentration (3 ng/ml) enhanced the expression of the first two markers. Combination of the ionophore with PMA induced the occurrence of Tac and further increased the expression of transferrin receptor and HLA-DR. A23187 similarly enhanced the PMA-mediated increase in cell size.

PMA and A23187 did not induce differentiation to Ig production. However, when cells were prestimulated with a combination of the two agents and were recultured in the presence of a preparation containing B cell differentiation factor, a strong increase in IgM, IgG, and IgA production was found. We conclude that PMA and A23187 synergistically trigger intracellular events in human B cells, leading to proliferation and to responsiveness to differentiation factors.

The tumor-promoting compound phorbol myristate acetate (PMA) produces a variety of effects on normal cellular functions, most likely mediated by its ability to activate the enzyme protein kinase C [1]. Thus, PMA has been reported to stimulate proliferation of human T lymphocytes [2–4]. Interestingly, a strong synergism between PMA and agents such as PHA, anti-T3 antibodies, or calcium ionophores was noted with respect to the induction of interleukin 2 (IL-2) production by the Jurkat cell line [5]. The same effect was observed in relation to proliferation of murine thymocytes [6] and human resting T cells [7]. These agents, synergizing with PMA, are all known to give rise to a rapid increase in free cytosolic calcium ([Ca\textsuperscript{2+}])\textsuperscript{3} in cells of T lineage [5, 8].

Human B cells proliferate in response to PMA [9]. This effect is augmented by anti-IgM [10], again an agent which reportedly raises [Ca\textsuperscript{2+}]\textsuperscript{3} [11, 12]. PMA also has been reported to increase la expression of murine B cells [13], Tac expression of human B cells [14], as well as immunoglobulin (Ig) production by a human B cell line [15].

The present study focuses on a synergism between PMA and the ionophore A23187 with respect to various aspects of human B cell activation, including proliferation, expression of activation markers, and differentiation to Ig-producing cells.

\textbf{MATERIALS AND METHODS}

\textit{Preparation of cells}. Human spleen and tonsil samples were dissociated in Tris-buffered minimal essential medium (MEM; GIBCO, Grand Island, NY) plus antibiotics. Heparinized blood was obtained from healthy volunteers. Mononuclear cells were obtained from these suspensions by density gradient centrifugation on Ficoll-Isoopaque (density = 1.077 g/cm\textsuperscript{3}) at 1000 × G for 20 min and were washed twice with MEM. The cells were resuspended at 2 × 10\textsuperscript{6} cells/ml in MEM containing 10% fetal calf serum (GIBCO). Adhering cells were removed from the mononuclear cell suspensions by a 60-min incubation in 250-ml plastic flasks (Nuncl, Kamstrup, Denmark), after which time the nonadhering cells were gently poured off. T cells were removed by two rounds of rosette formation with sheep erythrocytes treated with 2-aminoethyl isothiouroniumbromide hydrobromide. Samples were further depleted of T cells by complement-dependent lysis with the use of the CD3 monoclonal antibody E7 [a generous gift of Dr. H. Kreeftenberg, National Institute of Health, Bihov, The Netherlands]. To this end, 10\textsuperscript{6} cells were incubated on ice in 1 ml of MEM containing 20 µl of E7 ascites. After 60 min, 1 ml of freshly reconstituted Low Tox-H rabbit complement (Cedarlane, Hornby, Ontario, Canada) was added, followed by a 90-min incubation at 37°C. Cell preparations thus obtained contained less than 0.25% T cells, as judged by immunofluorescence with the CD2 monoclonal antibody OKT-1 (Ortho Diagnostics, Beeze, Belgium). Even after a 4-day culture with PMA (1 ng/ml) and A23187 (250 nM), which induces vigorous T cell proliferation (7), less than 0.5% T cells were detectable.

\textit{Cell cultures}. The cells were cultured in 96-well, flat-bottomed microtiter plates (Nunc) at 2 × 10\textsuperscript{4} per well containing 200 µl of culture medium consisting of RPMI 1640 (GIBCO), supplemented with 10% fetal calf serum (GIBCO), glutamine, and antibiotics. Proliferation was determined by the addition of \textsuperscript{3}H(thymidine (1 µCi/well; specific activity 5 mCi/mmol) 20 hr before the end of the culture period. The cells were harvested and the incorporated activity was

\textsuperscript{3}Abbreviations used in this paper: BCGF, B cell differentiation factor; BCGF, B cell growth factor; [Ca\textsuperscript{2+}]; free cytosolic calcium; DG, diacylglycerol; MEM, minimal essential medium; SAC, Staphylococcus aureus Cowan I strain.

\\textsuperscript{1}Received for publication June 24, 1985.
\textsuperscript{2}Accepted for publication August 27, 1985.
\textsuperscript{3}The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
\textsuperscript{4}This work was supported by the Foundation "De Drie Lichten" and by the Foundation for Medical Research FUNGO, which is subsidized by the Netherlands Organization for the Advancement of Pure Research (ZWO) (Grant 900-506-091).
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determined in a liquid scintillation counter. All tests were performed in quadruplicate. The standard deviation never exceeded 10%. A23187 was purchased from Sigma Chemical Co., St. Louis, MO, was dissolved in dimethylsulfoxide at 10 mM and stored at -20°C. PMA was purchased from Sigma, dissolved in dimethylsulfoxide at 5 mg/ml, and was stored at -20°C. Anti-Tac ascites, recognizing the human IL 2 receptor, was kindly provided by Dr. T. Waldmann, Metabolism Branch, National Institutes of Health, Bethesda, MD. The human T cell hybrid Ko 2.9, generated in our laboratory, was used as a source of B cell differentiation factor (BCDF). This cell line has been shown to produce IL 2 as determined in the cytotoxic T lymphocyte proliferation assay, and BCDF as measured by the induction of Ig secretion in Staphylococcus aureus Cowan I strain (SAC)-pretreated B cells. Recombinant IL 2 (Sandzis, Basel, Switzerland) and Jurkat IL 2 could not substitute for Ko 2.9 supernatant in the latter assay (T. Logtenberg, manuscript in preparation).

Fluorescence studies. Cells were cultured in 24-well, flat-bottomed culture plates at 10⁶/well in 1 ml, were harvested, and were washed twice with MEM. Cells were then suspended in 10 μl of antibody: anti-Tac 1/1000 ascites; anti-HLA-DR-FITC (Becton Dickinson, Mountain View, CA); or anti-transferrin receptor-FITC (Becton Dickinson). Staining with anti-Tac was followed by a second step using FITC-labeled goat anti-mouse Ig (Becton Dickinson). The samples were then resuspended in 1 ml of MEM containing 1% bovine serum albumin, and were analyzed on an Ortho-Cytolourograph. Data analysis was based on reading 10,000 cells per sample.

The Spot-ELISA test. The Spot-ELISA, used to enumerate individual human B cells secreting IgM, IgG, and IgA class antibodies, has been described in detail elsewhere (16, 17). In brief, cultured cells were washed and were incubated for 18 hr at 2000 cells per well in 96-well plates which had been coated previously with affinity-purified goat anti-human IgM (Tago, Burlingame, CA; batch no. 011401), anti-IgG (Tago; batch no. 003701, 1/500), or anti-IgA (Tago; batch no. 011401, 1/1000). After removal of the cells, individual wells were incubated with affinity-purified goat anti-human IgM, IgG, or IgA conjugated to alkaline phosphatase (Tago; batch nos. 920920, 901402, and 910703, each diluted 1/1000). Finally, after rinsing the wells, the alkaline phosphatase substrate 5-bromo 4-chloro 3-indolyl phosphate (Sigma) was added in agaroase. After 30 min, the first antibody-secreting cells became visible as blue spots. The number of spots was routinely determined after 18 hr with the use of an inverted microscope.

RESULTS

Cell proliferation. As shown in Figure 1, the ionophore A23187 was not mitogenic for tonsil, spleen, and blood B cells. However, combination of the ionophore with PMA resulted in a strong proliferation, even if the phorbol ester was present in nonmitogenic concentrations (1 to 3 ng/ml). The proliferation induced by the combination of A23187 (500 nM) and PMA (3 mg/ml) could not be blocked by anti-Tac antibody at concentrations that did effectively inhibit T cell proliferation induced by the combination of two agents (Fig. 2).

B cell activation markers. The expression of three surface markers related to B cell activation was measured during the first 3 days of culture in the presence or absence of A23187 and PMA. Figure 3 shows the results obtained with tonsil B cells; similar responses were found for blood and spleen B cell fractions. Ionophore A23187 apparently does not modify the expression of the markers as compared to the controls. PMA induces a clear increase in HLA-DR and transferrin receptor expression. Combination of the two agents enhances the effect of PMA on these two markers and induces the appearance of Tac antigen. The ionophore does not affect cell size, but enhances the PMA-mediated increase of this parameter (Fig. 4).

Ig production. The effect of the combined action of PMA (3 mg/ml) and A23187 (500 nM) on a third aspect of B cell activation, i.e., Ig production, was analyzed in the Spot-ELISA. The combination of the two agents did not induce Ig production, as assessed on days 3, 4, and 5 of culture. We next investigated whether B cells prestimulated with PMA and the ionophore would acquire responsiveness to BCDF. To that end, purified blood B cells were

Figure 1. Cell proliferation of spleen, tonsil, and blood B cells was measured after a 4-day stimulation with the indicated concentrations of PMA and A23187.

Figure 2. T lymphocytes were optimally stimulated with A23187 (100 nM) and PMA (1 mg/ml); tonsil B cells by A23187 (500 nM) and PMA (3 mg/ml). To the cultures was added anti-Tac ascites at the indicated dilutions, or control ascites from the hybridoma fusion partner AG8 at 10⁻⁶ dilution. Proliferation was assessed on day 4 of culture.

Figure 3. The expression of the surface markers transferrin receptor (Trf-Rec), Tac, and HLA-DR by tonsil B cells was determined on each day of a 3-day culture period in the absence of stimuli (I), and in the presence of A23187 (500 nM) (II), of PMA (3 mg/ml) (III), and of both agents (IV). The results are presented as the cumulative percentage (y-axis) of fluorescence in arbitrary units on a linear scale (x-axis).
prestimulated with PMA and A23187 for 24 hr, were washed, and were recultured in the presence or absence of 10% (v/v) of supernatant of the hybrid T cell hybridoma Ko 2.9, used as a source of BCDF. As shown in Figure 5, B cells prestimulated with PMA and A23187 clearly become responsive to the differentiation signal provided by the Ko 2.9 supernatant, as measured by IgM, IgG, and IgA production. Similar results were obtained with tonsil B cells.

**DISCUSSION**

Hydrolysis of phosphatidyl inositol lipids is a ubiquitous event accompanying many receptor-ligand interactions. One of these lipids located in the cell membrane, phosphatidyl inositol biphosphate, is hydrolyzed to diacylglycerol (DG) and inositol trisphosphate. DG acts as an activator of protein kinase C, a calcium-dependent key enzyme in cell activation. PMA, having a DG-like structure, activates this enzyme by increasing its affinity for calcium (reviewed in Reference 18). Inositol trisphosphate has been postulated to mediate the rise in (Ca**++)**, which occurs after receptor-ligand interaction (19). The use of PMA and calcium ionophores such as A23187 thus allows for separate investigation of each of the two branches of this bifurcating messenger system. There is accumulating evidence that B lymphocytes utilize this second messenger mechanism for transmembrane signaling after cross-linking of membrane-bound antigen receptors, i.e., surface Ig (20, 21). Cross-linking of surface Ig by SAC provides a trigger which leads to cell prolifer-