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Platelet Activating Factor's Role in Regulating Apoptosis in Immature B Cells

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A thesis submitted to the faculty of Graduate Studies and Research in partial fulfillment of the requirement of the degree of Master in Sciences

Key Words

B lymphocytes, Apoptosis, Lipid mediators, Co-stimulatory molecules, Oxidative free radicals, Protein synthesis, sIgM, VLA-4

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Contribution of Authors

As first author in the two manuscripts included in this thesis document I performed all the experiments except for glutathione measurement performed by Dr. Sylvain Baruchel and assessment of DNA laddering by gel electrophoresis performed by Dr. M. Newkirk in the initial manuscript. All experiments in the second manuscript were performed by myself. The advice and leadership of Dr. Bruce Mazer was provided during both the experimental and manuscript preparation of both manuscripts.

Abbreviations used thesis:

αIgM	anti-IgM antibodies
BCR	B cell receptor
DCF	2'7'-dichlorofluorescein (oxidized, fluorescent)
DCFH	2'7'-dichlorofluorescein (reduced, non fluorescent)
DCFH-DA	2'7'-dichlorofluorescein diacetate
ESA	ELISA spot assay
GSH	reduced glutathione.
PAF	platelet-activating factor
PCD	programmed cell death
PI	Propidium Iodide
PS	phosphotidylserine.
sIgM	surface IgM

TABLE OF CONTENT

Abstract (English)

B lymphocyte development is characterized by deletion, via apoptosis, of immature cells that are stimulated via the BCR in the absence of a second signal. We have investigated whether platelet-activating factor, a potent B lymphocyte activator, can provide a complementary signal with BCR ligation in order to abrogate apoptosis. Cross-linking of the sIgM on Ramos B lymphoblastoid cells using anti-IgM antibodies (2μg/ml) caused PCD in 34±5.4% of the cells. Co-incubation of PAF (10^{-7}M) with αIgM led to a significant decrease in apoptotic cells as measured by DNA laddering and TUNEL assay (13.8±3%). The effect of PAF was dose dependent (10^{-7}-10^{-9}M), and was inhibited by the specific PAF receptor antagonist, WEB 2170. PAF protected cells from the effect of αIgM for up to one hour after it was added.

αIgM-induced PCD in Ramos cells is blocked by catalase, and therefore PCD is caused in part by the production of toxic hydroxyl radicals from hydrogen peroxide. We investigated the action of PAF on markers of intracellular oxidation. H_2O_2 in low doses induced apoptosis, via production of $OH^{\cdot}$ radicals. PAF inhibited H_2O_2 induced apoptosis in Ramos cells; it also attenuated H_2O_2 and αIgM-mediated increases in hydroxyl radical ($OH^{\cdot}$) as measured by the oxidation of 2',7'-dichlorofluorescein diacetate (DCFH-DA) to DCF, and blocked the depletion of GSH induced by αIgM. PAF maintained IgM secretion, greatly inhibited by incubation with αIgM alone.

We characterized the phenotype of Ramos cells that have not become apoptotic following BCR stimulation. In these cells, there is a significant decrease in the surface expression of the VLA-4 adhesion molecule (31% of control expression) and surface IgM expression (sIgM) (53% of control expression). Significantly fewer cells co-incubated with PAF underwent apoptosis, and the remaining cells maintained control levels of VLA-4 (104% of control expression) and sIgM expression (104% of control). All of these protective effects were inhibited by the specific PAF receptor antagonist, WEB 2170. The ability of PAF to maintain sIgM expression at control levels was inhibited by cycloheximide (7.5 μg/ml), an inhibitor of protein synthesis while cytochalasin B, (5 μg/ml), an inhibitor of microtubular function, had no effect on sIgM expression. In contrast neither cycloheximide nor cytochalasin B altered PAF's ability to abrogate apoptosis.

These data indicate that PAF provides an important co-signal to sIgM stimulated Ramos cells by inhibiting apoptosis. This is in part due to the activity of PAF in the oxidant/antioxidant pathway. PAF's effect on rescuing and maintaining αIgM stimulated Ramos B cells is mediated via at least two pathways. Abrogation of apoptosis does not require de novo protein synthesis, while maintenance of sIgM expression requires proteins synthesis.

Abstract (French)

Lorsque la maturation de cellules B en plasmocytes est déclenchée par une stimulation antigénique (liaison du sIgM), seulement 5% des lymphocytes B deviennent des cellules matures, le reste des cellules B disparaissant par apoptose. Nous avons démontré que le PAF, un médiateur phospholipidique libéré durant la réponse inflammatoire, inhibe l'apoptose initiée par la liaison du sIgM. Si on utilise un αIgM (2 µg/ml), la liaison du sIgM avec des cellules lymphoblastoïdes B Ramos induit l'apoptose de 34 ± 5.4% d'entre elles. La co-incubation de l'αIgM et du PAF (10^{-7} M) entraîne une diminution significative du taux de cellules apoptotiques évalué par la méthode TUNEL et laddering de l'ADN (13.8 ± 3%). Les effets du PAF dépendent de la dose utilisée (10^{-7}-10^{-9} M); ils sont contrecarrés par WEB 2170, un antagoniste du récepteur du PAF. Le PAF protège les cellules s'il est ajouté moins d'une heure après l'addition d'αIgM.

La catalase inhibe l'apoptose induite par l'αIgM de façon efficace, ce qui suggère que le radical libre dérivé de l'oxygène OH· est un étape importante dans l'induction de l'apoptose. Le mécanisme intracellulaire qui initie l'apoptose implique la production de radicaux libres dérivés de l'oxygène (OH·, O_2^- et H_2O_2). H_2O_2 en petite dose induit l'apoptose par la production d'OH·. Le PAF empêche l'apoptose des lymphocytes immatures stimulés par l'H_2O_2 chez les cellules Ramos. Le PAF diminue l'accumulation d'OH· induite par l'αIgM ou le H_2O_2, telle qu'évaluée en utilisant du 2'7'-dichlorofluorescie diacétate (DCFH-DA) au

DCF. L'administration d'αIgM diminue le niveau de GSH et la sécrétion d'IgM, le PAF maintient le niveau de GSH et la sécrétion d'IgM malgré l'administration d'αIgM.

Nous avons caracterisé le phénotype des cellules Ramos qui ne sont pas apoptotiques après la stimulation d'αIgM. Il y a une diminution significatif de l'expression de la molécule d'adhésion VLA-4 (31% du témoin) et du sIgM (53% du témoin). Après co-incubation avec du PAF, celui-ci diminue significativement le taux de cellules apoptotiques et les cellules maintiennent leur niveau de VLA-4 (104% du témoin) et de sIgM (104% du témoin). Les effets du PAF sont inhibés par WEB 2170, un antagoniste du récepteur du PAF. L'habilité du PAF à maintenir l'expression du sIgM est inhibé par le cycloheximide (7.5 μg/ml), un inhibiteur de la synthèse de protéine, tandis que le cytochalasin B (5 μg/ml), un inhibiteur du squelette cellulaire, n'a pas d'effet sur l'expression de sIgM. Les deux agents aux doses utilisées n'ont pas d'effet sur l'habilité du PAF à inhiber l'apoptose.

Le PAF fournit un signal potentiellement important aux cellules stimulées par des antigènes menant à une inhibition de l'apoptose; ceci est dû en partie à son activité anti-oxidante. En fait, le PAF fonctionne par au moins deux voies métaboliques, l'inhibition de l'apoptose n'a pas besoin de synthèse de protéine et l'expression du sIgM nécessite la synthèse de protéine.

INTRODUCTION

The role of apoptosis in the immune system

There are two ways by which a eukaryotic cell can die. The most commonly recognized is accidental death or necrosis where due to an interruption of the blood supply or exposure to a toxin there is physical injury to the cell. The other is programmed cell death which is a genetically determined, biologically active process that plays the opposite role to mitosis in tissue size regulation (1, 2).

Apoptosis, derived from the Greek for falling off, can be compared to leaves falling off of a tree in autumn. Cells die without causing their neighbors trouble, unlike necrotic cell death which is accompanied by an inflammatory response. During apoptosis cells neatly package their contents and offer them to their neighboring cells which take up these cells by phagocytosis and use them in an environmentally conscious cellular recycling (2, 3).

Apoptotic cells are quite different from cells dying necrotically as can be seen in table 1. Necrosis is hallmarked by cell swelling and cell lysis with the nuclear material being excluded from any obvious pathological effect. In contrast, apoptosis involves loss of cell volume and cleavage of nuclear material in a cell that retains its surface membrane integrity. As a result of necrosis there is a release of cell contents with an inflammatory response while the apoptotic cell retains its membrane integrity and is phagocytosed without an inflammatory response (3, 4).

Table 1

	NECROSIS	APOPTOSIS
Morphology		
Cell	Swells	Shrinks
Nuclear Material	Disintegrates	Condenses and becomes packed
Other organelle	Swell	Normal
Cell membrane	Ruptures	Remains intact, Blebs
Surrounding tissue	Inflammation	Phagocytosis
DNA Changes		
Onset	Late	Early
Fragmentation	Random	Organized
Breakdown	Lysososmal enzymes	Endonucleases
End stage	Leak out	Packaged
Biochemistry		
Energy Supply	Fails	Required
Protein synthesis	Stops	Required

Table 1 Differences Between Morphological and Biochemical Characteristics of Necrotic and Apoptotic Cells.

Apoptosis is crucial part of development and of adult life as a balance between proliferation and apoptosis is necessary for homeostasis. In the developing nervous system more neurons are initially produced than are eventually required. Therefore a neuron must interact with its target area in order to receive growth factors and survive. If the neurons

were to die via necrosis the result would be unfortunate for the developing nervous system (3). Apoptosis shapes organs during mammalian morphogenesis and removes cells that are immunologically reactive against self, infected or genetically damaged, whose survival pose a danger to the host.

Apoptosis is a fundamental component of the development and regulation of the immune system, which is due to the special place of cell death in cell selection and immune cell cytotoxicity, key processes in immune function (5). Yet it has also been implicated in the pathogenesis of important human disease as a result of either immunosuppression or immune hyperactivity. It is involved in the development and shaping of the immune receptor repertoire at the clonal level (6, 7), cytotoxic T cell killing and modulation of the immune response (8). In B lymphocytes, antigen binding specificity is generated in an undirected fashion therefore the repertoire has to be modified both to increase the proportion of potentially useful receptors and deleting potentially harmful self-reactive receptors Lymphocyte development and selection involves the deletion of 95% of immature lymphocytes. This is exemplified by the deletion of autoreactive B and T lymphocytes by apoptosis in order to avoid the generation of autoantibodies (9).

A major defense against viruses, transformed cells and foreign grafted cells is conferred by cytotoxic T lymphocytes (10), as these cells are able to recognize virus bearing cells and cause them to undergo apoptosis by transferring enzymes via granules through cellular pores or by engaging and signaling specific receptors on target cell surfaces such as FAS ligand (11). This results in the deletion of infected host cells without inducing an

inflammatory response. There is additional evidence that modulation of the immune response is by neutrophil apoptosis as a downregulation of granulocyte function in peripheral tissue is involved in ARDS (12).

Programmed cell death plays an important role in many stages of B cell development. It can result in from certain requirements of the immune system not being met at the right time or from other requirements being met appropriately signaling the need to delete a particular cell (13). The pre B cell that fails to rearrange its heavy or light chain in such a way as to produce a complete transcript is deleted via apoptosis (6). Rearrangement of heavy and light chains leads to the expression of a surface IgM molecule. Antigenic stimulation of these immature, sIgM+, sIgD- B cells will result in their deletion via apoptosis. On the other hand splenic B cells will undergo apoptosis unless they are stimulated by antigen. During the period of antibody hypermutation, the period when high affinity antibodies are formed, the strength of antigen-sIgM interaction will determine if the cell will mature in order to become a plasma cell or undergo apoptosis.

Platelet-activating factor

Platelet-activating factor is a member of a family of phospholipid mediators produced by many cells as part of their response to infection or inflammation (14). PAF is synthesized by enzymatic cleavage of membrane phospholipids by at least two pathways, utilizing enzymes such as phospholipase A_2 and acetylhydrolase. The cells that can produce PAF include endothelial cells, stromal cells, neutrophils, monocytes, mast cells, dendritic cells

and B lymphocytes (15). PAF is released in close proximity to where it is synthesized and exerts its effects in either a paracrine or autocrine fashion.

Platelet activating-factor receptors and PAF in pathology

All members of the PAF family send intracellular signals via the common PAF receptor (PAFR). This receptor was the first lipid mediator receptor to be cloned and sequenced; it is made up of 342 amino acids and is a member of a group of chemoattractant receptors that are linked to GTP-binding proteins and have seven hydrophobic transmembrane domains (16). Once the PAFR is stimulated by PAF, the receptor initiates multiple signal transduction events including protein phosphorylation, hydrolysis of membrane lipids to produce inositol-3-phosphates (17) and diacylglycerol, and elevation of intracellular calcium $[Ca^{2+}]_i$ (14, 18, 19). This implies that some, but perhaps not all of the effects mediated through the PAFR can be inhibited by pre-incubation of the cells with pertussis toxin and, in some cases, cholera toxin (20). The PAFR signal transduction pathway includes the crucial signaling molecule Mitogen Activation Protein (MAP) kinase, but there is still poor understanding in general of how GTP-binding protein receptors activate MAP kinase. Another important enzyme for progression of cell maturation phosphatidyl inositol 3′-kinase, may be up-regulated as a result of PAF signaling (21-23). The PAFR is expressed constitutively on many mature blood cells such as neutrophils and platelets (14, 24), and its expression on other cells can be induced by inflammation, by differentiation of cells, or by cytokines such as INF-γ or TGF-β (24-26).

PAF has been implicated in the pathogenesis of many disease states. In vitro and in animal models, PAF causes vascular leakage and hypotension (14), increases smooth muscle tone and bronchial smooth muscle hyperresponsiveness (14), and is a potent chemoattractant for neutrophils and eosinophils (14, 27). PAF has been implicated in acute and chronic inflammatory diseases, such as septic shock, asthma and rheumatoid arthritis (28). Other investigators suggest that regulation of the PAFR can influence the development of pathologic states. Individuals with hypertension have elevated levels of immunoglobulin, and their B cells are hyperesponsive to PAF. Recently, the site of pneumococcal infection was determined to be the PAFR, as lipid structures in the bacterial cell wall mimic PAF and attach to PAFR on pulmonary cells activated by the infection (29). In contrast to its role in pathology, PAF also regulates normal cellular processes and plays a key role in intracellular communication. PAF plays an essential role for chemotaxis and activation of neutrophils and platelets (30-32). Vascular endothelium, activated due to local infection, will synthesize and secrete lipid mediators (33), and monocytes (34) and other antigen presenting cells produce PAF, which can attract B lymphocytes to sites that require antibody synthesis. PAF is secreted as part of the immune response for cell recruitment in infectious as well as inflammatory states. B lymphocytes can be affected by this mediator under many conditions (35). The regulation of normal antibody synthesis, and antibody dysregulation in asthma or rheumatoid arthritis may be influenced by PAF (36). Our studies focus on the interaction of PAF with B cells, specifically as it relates to the maturation of a B cell stimulated through its antigen receptor.

Research Summary

In our current laboratory studies, we have shown prevention of programmed cell death in B lymphocytes. Crosslinking of the surface IgM using antibodies to sIgM in RAMOS B lymphoblastoid cells causes deletion of a large percentage of cells by apoptosis. Co-incubation of PAF, a potent B lymphocyte pro-inflammatory mediator, with αIgM stimulated RAMOS cells leads to a significant decrease in the number of apoptotic cells as measured by TUNEL assay. Therefore the concept of apoptosis resulting in downregulation of the immune response and inflammatory mediators counteracting the effects of apoptosis by prolonging immune effector cell survival which is prevalent in the immune system (37) is demonstrated in our system.

Platelet activating-factor abrogates apoptosis induced by cross-linking of the surface IgM receptor in a human B lymphoblastoid cell line

Baruch J. Toledano, Yolande Bastien, Francisco Noya, Sylvain Baruchel, Bruce Mazer

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Abstract

B lymphocyte development is characterized by deletion, via apoptosis, of immature cells that are stimulated via the BCR in the absence of a second signal. We have investigated whether platelet-activating factor, a potent B lymphocyte activator, can provide a complementary signal with BCR ligation in order to abrogate apoptosis. Cross-linking of the sIgM on Ramos B lymphoblastoid cells using anti-IgM antibodies (2μg/ml) caused PCD in 34±5.4% of the cells. Co-incubation of PAF (10^{-7}M) with αIgM led to a significant decrease in apoptotic cells as measured by DNA laddering and TUNEL assay (13.8±3%). The effect of PAF was dose dependent (10^{-7}-10^{-9}M), and was inhibited by the specific PAF receptor antagonist, WEB 2170. PAF protected cells from the effect of αIgM for up to one hour after it was added.

αIgM-induced PCD in Ramos cells was blocked by catalase, and therefore is caused in part by the production of toxic hydroxyl radicals from hydrogen peroxide. We investigated the action of PAF on markers of intracellular oxidation. H_2O_2 in low doses induced apoptosis, via production of $OH^{\cdot}$ radicals. PAF inhibited H_2O_2 induced apoptosis in Ramos cells; it also attenuated H_2O_2 and αIgM-mediated increases in hydroxyl radical ($OH^{\cdot}$) as measured by the oxidation of 2',7'-dichlorofluorescein diacetate to DCF, and blocked the depletion of GSH induced by αIgM. PAF maintained IgM secretion, greatly inhibited by incubation with αIgM alone. These data indicate that PAF potentially provides an important co-signal to sIgM stimulated Ramos cells by inhibiting apoptosis. This is in part due to the activity of PAF in the oxidant/antioxidant pathway.

Introduction

Ligation of the antigen receptor on B lymphocytes can result in such varied responses as maturation or deletion via apoptosis (38, 39). The response is dependent on the stage of B cell maturity, the antigen load, and the milieu in which the antigen-antibody contact occurs (13). While B cell maturation to an antibody secreting cell is initiated by antigen stimulation, this alone is insufficient as cytokines or cell-cell contact are required in order to drive B lymphocytes to full maturity.

Apoptosis is an active cellular process that is initiated for the benefit of the organism and aids in morphogenesis in the embryonic period, and cell turnover of aging or abnormal cells (5). Maturation of B lymphocytes to antibody secreting cells involves a stringent process of selection. Only 5% of developing B lymphocytes will become mature plasma cells, whereas the other 95% are deleted by apoptosis (4). B lymphocytes that are prone to deletion include those which have faulty rearrangement of heavy and light chains genes, or those for which the sIgM receptor is cross-linked while the cell is immature. The intracellular processes that come into play in order to cause cell death involve production of toxic reactive oxygen substances, and activation of enzymes such as endonucleases (40). It has been previously shown that cytokines or ligation of adhesion molecules can prevent programmed cell death in immature antigen stimulated B lymphocytes (41), through mechanisms such as induction of bcl-2, or by decreasing the production of hydroxyl radicals (42).

Platelet activating factors are a family of ubiquitous phospholipid mediators that are released during the inflammatory response. PAF (15) is synthesized by a variety of cells including endothelial cells, basophils, monocytes, neutrophils and B lymphocytes (15). PAF has also been detected in bone marrow (43) and in lymphoid tissue (44), the sites of B lymphocyte maturation. In B lymphoblastoid cell lines, the addition of PAF results in an increase in intracellular calcium, hydrolysis of membrane lipids (18), and tyrosine phosphorylation of proteins including MAP kinase (19) and PI-3 kinase (22). We have demonstrated that PAF increases immunoglobulin synthesis in B lymphoblastoid cell lines (45). We have also shown that in cells that express both PAF receptors and sIgM there was no heterologous desensitization between these receptors; neither ligand altered the ability of the second to elevate intracellular calcium. This indicates distinct signaling pathways for both the PAFR and the BCR (18). Because of these properties, the signal initiated by PAF can synergize with sIgM stimulation, and PAF may therefore be able to influence B cell maturation events.

In keeping with PAF's ability to elicit potent responses in B lymphoblastoid cells, its synergy with distinct signaling pathways via BCR and its synthesis at sites of B lymphocyte maturation and apoptosis, we investigated the effect of PAF on the signals initiated by ligation of sIgM that induce apoptosis. We demonstrate that PAF is able to abrogate αIgM mediated apoptosis in the Ramos B cell line by decreasing the production of reactive oxygen substances, maintaining the integrity of the surviving B lymphocytes and allowing them to function as antibody secreting cells.

Material and Methods

Reagents

PAF (1-alkyl-2-acetyl-sn-glycero-3-phosphocholine, C-16), purchased from Biomol, (Plymouth Meeting, PA) was resuspended in ethanol and stored at -20°C. Before use, the ethanol was evaporated under nitrogen and PAF was reconstituted in PBS containing 0.125% BSA. Web 2170 was obtained through the generosity of Boehringer-Ingelheim, (Ingelheim en Rhein, Germany). αIgM (IgG BU1 clone) was purchased from The Binding Site (San Diego, CA). H_2O_2 (30% solution) and catalase were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell lines and culture

The human B-lymphoblastoid cell line Ramos (RA-1, ATCC, Rockville, MD) expressed surface markers sIgM+, sIgD- CD10+, CD38+. It was maintained in complete medium consisting of RPMI 1640 (Gibco BRL, Burlington, ON) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 50 U/ml penicillin, 50 ng/ml streptomycin, 10 μg/ml L-glutamine and 5 μg/ml sodium pyruvate (all from Gibco BRL). Frozen aliquots were thawed every 8 weeks to minimize inter-assay variability. Cells were prepared for culture by harvesting and washing three times in RPMI 1640 alone, and then resuspended in serum free medium as described (45) at a concentration of 3×10^5 cells/ml. Serum free medium was prepared by the method of Kovar and Franek (46). All cultures were performed in cell culture tubes (Falcon 2054, Becton Dickinson, La Jolla, CA). PAF, αIgM, WEB 2170 and/or other reagents were added at the appropriate dilutions to the cell suspensions at

indicated times and the cell culture was maintained for 24 hours in a humidified incubator at 37°C in 5% CO_2.

Assessment of membrane integrity with propidium iodide

Following cell culture for the indicated times the cells were washed twice with PBS by centrifugation at 1200 RPM for 10 minutes. The cells were then resuspended in 0.5 ml PBS with Propidium Iodide (PI) (Molecular Probes, Eugene, OR) 20 µg/ml and incubated in the dark for 5 minutes. Analysis of PI uptake or exclusion was performed by flow cytometry (Facscan, Becton Dickinson) using Lysis II software; fluorescence was measured at >650nm and analyzed via a histogram of log fluorescence (FL3).

Cell cycle analysis

3 x 10^5 cells were cultured for the indicated times and were washed twice with PBS by centrifugation at 1200 RPM for 10 minutes. The cells were then resuspended in 250 µl FCS and 250 µl RPMI 1640 and fixed in 1.5 ml 70% ethanol at 4° C for a minimum of 30 minutes. The cells were washed twice with PBS by centrifugation at 4° C, 2400 RPM for 5 minutes, resuspended in 1 ml PBS with 0.05 mg/ml RNase A (50 units/mg) (Boehringer Manheim, Laval, QC) and incubated for 30 minutes at 37° C. Following this, 50 µg/ml propidium iodide was added and the cells were analyzed by flow cytometry utilizing Cell Fit Software (Becton Dickinson).

Detection of DNA breaks via fluorescent tagging

The Apoptag kit (Oncor, Gaithersburg, MD) was utilized according to the manufacturer's recommendations. Briefly, following cell culture for the indicated time, cells were fixed for 15 minutes in 1% paraformaldehyde. The cells were washed twice with PBS (1000 RPM for 5 minutes), then resuspended in 70% ethanol and stored for a maximum of 2 days. Cells were resuspended in 50 µl TDT enzyme with digoxigenin d-UTP and incubated for 30 minutes at 37°C, then washed twice with wash buffer and incubated with fluorescein conjugated anti-digoxigenin antibody for 30 minutes at room temperature. Following two washes in 0.1% triton X-100 the cells were resuspended in PI solution (5 µg/ml) containing 50 µg/ml RNase A and incubated for 15 minutes in the dark. The cells were analyzed by flow cytometry using Lysis II software, with fluorescein intensity measured at 510-550nm and PI fluorescence measured at >650nm.

Assessment of DNA laddering by gel electrophoresis

DNA laddering was performed as per Perandones et al (47). Briefly, following cell culture for 16-24 hours the cells were lysed in 500 µl 0.2% triton X-100, 10 mM TRIS and 1 mM EDTA. The cells were then centrifuged at 13,800 g at 4° C for 15 minutes and 250 µl of the supernatant was utilized to make a 0.5 M NaCl solution to which an equal volume of isopropyl alcohol was added. DNA was precipitated overnight at -20° C, then centrifuged at 13,800 g for 15 minutes and the pellet was washed with 200 µl of 70% ethanol and dried by vacuum centrifugation. The DNA was resuspended in 12 µl TE solution (10 mM Tris HCl, 1 mM EDTA, pH 7.4) and 3 µl loading buffer (50% glycerol, 1X TAE, 10% saturated bromophenol blue, 1% xylene cyanol), and incubated at 37° C for 20 minutes. The sample

was then electrophoresed on 1% agarose gel containing 0.71 μg per ml ethidium bromide for 1 hour. Gels were photographed using UV trans-illumination.

Measurement of intracellular hydroxyl radical ($OH^{\cdot}$) via DCFH

Assay of the production of intracellular oxidants was performed using 2'7'-dichlorofluorescein diacetate. DCFH-DA was purchased from Molecular Probes (Eugene, OR) and diluted to 10mM in ethanol prior to each experiment according to the manufacturer's instructions. Ramos cells were incubated with αIgM, PAF or H_2O_2 for the indicated times, resuspended in PBS at 5 x 10^6/ml, and incubated with 10μM DCFH-DA at 37°C for 20 minutes. Cells were washed twice in PBS, and kept on ice until use. Fluorescence was measured using a Shimadzu RF-5000 spectrofluorimeter, with an excitation wavelength of 488λ and emission wavelength of 525λ (48).

Measurement of IgM synthesis

Cell culture supernatants were harvested and frozen at -20°C until use. Supernatant IgM levels were measured by ELISA, as described by Mazer et al. (45). Briefly, 96 well round bottom ELISA plates (Maxisorb, NUNC, Burlington, ON) were coated with 3 μg/ml goat anti-human IgM (Tago, Burlingame, CA). Supernatant (20 μl) was added to each well and diluted 1:5 with 0.1 M TRIS plus 1% BSA buffer. After a 2 hour incubation at 37°C, the plates were washed and goat anti-human IgM antibody conjugated with alkaline phosphatase (Tago, Burlingame, CA) was added diluted appropriately with 0.1 M TRIS plus 1% BSA. After a 2 hour incubation, the plates were developed by adding a 2 mM

solution of para-nitrophenol phosphate (Sigma Chemical Co., St. Louis, Mo.) in 1 M TRIS buffer; colorimetric analysis at wavelength 410 nm was performed using an automated ELISA reader (Anthos Microplate Reader 2001, Salzburg, Austria).

ELISA Spot Assay

The ESA was performed by the method of Mazer at al. (49). Briefly, 96-well flat bottom plates (Corning Glassware Co., Corning, NY) were coated with 3 μg/ml of goat-antihuman-IgM (Tago, Burlingame, CA). After the cells were cultured for the appropriate time they were washed and plated on ESA plates and incubated for a further 16 hours at 37° C. Cells were then removed from the plates by washing three times and alkaline phosphatase conjugated goat anti human IgM (Tago) was added at the optimal concentration. After a 2 hour incubation at 37° C, the plates were washed, and substrate was added as a combination of 5-bromo-4-chloro-3-indoyl phosphate and 1.2% agarose (both purchased from Sigma). The plates were incubated at 37° C for 1 hour and then at room temperature overnight. Spots were hand counted by microscopy.

Measurement of reduced glutathione

GSH measurement was performed by the method of Tietze et al. (50). After a 5 hour incubation, 10^6 cells were centrifuged at 5000 RPMI for 10 minutes at 4°C and lysed with 0.1% sulfosalicylic acid in water (Sigma). The lysate was incubated 15 minutes on ice, then frozen at -70°C and stored until use. For determination of GSH, 100 μl of the lysate was added to 700 μl 0.3 M NADPH (Boehringer Mannheim), 100 μl of 1 M triethanolamine

(BDH, Ville St-Laurent, QC), 100 µl 6 mM dithionitrobenzoic acid (ICN, Toronto, ON) and 10 µl of glutathione reductase (120 U/ml) (Boehringer Mannheim). The sample was mixed by inversion three times and the optical density was read after 2 minutes at 412 nm (Spectronic 1001 Plus, Fisher Scientific, Montreal, QC). A standard curve was constructed from serial dilutions of GSH, and results expressed in nmole of GSH per 10^6 cells.

Graphical and Statistical Analysis

All graphical analysis including mean and SEM were performed using Excel software (Microsoft Corp., Port Redmond, WA) and other statistical analysis was performed with Instat (Graphpad Software, San Diego, CA). p values were obtained by Tukey Multiple Comparison Test.

Results

PAF inhibits αIgM induced programmed cell death in Ramos B lymphocytes

The Ramos cell line, an EBV negative Burkitt lymphoma line, has been used as a model to study the growth and development of germinal center B cells (17). Ligation of sIgM on Ramos leads to programmed cell death or apoptosis, which is presumed to be the mechanism of deletion of cells that are insufficiently stimulated to mature (38, 39). Addition of a second stimulus, such as simultaneous ligation of cell surface adhesion molecules CD40 or CD20 in conjunction with sIgM stimulation (51) has been shown to abrogate the initiation of PCD. Our previous studies have indicated that the Ramos cell line expresses high levels of the PAFR (18, 20) and PAFR mRNA (52); further, we have demonstrated via signal transduction experiments that the signals of PAF and αIgM on Ramos cells may be complementary (18, 20). We employed this well defined model of αIgM induced apoptosis to examine the effects of PAF on PCD in Ramos cells.

Incubation of Ramos cells with αIgM (2μg/ml) for 24 hours led to marked morphologic changes compared to control cells, with a population of small, dense cells emerging (data not shown). These populations were characterized for viability by propidium iodide inclusion. For the αIgM treated cells, only 55% ± 2.7% remained viable at 24 hours compared to 92% ± 2.6% of control cells ($p < 0.001$)(Figure 1). Addition of PAF (10^{-7}M) simultaneously with αIgM, diminished the number of cells that had lost membrane integrity (80% ± 1.1% viable cells, n.s. compared to control, $p < 0.01$ compared to αIgM) (Figure 1). This effect was

completely blocked by the pharmacologic PAF receptor antagonist Web 2170 (10^{-6}M) (data not shown).

Figure 1

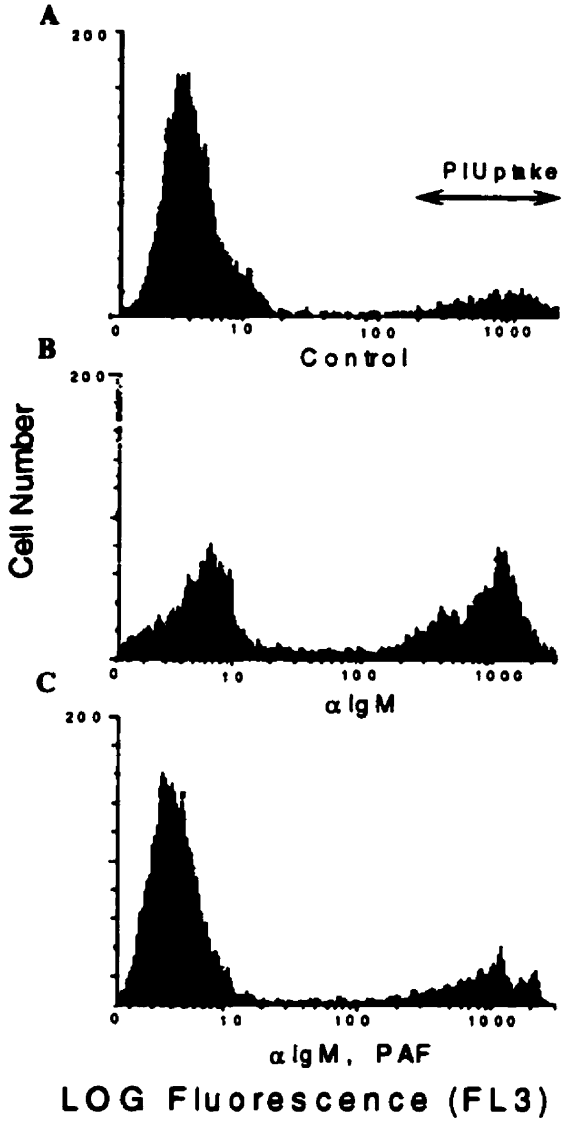


Figure 1 *Representative histogram of 4 experiments demonstrating cell viability by propidium iodide exclusion. A: represents control cells. B: cells following incubation with αIgM (2 μg/ml); leading to a decrease in the viable (PI excluding) cells. C: simultaneous addition of PAF (10^{-7} M) and αIgM (2 μg/ml) restores number of viable cells.*

Progression of the cells through the cell cycle mirrored the changes suggested by PI staining. As shown in Table 2, ligation of the BCR by αIgM significantly diminished the number of cells in G_0-G_1 and S phase. Ramos cells simultaneously incubated with αIgM and PAF were able to progress through the cell cycle in a manner similar to control cells. A significant number of αIgM treated cells appeared in the gate to the left of G_0 (31.6 ± 3%). These cells were predicted to be apoptotic, according to the method of Darzynkiewicz. (53) Co-culture of αIgM with PAF reduced the number of cells in this region to 10 ± 2% (Table 2).

Table 2.

	Apoptosis	G1	S	G2/M
Control	6.5 ± 2	30 ± 5	58 ± 5	6 ± 3
αIgM	32 ± 5★	24 ± 6	40 ± 83	5 ± 2
αIgM, PAF	10 ± 2	30 ± 5	53 ± 6	6.5 ± 2
αIgM, PAF, Web	33 ± 2★	21 ± 6	44 ± 9★	3.5 ± 2

Table 2.
Cell cycle analysis of αIgM stimulated Ramos cells. Number of cells (%) in each phase of the cell cycle as assessed by PI staining. Apoptotic cells were those with less than 2n DNA content. (N = 5). Results are expressed as mean ± SEM. ★ represents p <0.01 as compared to Control.

Loss of membrane integrity occurs 4-6 hours following PCD and therefore propidium iodide inclusion may represent the termination of ongoing apoptosis in a cell population. DNA laddering, a specific measure of PCD, was performed to directly visualize the appearance of non random 180-200 bp breaks which hall-mark apoptosis (54). Figure 2 demonstrates the effects of αIgM cross-linking (lane 2) compared to control (lane 1), and the

marked diminution of DNA laddering following addition of PAF (10^{-7}M) (lane 3). The effects of PAF are reversed by pre-incubation of the cells with the specific PAF antagonist Web 2170 (data not shown).

Figure 2

Figure 2 *A: Representative gel electrophoresis demonstrating DNA laddering. Lane 1: Control, unstimulated cells. Lane 2: Cells following incubation with αIgM (2 μg/ml). Lane 3: Cells incubated with PAF (10^{-7}M) and αIgM.*

Figure 3

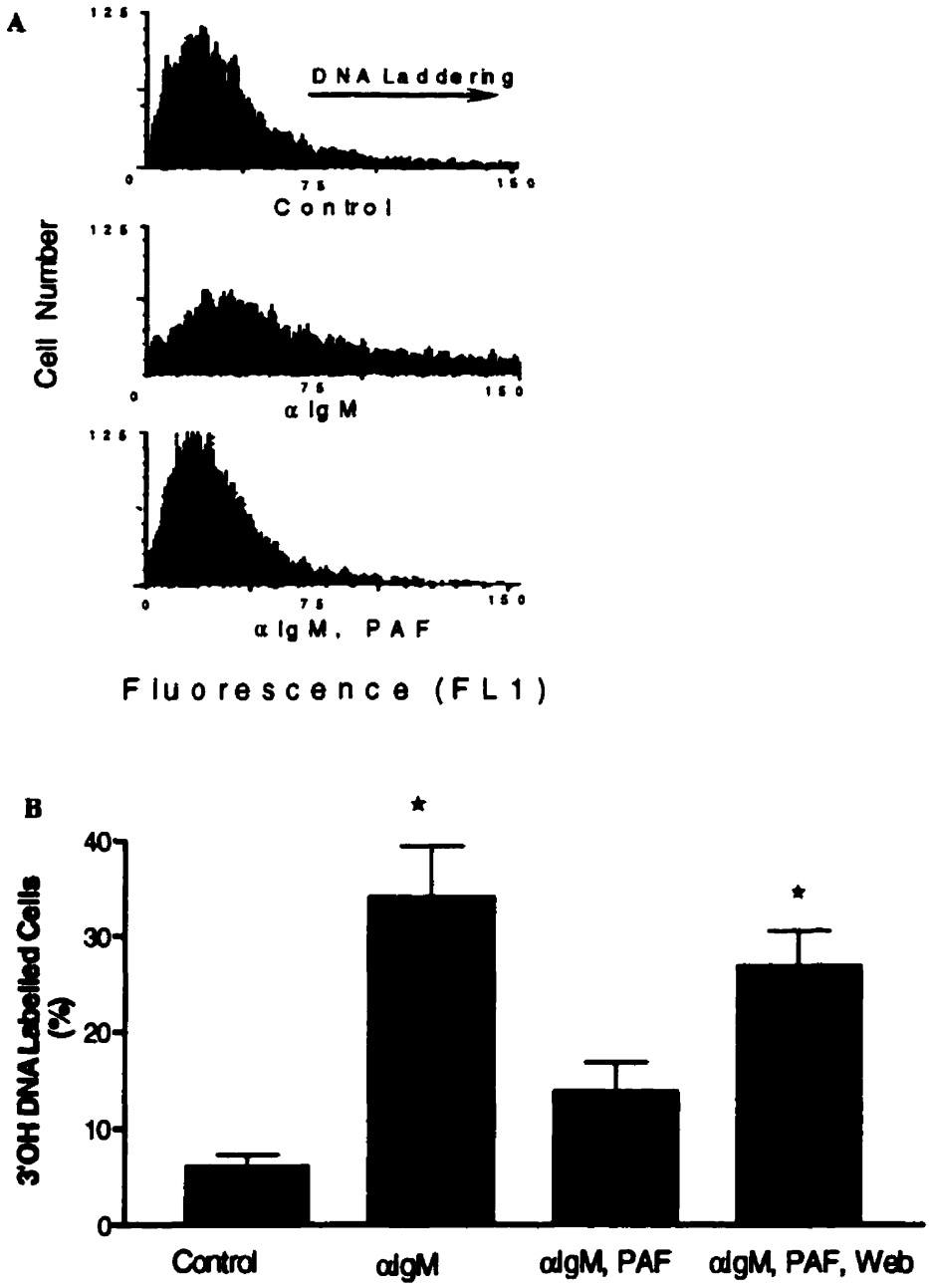


Figure 3 *A: Representative histogram of 4 experiments assessing Ramos cells for 3'OH DNA labeling representing apoptotic cells (TUNEL assay). Top Panel: Control cells with minimal DNA breaks. Middle Panel: Cells following incubation with αIgM (2 μg/ml) demonstrating a marked increase in the number of cells demonstrating DNA fragmentation. Lower Panel: Cells following simultaneous addition with PAF (10^{-7} M) and αIgM (2 μg/ml) demonstrating a decrease in the number of cells with DNA fragmentation. B: A graphical representation of the percent of Ramos cells demonstrating*

evidence of DNA breaks (TUNEL Assay). Incubation with αIgM (2 μg/ml) resulted in a significant increase in the number of cells with DNA fragmentation, which is reversed by the simultaneous addition of PAF (10^{-7} M). PAF's effects were blocked by WEB 2170 (10^{-6} M). Results are expressed as mean ± SEM. (N = 4). ★ represents p<0.01 compared to Control.

In order to enumerate the specific cell populations undergoing apoptosis, we employed the TUNEL assay. This procedure labels fragmented DNA by binding digoxigenin linked nucleotides to 3′ OH DNA segments, which are then coupled to fluorescein conjugated anti-digoxigenin. 3′ OH DNA labeled cells that exclude PI are visualized and quantitated by flow cytometry (55, 56). In this cell population, αIgM induced DNA fragmentation in an average of 34% ± 5.4% of Ramos cells, compared to 6.0% ± 1.9 of control cells (p<.001) (Figure 3A and B). Co-incubation of PAF with αIgM diminished the population of apoptotic cells, with only 13.8% ± 3.1 of the viable cells taking up the conjugated digoxigenin label (n.s. compared to control) (Figure 3A, B). PAF alone modestly decreased the number of cells that spontaneously underwent apoptosis (1.8% ± 1.1 in PAF treated cells, compared to 6.0% ± 1.9 for control cells; data not shown). There was a significant decrease of the action of PAF when cells were pre-incubated with the specific PAF antagonist Web 2170 (Figure 3B), yet co-incubation of Ramos cells with αIgM and the vehicle for PAF, 0.125% BSA in PBS, had no effect on αIgM mediated induction of apoptosis.

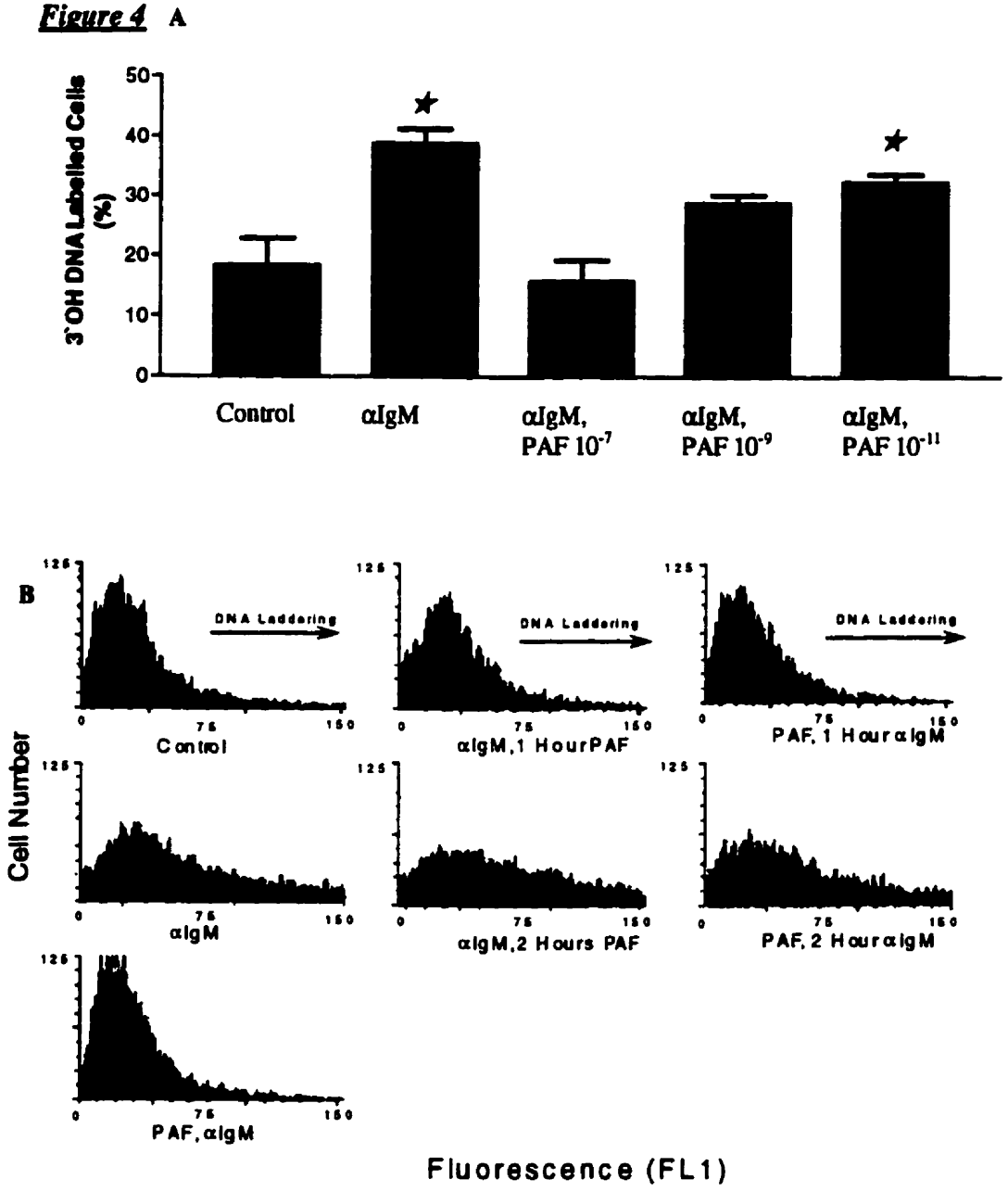


Figure 4 *A: Dose response to PAF. Cells were incubated for 24 hours with αIgM and decreasing doses of PAF and DNA fragmentation was assessed by TUNEL assay. Results are expressed as mean ± SEM. (N = 5). ★ represents p<0.05 compared to αIgM.*

B: Time course of PAF effect. Cells were incubated with αIgM and PAF (10^{-7} M) was added at indicated times. Representative histograms of TUNEL assay. The effect of PAF was maintained if it was added up to one hour following αIgM incubation but it no

longer had any protective effect beyond 1 hour. The PAF effect was maintained if it was added up to one hour prior to addition of αIgM; if it was added beyond this time there was no longer any protective effect

Dose response studies indicated that PAF consistently diminished αIgM-mediated PCD at concentrations between 10^{-7} to 10^{-9}M, while 10^{-11}M and below did not cause significant rescuing of cells (Figure 4A). Additionally, the effect of PAF was sustained if it was added up to one hour prior to the addition of αIgM (Figure 4B, 5); if PAF was added earlier there was a reduction in the protective effect. Addition of PAF up to 1 hour following αIgM incubation was equally effective in decreasing the proportion of apoptotic cells; its effectiveness again was lessened if added at 2 hours or later (Figure 4B, 5).

Figure 5

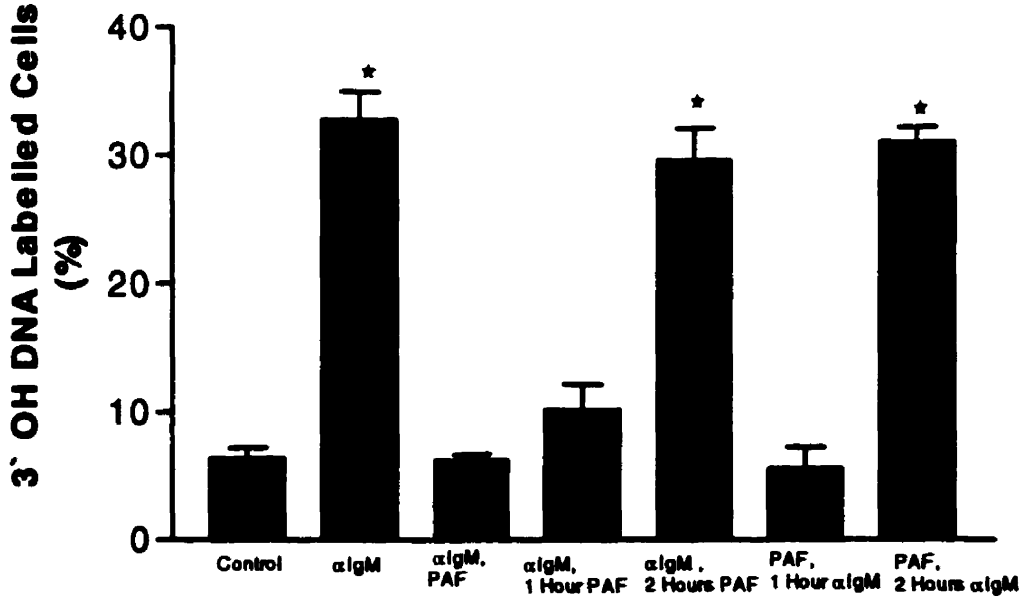


***Figure 5**: A graphical representation of the time course of the PAF effect (TUNEL assay). Results are expressed as mean ± SEM. (N = 3). ★ represents p<0.001 compared to Control.*

Anti-IgM stimulation increases reactive oxygen substances such as $OH^{\cdot}$ radicals in WEHI-231 murine B cells (57), and oxidative stress has been suggested as a mechanism by which αIgM mediated PCD occurs in these cells. Additionally, apoptosis has been associated with significant accumulation of reactive oxygen substances and intracellular oxidative stress in other cells (58, 59). We sought to determine whether this occurs in Ramos cells. The first stage in the elaboration of toxic $OH^{\cdot}$ radicals is the production of H_2O_2. Therefore, we preincubated Ramos cells with catalase, which scavenges H_2O_2 and does not allow for its further breakdown (57). The addition of 16,000 units of catalase completely inhibited apoptosis induced by αIgM, measured by the TUNEL assay (Figure 6). Thus, αIgM induced PCD in Ramos cells is associated with significant elevation in H_2O_2.

Figure 6

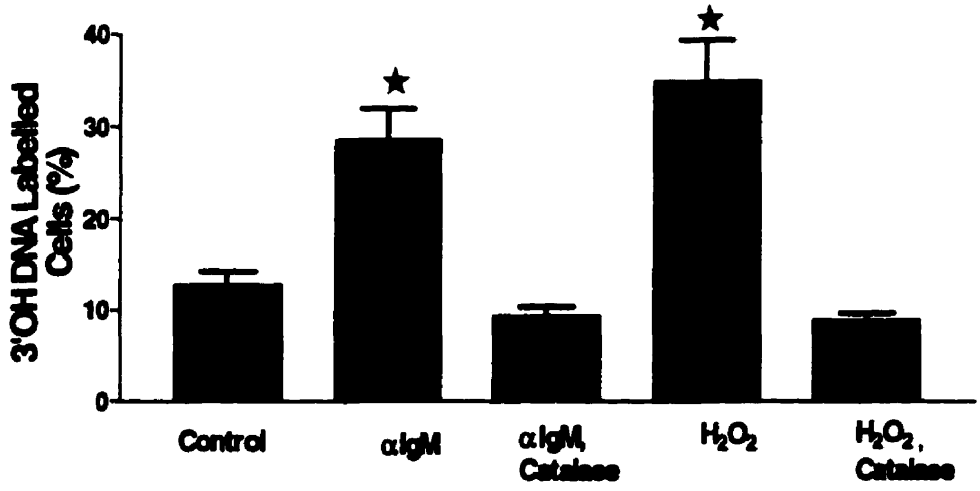


Figure 6 *A: Effect of the H_2O_2 scavenger catalase on apoptosis. Ramos cells were incubated for 24 hours with αIgM (2 μg/ml) or H_2O_2 (10 μM) ± catalase(16 000 units/ml). Incubation of the cells with catalase (16 000 units/ml), a potent H_2O_2 scavenger resulted in a significant decrease in the number of cells with 3'OH DNA labeling whether apoptosis*

was induced by incubation with αIgM (2 μg/ml) or H_2O_2 (10 μM). Results are expressed as mean ± SEM. (N = 5). ★ represents p<0.01 compared to Control.

Figure 7

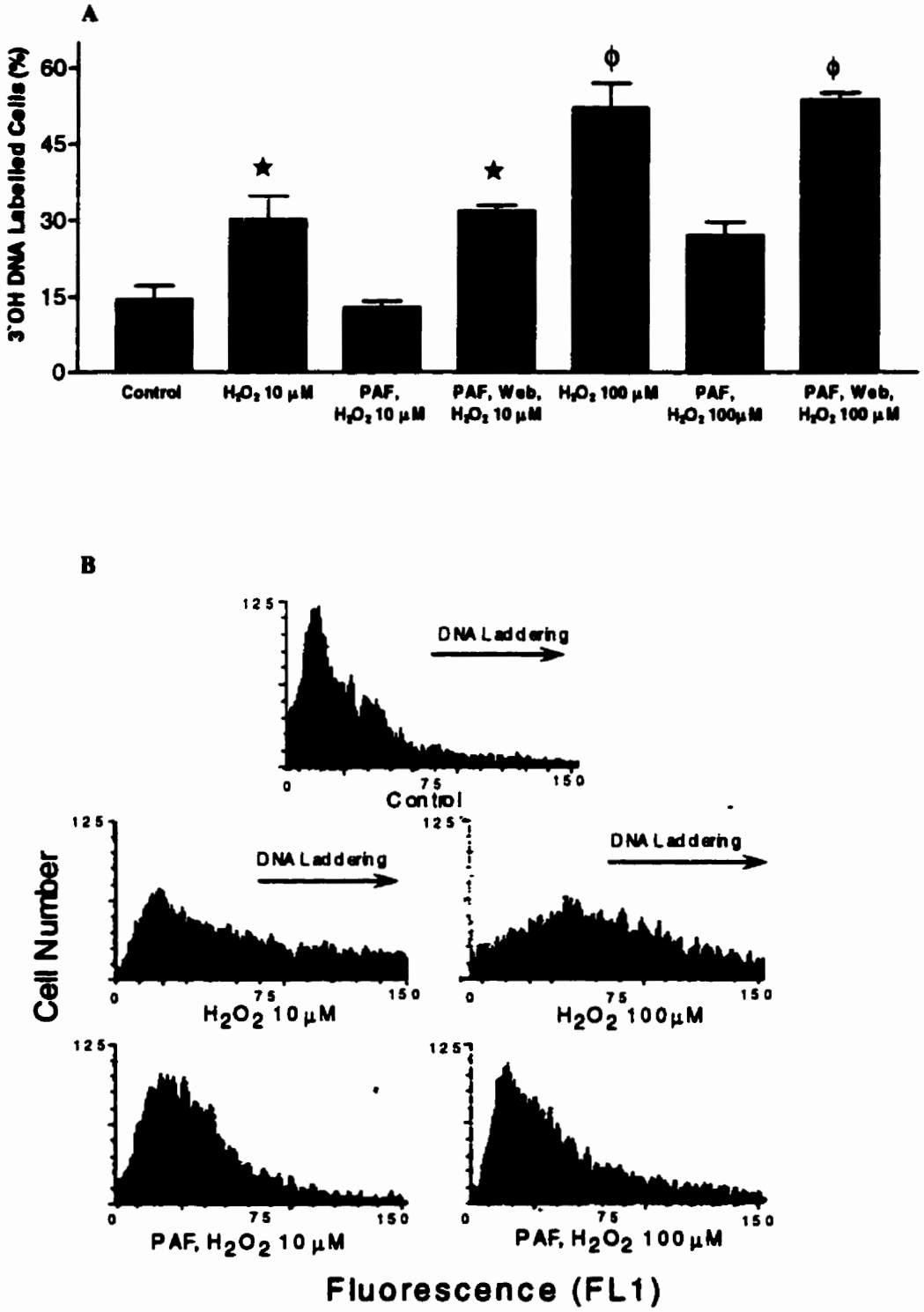


Figure 7 *A: A graphical representation of the data represented in the above panel. Results are expressed as mean ± SEM. (N = 5).* ★ *represents p<0.01 compared to Control.* ϕ *represents p<0.001 compared to Control. B:* H_2O_2 *induces apoptosis in Ramos cells. Cells were incubated for 24 hours with* H_2O_2 *and assessed for 3'OH DNA labeling (TUNEL assay). 10 and 100 μM* H_2O_2 *significantly increase 3'OH DNA labeled cells. This effect was decreased by co-incubation of the cells with PAF.*

While H_2O_2 causes necrotic cell injury at doses above 1mg/ml, low dose H_2O_2 has been shown to induce apoptosis, likely via its metabolism to $OH^{\cdot}$ radicals causing lipid peroxidation, protein modification and ultimately DNA damage (60). We determined that 10μM H_2O_2 produced DNA laddering in 30 ± 4.5% of Ramos cells, similar in effect to 2 μg/ml of αIgM (Figure 7B). Like αIgM induced PCD, preincubation of the cells with 16,000 units of catalase completely inhibited H_2O_2 induced apoptosis (Figure 6). 100μM H_2O_2 also caused 52 ± 4.8 % of Ramos cells to undergo apoptosis (Figure 7B) , whereas 1 mM or more caused cell necrosis (data not shown). We performed the TUNEL assay with cells co-incubated with both PAF and H_2O_2. PAF significantly decreased apoptosis induced by H_2O_2 at both concentrations (Figure 7A), an effect that was inhibited by Web 2170 (Figure 7 A).

A consequence of H_2O_2 formation is the enzymatic production of $OH^{\cdot}$ via the Fenton Reaction (61). We therefore studied the effects of PAF on accumulation of intracellular $OH^{\cdot}$ radicals in αIgM stimulated Ramos cell, using DCFH-DA. As this fluorochrome is taken up into cells, intracellular esterases cleave the diacetate group, preventing it from diffusing out of the cell membrane. When $OH^{\cdot}$ radicals (but not $O^{\cdot}$ radicals) are produced (62), DCFH is converted to DCF resulting in fluorescence proportional to the presence of the $OH^{\cdot}$ radicals. Cells incubated with αIgM show a steady increase in DCF fluorescence over a 5 hour incubation period (Figure

8 A). In comparison, H_2O_2 caused a more rapid rate of rise in DCF fluorescence than αIgM (Figure 8 B). Addition of PAF to Ramos cells caused a marked decrease in the oxidation of DCFH to DCF produced by either αIgM or H_2O_2 (Figure 8A and B). DCF fluorescence also remained at or near baseline when cells were preincubated with catalase (Figure 8A). This was not observed in cells pretreated with the PAFR antagonist WEB 2170 prior to addition of PAF, or when the BSA vehicle alone was added to cells (data not shown).

Figure 8

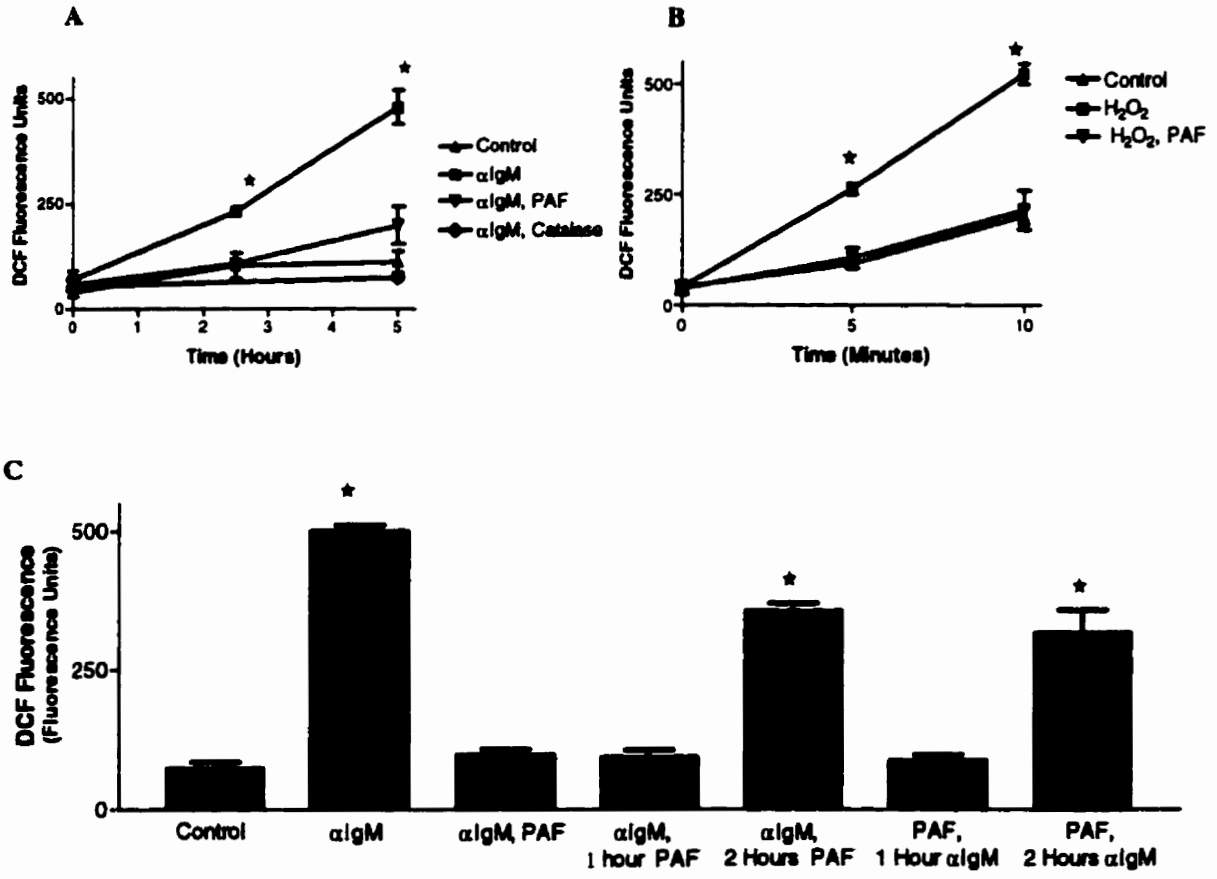


Figure 8 *A: Assessment of OH· production via DCF fluorescence. Following incubation with αIgM, Ramos cells demonstrated a significant increase in DCF fluorescence over 5 hours; while co-incubation with PAF caused a marked decrease in the rate of rise of DCF fluorescence. Results are expressed as mean ± SEM. (N = 4). ★ represents p<0.01*

compared to Control. B: Following incubation with 10 μM H_2O_2 there was a rapid rise in DCF fluorescence over 10 minutes; while co-incubation with PAF caused a marked decrease in the rate of rise of DCF fluorescence. Results are expressed as mean ± SEM. (N = 5). ★ represents p<0.001 compared to Control. C: Time course of action of PAF on OH· production by Ramos cells. Following 5 hour incubation with αIgM (2 μg/ml) Ramos cells demonstrate a significant increase in DCF fluorescence. The addition of PAF within one hour of αIgM incubation is effective in decreasing DCF fluorescence; beyond 2 hours of incubation the protective effect is lost. Results are expressed as mean ± SEM. (N = 5). ★ represents p<0.001 compared to Control.

Additionally, we followed changes in the fluorescence of DCFH in cells incubated with 2 μg/ml of αIgM for 6 hours, with PAF added at various times after the initiation of culture. As was previously noted using the TUNEL assay for apoptosis, addition of PAF within one hour of culture initiation was as effective as if PAF was added simultaneously with αIgM (Figure 5C). The effectiveness of PAF subsequently diminished, and after 2 hours PAF was unable to prevent the accumulation of OH· radicals and rescue the cells from αIgM-induced apoptosis. If PAF was added up to one hour after sIgM ligation, there was a similar abrogation of αIgM-induced apoptosis, but not if it was added after 2 hours (Figure 8C).

Glutathione, a thiol derivative of cysteine, scavenges reactive oxygen substances and represents one of the principle antioxidants present in the cell. Depletion of GSH is thought to reflect the level of oxidative stress on the cell (63). Since GSH acts distally to the production of OH·, we measured intracellular GSH as a further indicator of the action of PAF on reactive oxygen substances. αIgM incubation alone (Figure 9) significantly decreased the GSH level to 2.6 ± 0.7 nmoles/10^6 cells as compared to control (8.0 ± 1.2 nmoles/10^6 cells) ($p < 0.01$). Intracellular GSH was preserved by simultaneous incubation

of Ramos cells with PAF and αIgM (7.8 ± 0.4 nmoles/10^6 cells) (n.s compared to control). WEB 2170 preincubation was again able to block the effects of PAF ($p < 0.01$ compared to control) (Figure 9).

Figure 9

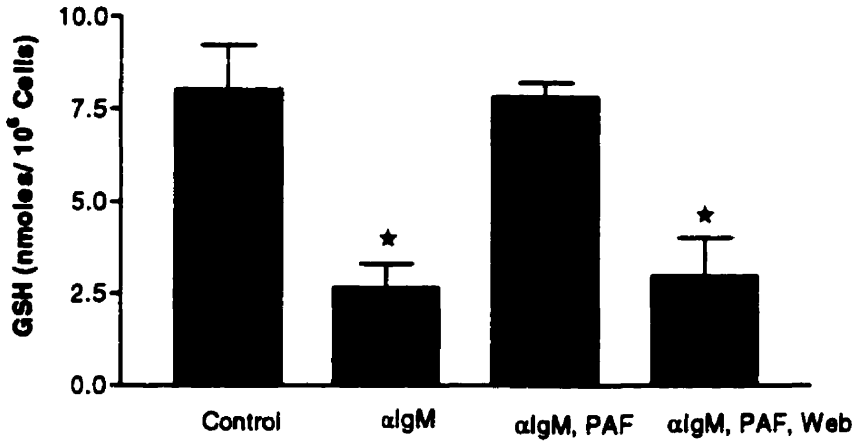


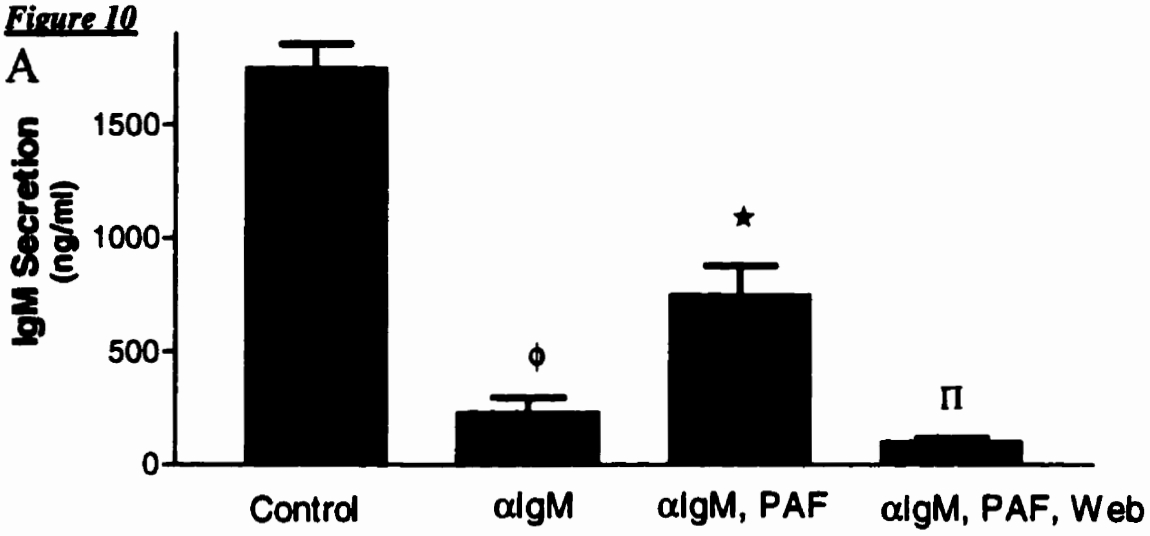
Figure 9 *Measurement of intracellular GSH. Cells incubated with αIgM (2 μg/ml) for 24 hours demonstrate a significant decrease in GSH level. Simultaneous addition of PAF (10^{-7} M) restores the GSH; WEB 2170 (10^{-6} M) antagonizes PAF's effect. Results are expressed as mean ± SEM (N = 5). ★ represents p<0.01 compared to Control.*

PAF sustains the functional capability of αIgM stimulated Ramos cells

Having demonstrated that PAF abrogates αIgM-induced apoptosis and preserved the viability of the cells, we then assessed the ability of PAF to influence immunoglobulin secretion. Cultured Ramos B lymphocytes constitutively secrete IgM. As would be expected, the population that was stimulated with αIgM was incapable of IgM production (Figure 10A). Addition of αIgM for 24 hours resulted in a significant decrease of IgM synthesis to 261 ± 112 ng/ml compared to control (1819.5 ± 161 ng/ml) ($p < 0.001$). IgM

secretion was restored significantly (890 ± 75 ng/ml) by simultaneous incubation of αIgM with PAF ($p < 0.01$ compared to αIgM alone) (Figure 10A). Preincubation with WEB 2170 resulted in a complete block of the PAF effect (Figure 10A).

Figure 10



B

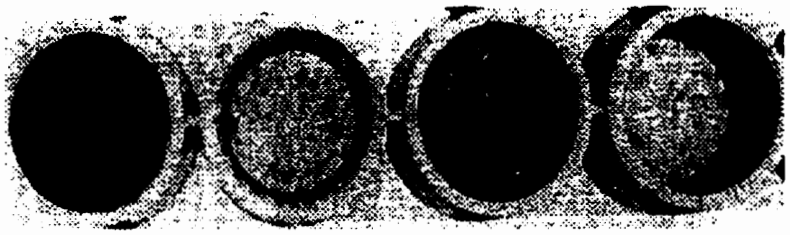


Figure 10 *A: IgM production by Ramos cells. Following incubation with αIgM (2 μg/ml) for 24 hours there is a significant decrease in the secretion of IgM detected in cell supernatants. Simultaneous addition of PAF (10^{-7} M) results in a significant increase in the secretion of IgM while the effect of PAF was blocked by WEB 2170 (10^{-6} M). Results are expressed as mean ± SEM (N = 8). ϕ represents p value <0.001 compared to Control.*

★: p value <0.001 compared to αIgM. Π: p value <0.001 compared to αIgM, PAF. B: Enumeration of immunoglobulin secreting cells by ESA. Ramos cells incubated with αIgM (2 μg/ml) for 24 hours demonstrate a significant decrease in the number of immunoglobulin secreting cells. Simultaneous addition of PAF (10^{-7} M) restored to control value the number of immunoglobulin secreting cells. PAF's effects were blocked by WEB 2170 (10^{-6} M).

Figure 11

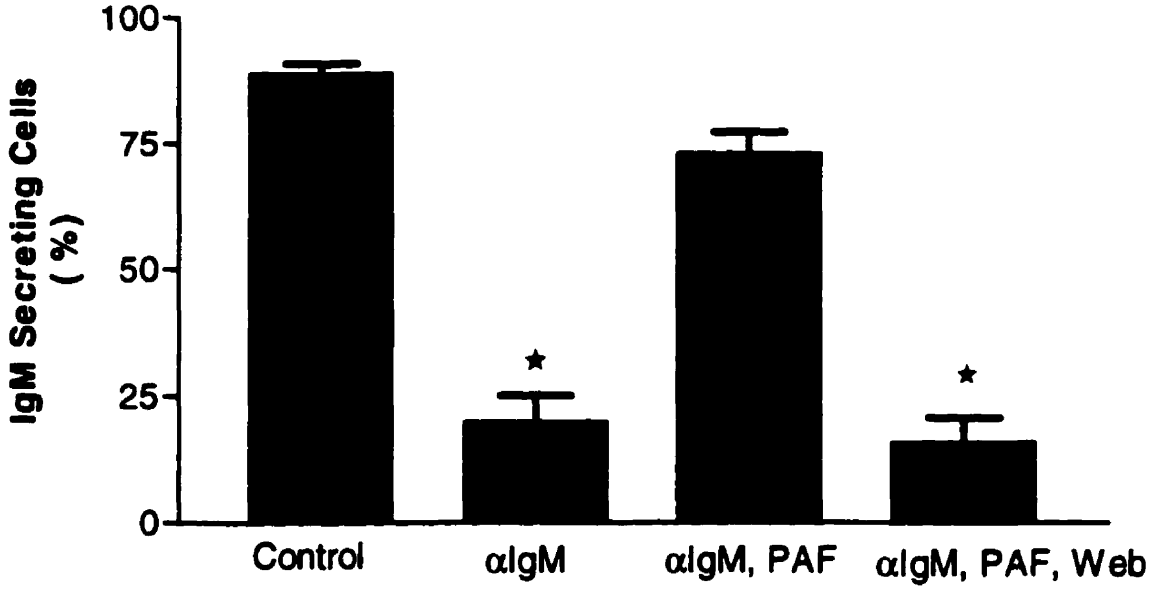


Figure 11 Graphical representation of ESA data. Results are expressed as mean ± SEM (N = 6). ★ represents p value <0.001 compared to Control.

ESA's were performed to examine the population of immunoglobulin secreting cells rescued by PAF. Incubation with αIgM alone resulted in a significant decrease in Ramos cells being capable of immunoglobulin secretion (20% ± 12%) as compared to control (89% ± 1%, p <.001). Simultaneous incubation of PAF with αIgM resulted in normalization of the number of IgM secreting cells (72% ± 11%) (n.s. compared to control) (Figure 10B, 11). The protective effect of PAF was blocked by WEB 2170 with only 16% ± 11% (p

<0.05) of plated cells being capable of secreting IgM (Figure 10B, 11). When immunoglobulin secreted per cell was calculated as described in Mazer et al. (49), it revealed that there was no significant difference in per cell immunoglobulin secretion between the conditions (data not shown). This demonstrated that incubation with PAF maintained the ability of surviving Ramos cells to secrete immunoglobulin.

Discussion

In these studies, we have examined the action of PAF on programmed cell death initiated through BCR signaling in Ramos cells. PAF is a highly potent, pro-inflammatory phospholipid mediator involved in leukocyte recruitment, cell adhesion and cellular activation (14). We and others have demonstrated that B lymphoblastoid cell lines have functioning PAF receptors (18, 20, 22, 64), but the full impact of PAFR signaling in B lymphocytes remains obscure.

In immature B lymphocytes, ligation of the BCR in the absence of a second signal leads to the deletion of the cell by apoptosis, the postulated mechanism for removal of autoreactive B cells or those not sufficiently mature to appropriately respond (4). B cells that are stimulated via the BCR in the context of an infectious or inflammatory stimulus receive second signals that allow for maturity towards memory B cells or plasma cells. These second signals may be cytokines such as IL4 (41), T cell help via CD40 ligand (41, 65), or adhesion molecules such as VLA-4 (66). In our model of PCD, PAF fulfills the role of second signal. The lipid mediator is able to preserve the viability of αIgM stimulated Ramos cells, and abrogate the signals for apoptosis. PAF stimulated cells were able to progress in cell cycle comparably to control cells, and ultimately could secrete IgM. These actions were specifically inhibited by the PAF receptor antagonist Web 2170.

The signals that cause PCD in cells stimulated through the BCR are not completely understood. However apoptosis is associated with production of oxidants such as $OH^{\cdot}$ radicals. This leads to lipid peroxidation and DNA damage, initiating the action of endopeptidases that cause further

DNA cleavage (58-60) and ultimately cell death. $OH^{\cdot}$ radicals are produced most commonly by the reduction of H_2O_2 through transfer of an electron from a reduced metal such as ferrous iron or copper in a process known as the Fenton reaction (62). Intracellular antioxidants such as catalase are effective in scavenging H_2O_2 and inhibiting further toxic radical production. Apoptosis as a consequence of αIgM signaling in WEHI-231 cells has been shown to be at least partially due to oxidative stress within the cells, and can be inhibited with antioxidants (57). Similarly, we have shown in the Ramos cell line that catalase effectively inhibits αIgM induced PCD. This not only suggests that production of reactive oxygen substances is important in PCD associated with BCR ligation, but also implicates H_2O_2 as a key molecule in the initiation of apoptosis in B lymphocytes.

With this information we explored the mechanism by which PAF inhibited αIgM mediated PCD. This was accomplished in part by observing the effect of PAF on apoptosis induced by H_2O_2, which in low to medium doses induced nuclear changes that are comparable to αIgM. PAF was also able to inhibit apoptosis induced by H_2O_2. In fact, PAF inhibited the effects of H_2O_2 not only at a dose that gave equivalent results to αIgM (10 μM), but also at a dose that was tenfold greater (Figure 7A and B).

A consequence of the ligation of the BCR and the production of H_2O_2 is the biochemical conversion of H_2O_2 to $OH^{\cdot}$ (61). Using the intracellular fluorescent probe DCFH, we demonstrated the kinetics of $OH^{\cdot}$ radical production initiated by αIgM or H_2O_2 incubation. The main substrate for DCFH oxidation $OH^{\cdot}$ and not superoxides (O^{-}) (62), which allows this

indicator to estimate the production of $OH^{\cdot}$. In cells simultaneously incubated with PAF and either αIgM or H_2O_2, the rate of DCFH oxidation, and therefore the accumulation of $OH^{\cdot}$, was greatly diminished (Figures 8A and B).

To overcome the oxidative stress caused by αIgM or H_2O_2 treatment, cells scavenge $OH^{\cdot}$ radicals by enzymatic conversion of GSH to oxidized glutathione via GSH peroxidase (67). Not unexpectedly, depletion of GSH is a characteristic of Ramos cells that undergo apoptosis (Figure 9). In further support of PAF's role in maintaining the balance of oxidant and reactive oxygen substances, cells treated with PAF do not show any depletion of GSH (Figure 9).

These data suggest that PAF is able to rescue antigen stimulated Ramos B cells from apoptosis by decreasing intracellular oxidative stress. This action of PAF has not been previously described, and the pathway through which reactive oxygen substance accumulation is blocked needs to be elucidated. Much of the work on apoptosis and reactive oxygen substances has focused on the gene products of bcl-2 and bclx. The bcl-2 gene was first found overexpressed in B cell lymphomas and has been confirmed to be an anti-apoptotic gene. Overexpression of BCL_2 and Bcl_x proteins blocks apoptosis induced by oxidative stress (42, 60, 68) including PCD induced by αIgM (57). Although bcl2 is constitutively expressed in some immature B cells (65), this is not the case in our Ramos line (N. Sakata, personal communication). bcl_2 and bcl_x genes can be induced by receptor mediated stimuli such as anti-CD40 antibodies (65). The induction of these genes, and maximal expression of their protein takes 2 hours (65). We have performed time course experiments that have examined cell viability (data not shown), apoptosis (Figure

3B) and DCFH oxidation (Figures 8C). They demonstrate maximal efficacy if PAF is present within one hour of BCR stimulation. This time course is not compatible with gene induction and new protein synthesis, which would be suggested by a different kinetic of protection. This does not completely rule out induction of bcl_2 or bcl_x genes by PAF, and studies of this pathway are in progress. However, alternative protective mechanisms should be investigated.

Other possible ways that PAF may act include steric hindrance and/or via desensitization of the sIgM. We have previously demonstrated, using changes in intracellular calcium, that PAF and αIgM can act in parallel in B cell lines, suggesting that they neither interact with the same receptor, nor do they cause heterologous desensitization (18, 20). The ability of PAF to act if added one hour following stimulation with αIgM is also not supportive of a steric interaction. As well, the effect of PAF is not exclusive to αIgM, as it is equally effective in PCD induced by H_2O_2. This further suggests that the lipid mediator has specific effects within Ramos cells on the production of oxidants.

H_2O_2 is an important intracellular mediator (69) and its presence may influence the cell in a variety of ways. In contrast to its often deleterious effects, H_2O_2 can induce cell activation if the correct combination of signals is present (70). The addition of H_2O_2 to B lymphocytes, including Ramos cells, induces phosphorylation of $p72_{syk}$ and $p56_{lyn}$, and increases intracellular calcium levels (71, 72). Although it is clear that, in B lymphocytes, BCR signaling is linked to pathways that produce H_2O_2, the actual sequence of events is not known (57, 73). Prominent enzymatic pathways that may be involved include cytochrome P_{450} enzymes, xanthine

oxidases, mitochondrial peroxidases, amino acid oxidases, cyclooxygenases, and lipoxygenases, all of which liberate H_2O_2 in their metabolism of protein or lipid cellular constituents (61, 74). Antioxidants have been shown to completely inhibit αIgM-mediated cell growth in murine B cells (75), underscoring the importance of H_2O_2 and its metabolites in BCR signaling. The two seemingly opposing effects of peroxide and its metabolites suggest that, depending on the maturity of the cells or the presence of a second signal, a B cell may employ H_2O_2 as an intracellular messenger either positively or negatively, guiding the breakdown of H_2O_2 towards or away from the production of high concentrations of toxic reactive oxygen substances.

How does PAF modify the effects of H_2O_2 production in Ramos cells? PAF has been demonstrated to initiate transmembrane signaling through its receptor in a number of B cell lines (18, 20, 22). PAFR is a GTP binding protein linked receptor, with 7 transmembrane domains (14). Signals through PAFR induces increases in intracellular calcium and inositol phosphates and phosphorylation of ERK2, the p42 MAP kinase (19, 64) and PI-3 kinase (22). PI-3 kinase has been shown to be involved in anti-apoptotic signals in PC12 cells (76), and hematopoietic cells (77) and it also may be important in BCR mediated growth arrest in immature B cells (78). The role of the MAP kinase pathway in regulation of apoptosis is also under investigation (79, 80). Further, the rapidity of its action suggests that PAF is a candidate for modification of the activity of enzymes that influence the production of H_2O_2. PAF can liberate arachidonic acid and has been demonstrated to induce the activity of PLA_2 (21), cyclooxygenases (81, 82), and isoforms of lipoxygenases (14). Because immature B cells appear to be more prone to produce reactive oxygen substances following BCR ligation than mature

cells (83) the inducible enzyme pattern following αIgM activation of a cell such as Ramos may be different than that of a more mature B cells line. PAF itself may influence the generation of reactive oxygen substances differently in more mature cells than in cells such as Ramos. We are embarking on a series of investigations to determine at what level PAF may modify H_2O_2 production.

We have demonstrated a novel effect of PAF with its ability to protect Ramos B lymphocytes from the PCD initiated following BCR ligation. This is most likely due to an effect on intracellular oxidants, functioning by either decreasing H_2O_2 production or altering the metabolism of H_2O_2 to more toxic $OH^{\cdot}$ radicals. As PAF is secreted as part of the immune response for cell recruitment in infections (14) as well in inflammatory states (84) B lymphocytes can be profoundly affected by this mediator under many conditions. Studies examining complementary signal transduction pathways and enzymatic pathways are needed to determine the mechanism by which PAF can synergise with antigen signaling through the BCR. Studies must also be performed on B cell lines representing other stages of maturity, and extended to freshly isolated B lymphocytes in order to discern the full significance of PAF signaling in B cell maturation. Better definition of the role that H_2O_2 serves as a second messenger in B cells may clarify changes in susceptibility to PCD in maturing B lymphocytes.

Linking Text

Our previous study demonstrated that PAF can abrogate apoptosis in the Ramos B cell line. This was due to an effect on intracellular oxidants, by decreasing the production of $OH^{\cdot}$ radicals that are increased following αIgM stimulation of Ramos cells. In addition, we demonstrated that crosslinking of the BCR on Ramos cells by αIgM decreased their constitutive secretion of IgM antibodies. PAF maintained αIgM stimulated Ramos cells functional as they had increased levels of IgM secretion compared to those treated with αIgM alone.

In the present studies, we attempt to characterize the effect of PAF on events that follow sIgM crosslinking of Ramos cells. We have focused particularly on the population of cells that have not undergone apoptosis. We assessed changes in expression of the adhesion molecule VLA-4 and the BCR itself, which are both constitutively expressed on intact viable B lymphocytes. We demonstrate clearly that PAF is not only able to rescue Ramos cells from apoptosis, but maintains VLA-4 and BCR expression, essential features of B cell competence.

Characterization of B lymphocytes rescued from apoptosis by platelet-activating factor

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Submitted: Journal of Leukocyte Biology.

Abstract

B lymphocyte development is characterized by deletion via apoptosis of immature cells that are insufficiently stimulated. We have previously demonstrated that crosslinking of the B cell receptor (BCR) using αIgM (2 μg/ml) in Ramos B lymphoblastoid cells causes deletion of 30-40% of cells by apoptosis in 24 hours. Addition of the potent lipid mediator, platelet-activating factor (10^{-7} M) to αIgM stimulated Ramos cells significantly decreases the number of apoptotic cells as measured by Annexin V labeling. We have characterized the phenotype of Ramos cells that have not become apoptotic following BCR stimulation. In these cells, there is a significant decrease in the surface expression of the VLA-4 adhesion molecule (31% of control expression) and sIgM (53% of control expression). Significantly fewer cells co-incubated with PAF underwent apoptosis, and the remaining cells maintained control levels VLA-4 (104% of control expression) and sIgM expression (104% of control). All of these protective effects were inhibited by the specific PAF receptor antagonist, WEB 2170. The ability of PAF to maintain sIgM expression at control levels was inhibited by cycloheximide (7.5 μg/ml), an inhibitor of protein synthesis while cytochalasin B, (5 μg/ml), an inhibitor of microtubular function, had no effect on sIgM expression. In contrast neither cycloheximide or cytochalasin B altered PAF's ability to abrogate apoptosis. This data indicates PAF's effect on rescuing and maintaining αIgM stimulated Ramos B cells is mediated via at least two pathways. Abrogation of apoptosis does not require de novo protein synthesis, while maintenance of sIgM expression requires protein synthesis.

Introduction

Maturation of B lymphocytes to antibody secreting cells includes a stringent process of selection. Only 5% of developing B lymphocytes will become mature plasma cells, whereas the other 95% are deleted by apoptosis (4). B lymphocytes that are prone to deletion include those which have faulty rearrangement of heavy and light chains genes, or B cells where the receptor is crosslinked while the cell is immature (4). It has been previously shown that cytokines or ligation of adhesion molecules can prevent programmed cell death in immature antigen stimulated B lymphocytes (41, 66, 85).

The Ramos cell line, an EBV negative Burkitt lymphoma line, has been used as a model to study the growth and development of germinal center B cells (17). Ligation of sIgM on Ramos leads to PCD or apoptosis, which is presumed to be the mechanism of deletion of cells that are insufficiently stimulated to mature or insufficiently mature to be stimulated (38, 39, 86). We have demonstrated that platelet-activating factor (PAF), a potent B lymphocyte activator, can abrogate apoptosis in the Ramos B cell line (37). This was due to an effect on intracellular oxidants, by decreasing the production of $OH^{\cdot}$ radicals that are increased following αIgM stimulation of Ramos cells. In addition, we demonstrated that crosslinking of the BCR on Ramos cells by αIgM decreased their constitutive secretion of IgM antibodies. PAF maintained αIgM stimulated Ramos cells functional as they had increased levels of IgM secretion compared to those treated with αIgM alone.

We have previously shown that in B lymphoblastoid cell lines, the addition of PAF results in multiple signaling events including increases in intracellular calcium (18), generation of inositol phosphates, tyrosine phosphorylation of proteins including MAP kinase (19) and synthesis of immunoglobulin (45). In the present studies, we have attempted to characterize the effect of PAF on events that follow sIgM crosslinking of Ramos cells. We have focused particularly on the population of cells that have not undergone apoptosis. We assessed changes in expression of the adhesion molecule VLA-4 and the BCR itself, which are both constitutively expressed on intact viable B lymphocytes. VLA-4 promotes the interaction of B cells with the structural matrix in lymphoid tissues, and its ligation rescues B cells from PCD, and allows their progression to plasma cells (87). The BCR is essential for antigenic recognition and signaling. Downregulation of BCR expression would result in the inability of a B cell to interact with antigen, and therefore rescuing B cells from apoptosis with diminished BCR expression would suggest an impairment of normal B cell function (88). We demonstrate clearly that PAF is not only able to rescue Ramos cells from apoptosis, but maintains VLA-4 and BCR expression, essential features of B cell competence.

Material and Methods

Reagents

PAF (1-alkyl-2-acetyl-sn-glycero-3-phosphocholine, C-16) purchased from Biomol, (Plymouth Meeting, PA) was resuspended in ethanol and stored at -20°C. Before use, the ethanol was evaporated under nitrogen and PAF was reconstituted in PBS containing 0.125% BSA. Web 2170 was obtained through the generosity of Boehringer-Ingelheim, (Ingelheim en Rhein, Germany) and was resuspended in PBS containing 0.125% BSA. αIgM (IgG BU1 clone) was purchased from The Binding Site (San Diego, CA). Cycloheximide and cytochalasin B were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture

The human B-lymphoblastoid cell line, Ramos (RA1, American Type Culture Collection, Rockville, MD) is characterized by the expression of $sIgM^+$, $sIgD^-$, $CD10^+$, $CD38^+$ and $VLA\text{-}4^+$. It was maintained in complete medium consisting of RPMI 1640 (Life Technologies, Burlington, Canada) supplemented with 10% FCS (Hyclone, Logan, UT) and 50 U/ml penicillin, 50 ng/ml streptomycin, 10 µg/ml L-glutamine and 5 µg/ml sodium pyruvate (all from Life Technologies). Frozen aliquots were thawed every 8 weeks to minimize intra-assay variability. Cells were prepared for culture by harvesting and washing three times in RPMI 1640 alone, and then resuspended in serum free medium as previously described at a concentration of 3×10^5 cells/ml. Serum free medium was prepared by the method of Kovar and Franek (46). All cultures were performed in cell culture tubes (Falcon

2054, Becton Dickinson, La Jolla, CA). PAF, αIgM, WEB 2170 and/or other reagents were added at the appropriate dilutions to the cell suspensions at indicated times and the cell culture was maintained for 24 hours in a humidified incubator at 37°C in 5% CO_2.

Apoptosis assay

Following cell culture for the indicated times the cells were washed twice by centrifugation with PBS at 1200 RPM for 10 minutes, resuspended in 250 µl FCS and 250 µl RPMI 1640 and fixed in 1.5 ml 70% ethanol at 4° C for a minimum of 30 minutes. After fixation the cells were washed twice with PBS by centrifugation at 4° C, 2400 RPM for 5 minutes, resuspended in 1 ml PBS with 0.05 mg/ml RNase A (50 units/mg) (Boehringer Manheim, Laval, QC) and incubated for 30 minutes at 37° C. Following this, 50 µg/ml propidium iodide was added and the cells were analyzed by flow cytometry utilizing Cell Fit Software (Becton Dickinson). The cells whose DNA content was below the G_0 peak (<2n DNA content) were assessed as apoptotic.

Detection of apoptosis via Annexin V binding

The Apoptosis Detection Kit (R&D Systems, Minneapolis, MN) was utilized according to the manufacturer's recommendations. Following cell culture for the indicated time, cells were washed twice by centrifugation with cold PBS at 1200 RPM for 10 minutes and resuspended in 100 µl binding buffer at 10^6 cells/ml. Ten µl Annexin V and 10 µl propidium iodide (50 µg/ml) were added and the cells were incubated 15 minutes in the dark. Subsequently, 400 µl of binding buffer was added following the incubation period

and the cells were analyzed by flow cytometry using Lysis II software (Becton Dickinson). Following exclusion of the cells that stained with PI, the cells with Annexin V binding were assessed as apoptotic.

Assessment of surface expression of VLA-4 and sIgM

Following cell culture for the indicated times the cells were washed twice by centrifugation at 1200 RPM for 10 minutes. The cells were then resuspended in 45 μl PBS to which 5 μl fluorescein conjugated αIgM antibody (The Binding Site, San Diego, CA) or 5 μl phycoerythrin conjugated anti-VLA-4 (CD49d) antibody (Pharmigen, San Diego, CA) was added and subsequently incubated in the dark for 30 minutes at room temperature. The cells were washed twice with cold PBS and resuspended in 0.5 ml of PBS with 20 μg/ml PI solution. The cells were incubated in the dark for an additional 5 minutes at room temperature and analyzed by flow cytometry in the following manner: 1. Following exclusion of necrotic cells by PI uptake, presence of sIgM or VLA-4 was measured by analysis of the fluorescence at 510-550 nm (fluorescein spectrum) or 564-596 nm (phycoerythrin spectrum). 2. Following exclusion of apoptotic cells (<2n DNA content), sIgM expression was measured by analysis of the fluorescence at 510-550 nm (fluorescein spectrum). 3. Following exclusion of apoptotic cells (Annexin V^+ cells), VLA-4 expression was measured by analysis of the fluorescence at 564-596 nm (phycoerythrin spectrum). sIgM and VLA-4 mean channel fluorescence were expressed as a percentage of the control condition, mean channel fluorescence in addition to the isotype control in order standardize the evaluation of surface molecule density for the experiment.

Graphical and Statistical Analysis

All graphical analysis including mean and SEM were performed using Graphpad Prism (Graphpad Software, San Diego, CA) and all other statistical analysis was performed with Instat (Graphpad Software, San Diego, CA). p values were obtained by Tukey-Kramer Multiple Comparison Test.

Results

PAF abrogates αIgM mediated apoptosis in Ramos B cells

Involution of the cellular membrane, with exposure of phosphotidylserine (PS) residues, is an early event in apoptosis (89). Annexin V has a high affinity for PS residues on the cell membrane and this is thus used to measure early apoptotic cells (90, 91). αIgM stimulation of Ramos cells led to Annexin V labeling in an average of 16% ± 3.2% of the cells, compared to 4.7% ± 3.2% of unstimulated cells ($p<0.05$) (Figure 12A). Comparable to our previous studies using DNA laddering (37), co-incubation of PAF with αIgM diminished the population of apoptotic cells, with only 5.7% ± 2.5% of the cells demonstrating Annexin V labeling (ns compared to control). There was a significant decrease of the action of PAF when cells were pre-incubated with the specific PAF antagonist Web 2170 (Figure 12B).

Figure 12

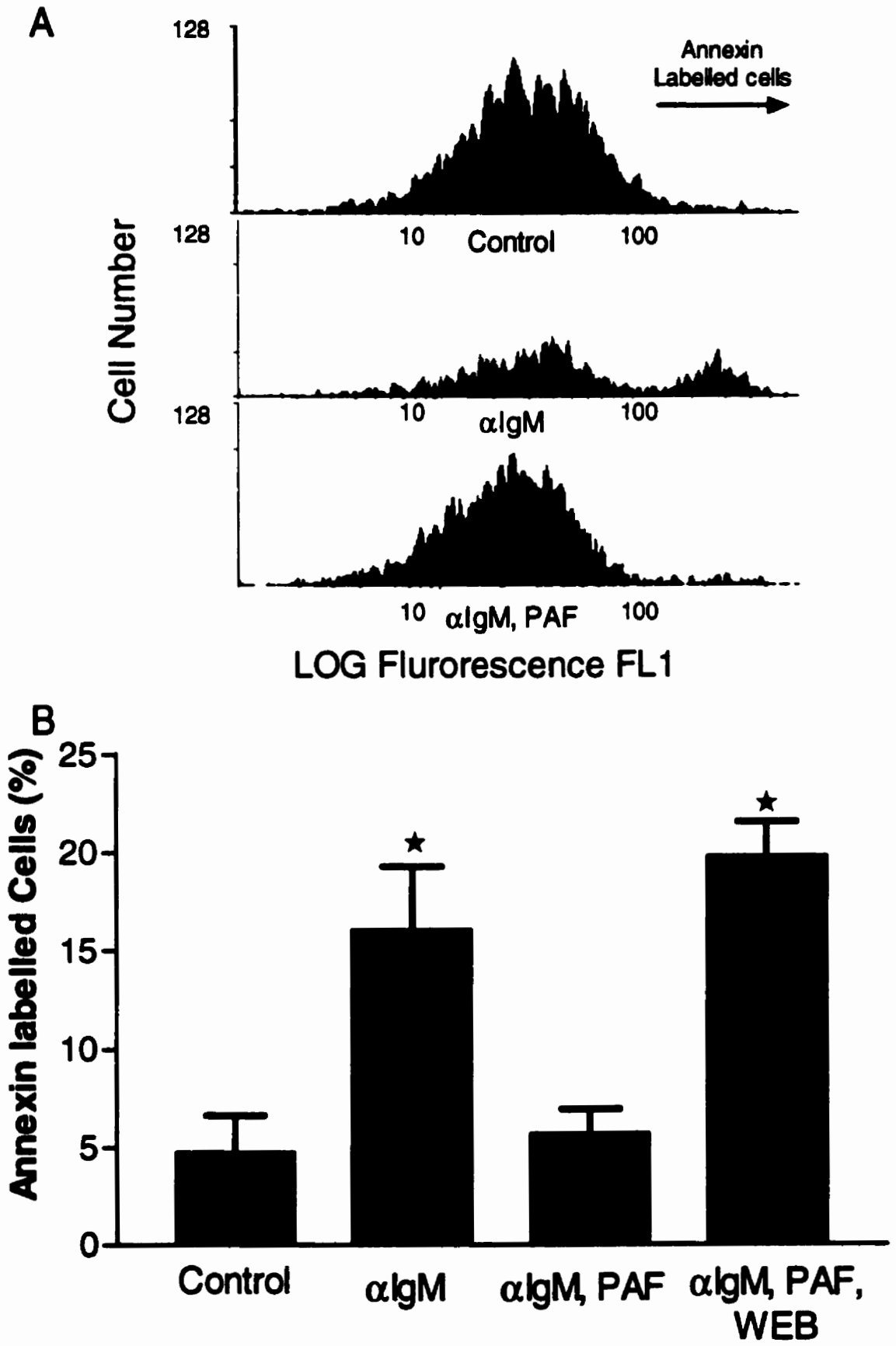

A
128
Annexin
Labelled cells
Cell Number
128
10
Control
100
128
10
αIgM
100
10
αIgM, PAF
100
LOG Flurorescence FL1
B
Annexin labelled Cells (%)
25
20
15
10
5
0
Control
αIgM
αIgM, PAF
αIgM, PAF,
WEB

Figure 12 *A: Apoptosis demonstrated by Annexin V labeling. A representative histogram of 3 experiments demonstrating PI Ramos cells with Annexin V labeling.* ***Top panel:*** *Control cells.* ***Middle panel:*** *Demonstrates that incubation with αIgM (2 μg/ml) resulted in a significant increase in the number of cells with Annexin V labeling.* ***Lower panel:*** *The increase in Annexin V labeled cells is reversed by simultaneous addition of PAF (10^{-7} M). B: Graphical representation of apoptotic Ramos cells (Annexin V labeled cells). Cells incubated with αIgM (2 μg/ml) resulted in a significant increase in the number of cells with Annexin V labeling, which is reversed by the simultaneous addition of PAF (10^{-7} M). PAF's effects were blocked by WEB 2170 (10^{-6} M). N=3 (★ p<0.01 as compared to control).*

PAF maintains cell surface marker integrity in αIgM stimulated Ramos cells

Little is known about the changes in expression of crucial surface markers following αIgM stimulation of Ramos cells. We therefore examined the integrity of cell surface molecules in those lymphocytes that had not undergone apoptosis following αIgM stimulation. VLA-4 is an adhesion molecule that is important in B lymphocyte homing to marrow stromal cells (92, 93), lymphoid tissue (94) and activated endothelial cells (95) and may be involved in regulating immunoglobulin synthesis (87). Following crosslinking of the sIgM, there was a significant decrease in VLA-4 expression in cells that excluded PI (Figure 13 A). Cells that were simultaneously incubated with αIgM and PAF maintained VLA-4 expression at baseline levels (Figure 13A). While αIgM crosslinking downregulated surface VLA-4 expression in the viable cells to 74% ± 3.4% of control (p<0.001 compared to control) (Figure 13B), PAF restored VLA-4 expression in αIgM stimulated cells, (93% ± 1% of control (ns compared to control)). PAF's protective effect was blocked by WEB 2170 preincubation (VLA-4 expression 81% ± 4% of control) (p<0.01 compared to control) (Figure 13B).

Figure 13

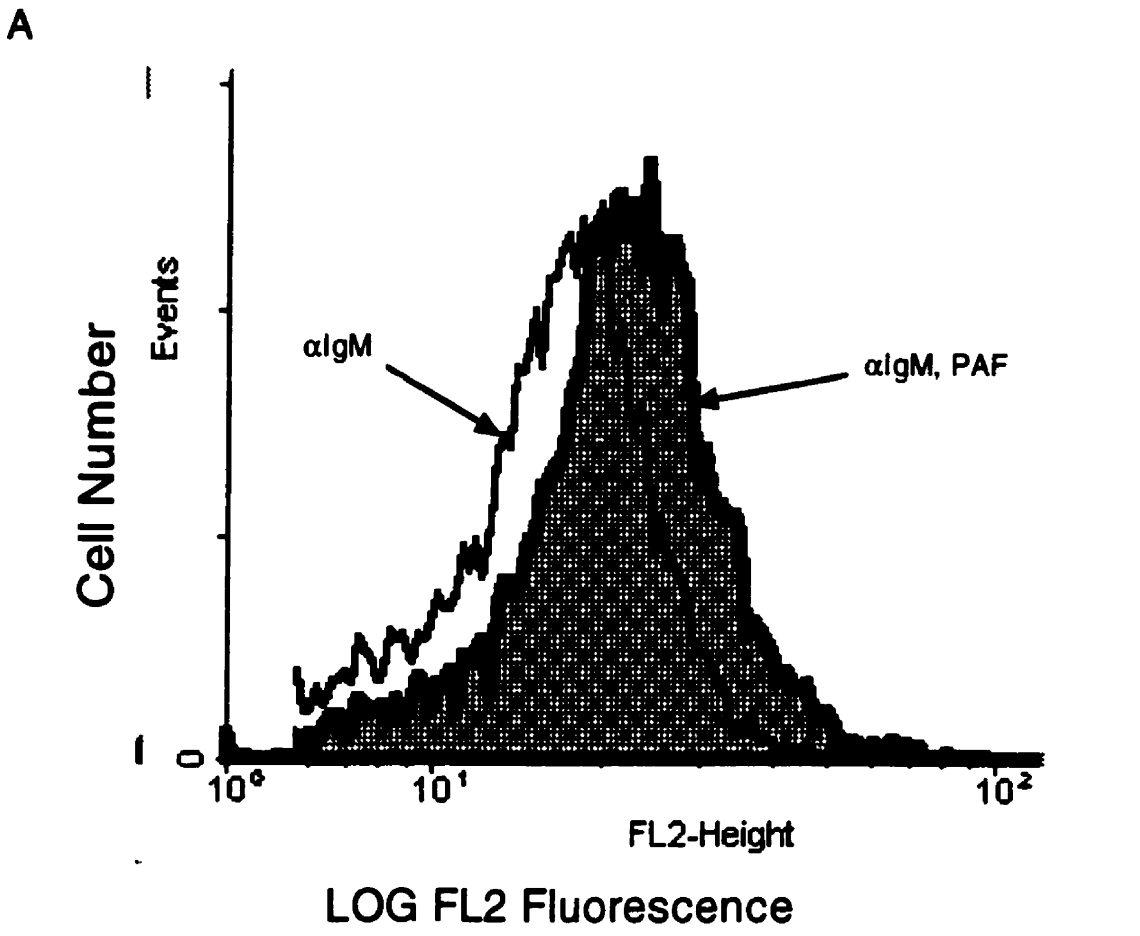

A
Cell Number
Events
αIgM
αIgM, PAF
0
10⁰
10¹
10²
FL2-Height
LOG FL2 Fluorescence
B
Percent of Control VLA-4 Mean Channel Fluorescence
100
50
0
Control
αIgM
αIgM, PAF
αIgM, PAF, Web

***Figure 13**: A: A representative histogram of 4 experiments demonstrating VLA-4 labeling in PI⁻ cells. Following incubation with αIgM (2 μg/ml) Ramos cells demonstrate a decrease in VLA-4 expression (clear region) that was blocked by simultaneous addition with PAF (10^{-7} M) (shaded region). B: Graphical representation of VLA-4 expression (VLA-4 mean channel fluorescence was expressed as a percentage of the control VLA-4 mean channel fluorescence). Following incubation with αIgM (2 μg/ml) Ramos cells demonstrate a significant decrease in VLA-4 expression that was blocked by PAF (10^{-7} M). The PAF effect was blocked by WEB 2170 (10^{-6} M). Results are expressed as mean ± SEM. (N = 4) (★ p<0.001 compared to Control).*

VLA-4 expression was also assessed following the exclusion of Annexin V labeled cells. We performed this to better define the non apoptotic population, as early apoptotic cells may have been among the non-apoptotic population following PI exclusion. In the Annexin V⁻/PI⁻ population there was a significant decrease in VLA-4 expression following sIgM crosslinking expression to 31.0% ± 15% of control (p<0.01 compared to control)(Figure 14A, B). Cells that were simultaneously incubated with αIgM and PAF maintained VLA-4 expression at baseline levels with VLA-4 expression in αIgM stimulated cells (104.5% ± 2.3% of control, ns compared to control)(Figure 14A, B). PAF's protective effect was blocked by WEB 2170 incubation (64% ± 10 of control, p<0.01 compared to control).

Figure 14

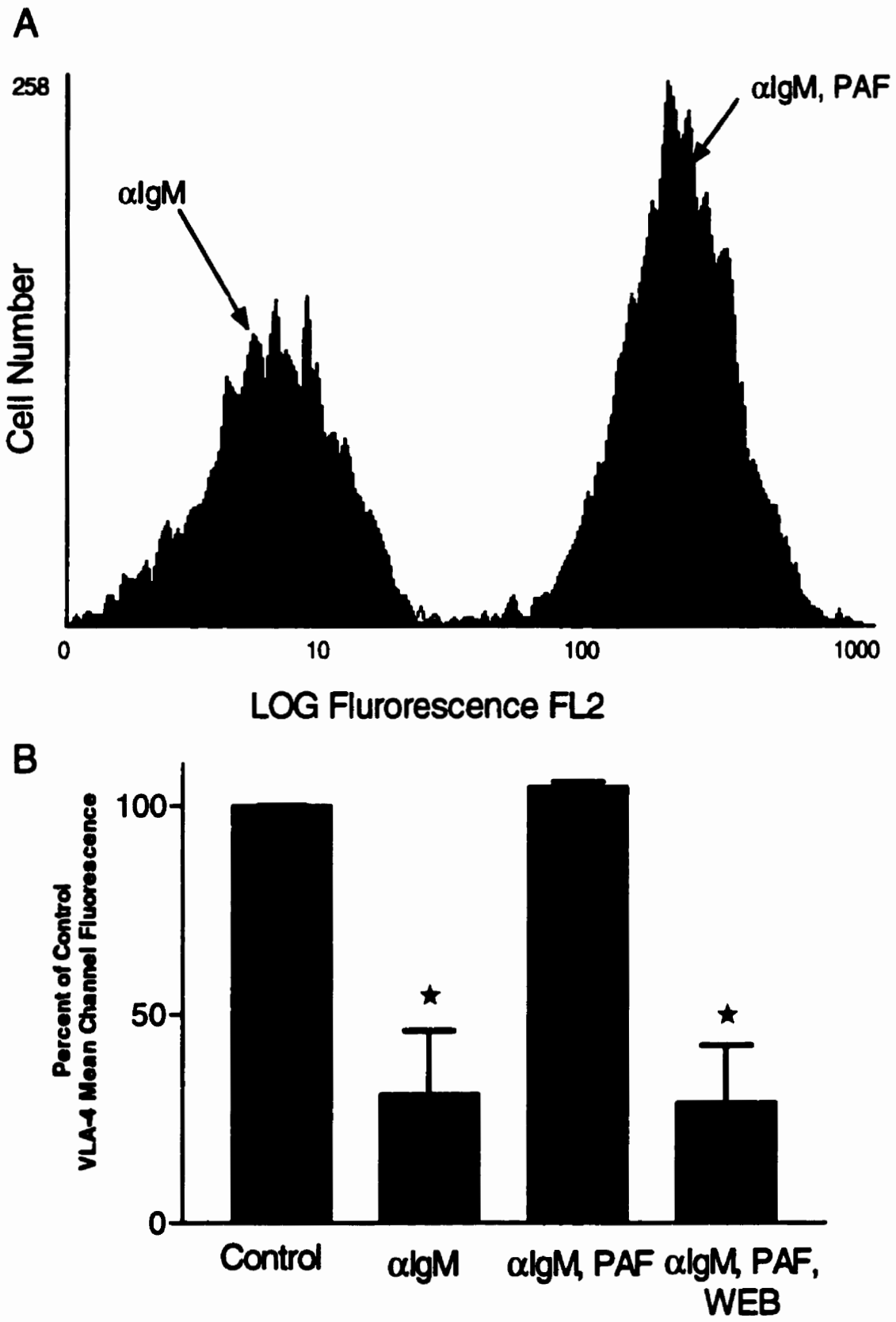
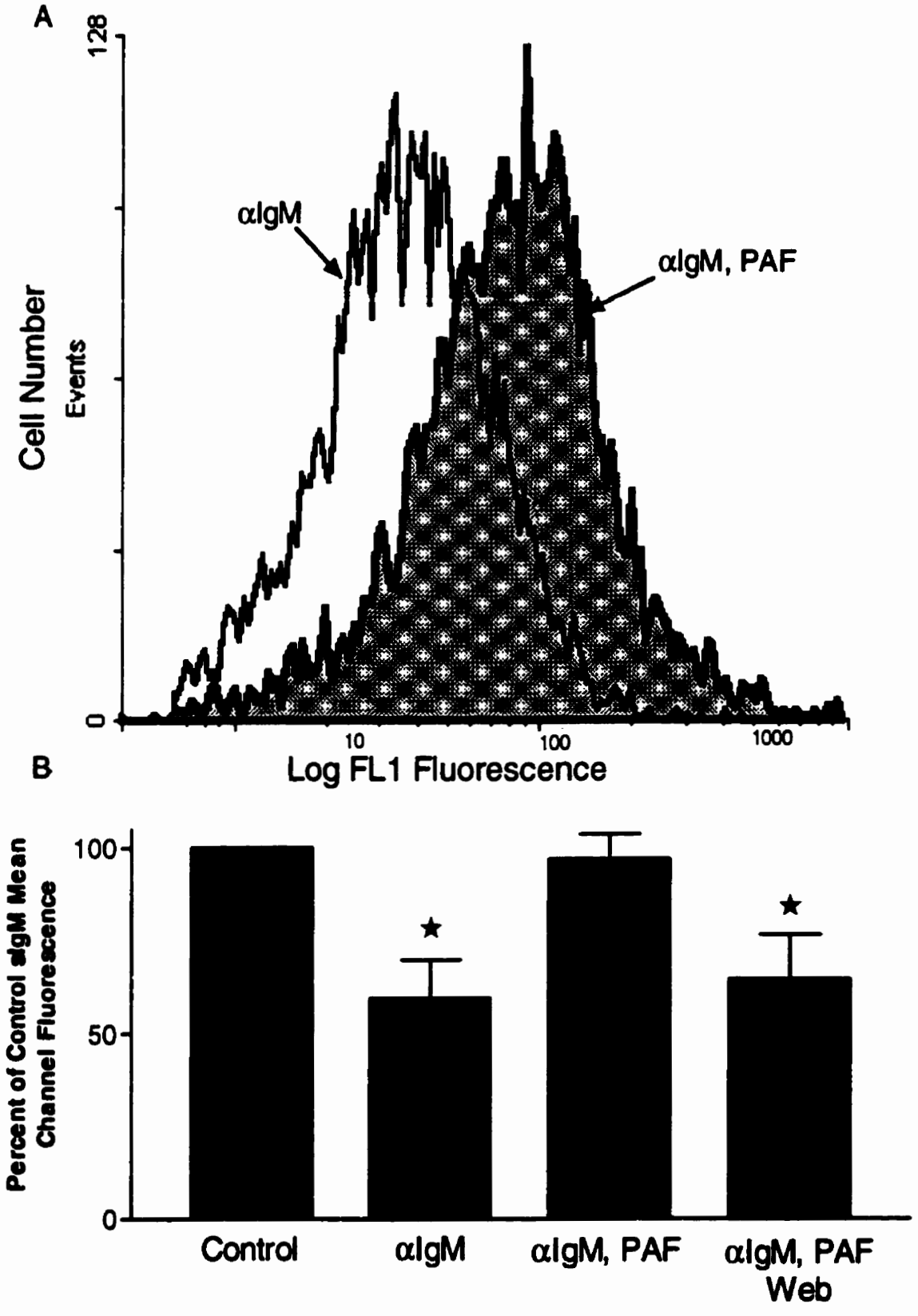

A
258
αIgM, PAF
αIgM
Cell Number
0
10
100
1000
LOG Fluorescence FL2
B
Percent of Control
VLA-4 Mean Channel Fluorescence
100
50
0
Control
αIgM
αIgM, PAF
αIgM, PAF, WEB

***Figure 14**: A: A representative histogram of 6 experiments demonstrating VLA-4 labeling in Annexin V cells. Following incubation with αIgM (2 μg/ml) Ramos cells demonstrate a decrease in VLA-4 expression that was blocked by simultaneous addition with PAF (10^{-7} M).*
B: Graphical representation of VLA-4 expression (VLA-4 mean channel fluorescence was expressed as a percentage of the control VLA-4 mean channel fluorescence). Following incubation with αIgM (2 μg/ml) Ramos cells demonstrate a significant decrease in VLA-4 expression that was blocked by PAF (10^{-7} M). The PAF effect was blocked by WEB 2170 (10^{-6} M). Results are expressed as mean ± SEM (N = 6) (★ $p<0.05$ compared to Control).

To further characterize PAF's effect on the cells that had not undergone apoptosis we assessed sIgM expression. As the BCR is crucial to B cell antigen interaction and initiates B cell activation we felt that assessing its expression would provide a measure of B cell competence. αIgM stimulation resulted in a significant decrease in sIgM expression in PI⁻ cells to 59% ± 10% of control ($p<0.05$ compared to control) (Figure 15A, B), while cells incubated simultaneously with PAF and αIgM demonstrated sIgM expression equal to control value (97% ± 7%, ns compared to control) (Figure 15B). PAF's effect was blocked with WEB 2170 incubation (sIgM expression 64% ± 10% of control $p<0.05$).

Figure 15


A
128
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Cell Number
Events
αIgM
αIgM, PAF
10
100
1000
Log FL1 Fluorescence
B
Percent of Control αIgM Mean Channel Fluorescence
100
50
0
Control
αIgM
αIgM, PAF
αIgM, PAF Web

Figure 15 A: Representative histogram of 6 experiments demonstrating sIgM labeling in PI cells. Following incubation with αIgM (2 μg/ml) Ramos cells demonstrate a decrease in sIgM expression that was blocked by simultaneous addition with PAF (10^{-7} M) (shaded region). B: Graphical representation of sIgM expression (sIgM mean channel fluorescence was expressed as a percentage of the control sIgM mean channel fluorescence). Following incubation with αIgM (2 μg/ml) Ramos cells demonstrate a significant decrease in sIgM expression that was blocked by PAF (10^{-7} M). The PAF effect was inhibited by WEB 2170 (10^{-6} M). Results are expressed as mean ± SEM (N = 6) (★ p< 0.05 as compared to Control).

PAF's ability to maintain sIgM expression requires new protein synthesis

The re-expression of the BCR following crosslinking of the sIgM is dependent on capping and endocytosis of the receptor (96) and the subsequent ability to regenerate a receptor via de novo protein synthesis (97). Therefore, we assessed sIgM re-expression following inhibition of cytoskeleton activity using cytochalasin B to inhibit endocytosis, as well as following inhibition of new protein synthesis by cycloheximide. αIgM stimulation decreased sIgM expression to 53% ± 3.9% of control sIgM expression ($p<0.01$ compared to control) in cells that did not demonstrate DNA fragmentation. This was not changed by preincubation with 7.5 μg/ml cycloheximide (61% ± 8.3%, ns compared to αIgM alone) or with 5 μg/ml cytochalasin B (62% ± 13.5%, ns compared to αIgM alone). Addition of PAF to αIgM treated cells restored sIgM expression (104.4% ± 2.2% of control expression, ns compared to control), which was not changed by preincubation with cytochalasin B (98.7% ± 1.7% of control sIgM expression, ns compared to control) (Figure 16A). However, cycloheximide preincubation resulted in PAF's effect being diminished (66.3% ± 2.6% of control sIgM expression, $p<0.001$ compared to control). The specific PAF antagonist WEB 2170 was also able to block the effects of PAF ($p< 0.01$ compared to control) (Figure 16B). The inability to inhibit the decrease in BCR expression by adequate

doses of cytochalasin B implies that F-actin mediated endocytosis of the BCR is not likely to be the mechanism of BCR expression downregulation (98, 99). The re-expression of the BCR following αIgM and PAF co-incubation is dependent on de novo protein synthesis as it was blocked by cycloheximide.

Figure 16

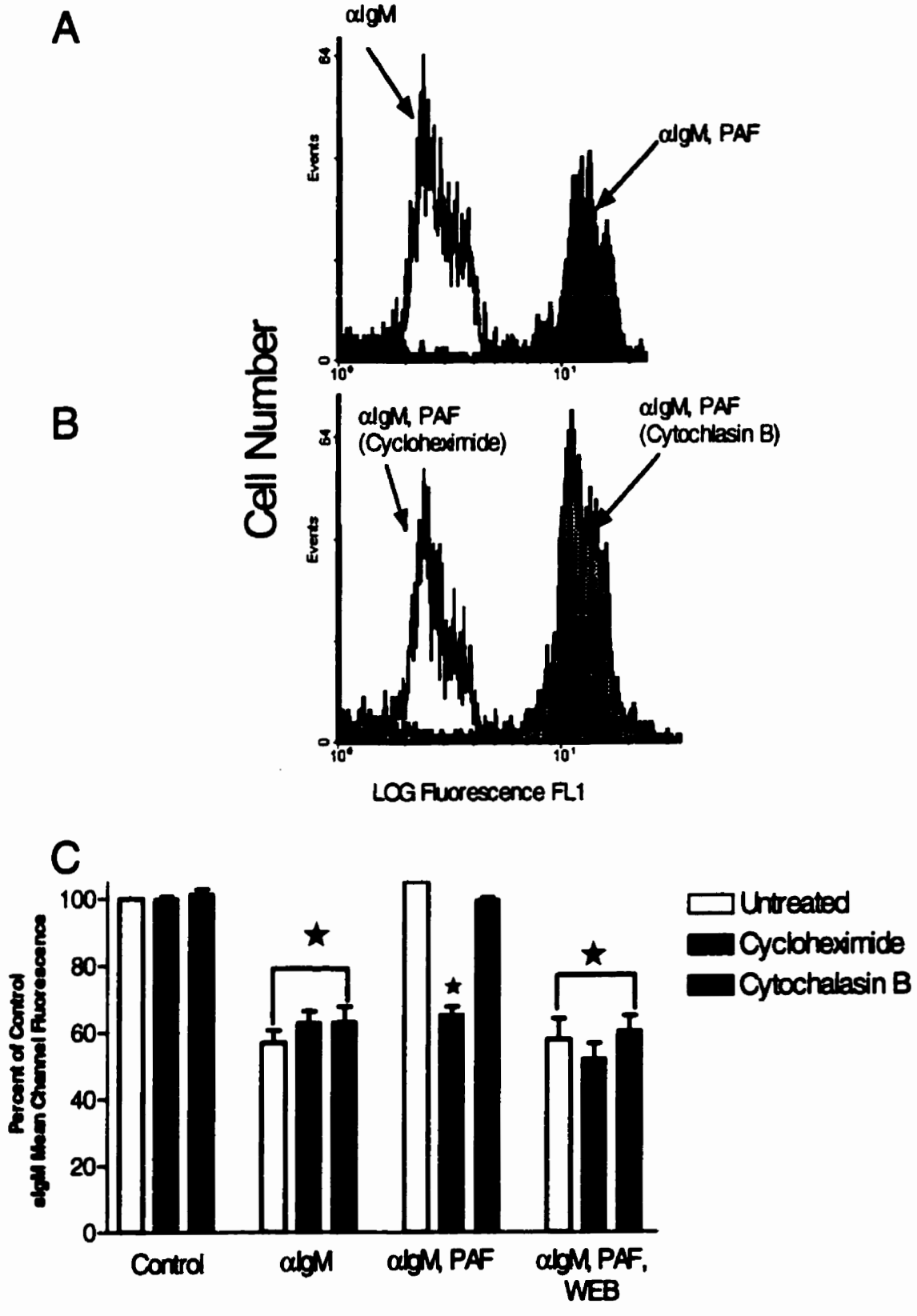

A
αIgM
αIgM, PAF
Events
64
0
10⁰
10¹
B
Cell Number
αIgM, PAF
(Cycloheximide)
αIgM, PAF
(Cytochlasin B)
LOG Fluorescence FL1
C
Percent of Control
αIgM Mean Channel Fluorescence
100
80
60
40
20
0
Control
αIgM
αIgM, PAF
αIgM, PAF,
WEB
Untreated
Cycloheximide
Cytochalasin B

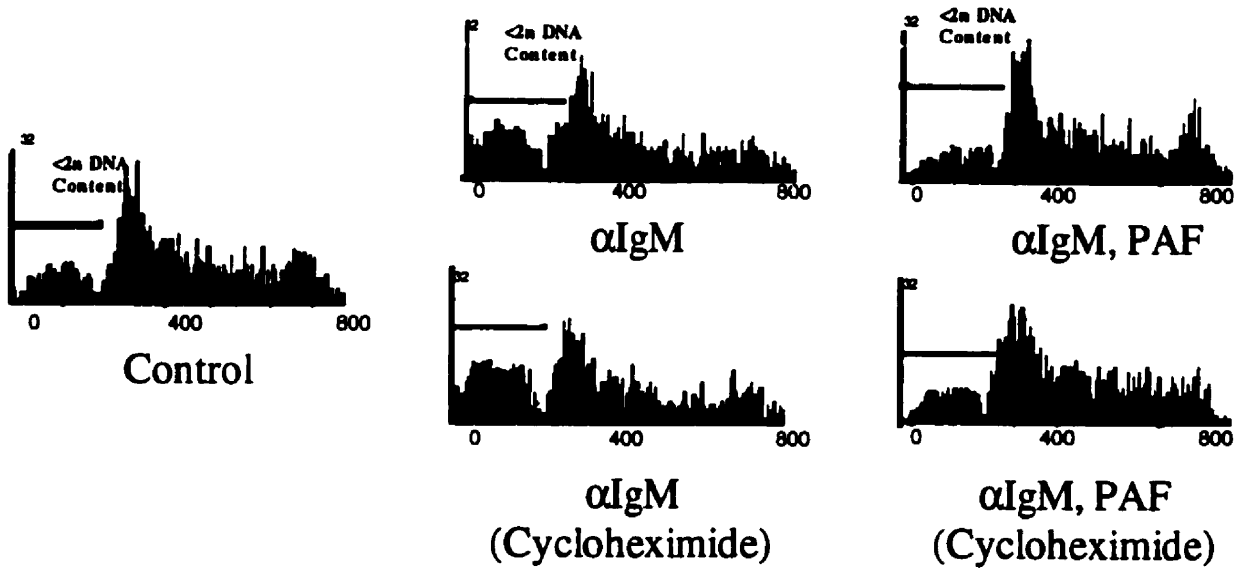
Figure 16 *A and B: Representative histogram of 7 experiments demonstrating sIgM expression in cells excluding <2n DNA content. A: Following incubation with αIgM (2 μg/ml) Ramos cells demonstrate a decrease in sIgM expression (clear region) that was blocked by simultaneous addition with PAF (10^{-7} M) (shaded region). B: Simultaneous incubation of αIgM (2 μg/ml) and PAF (10^{-7} M) of Ramos cells pretreated with cycloheximide or cytochalasin B. Cycloheximide inhibited the increase in sIgM expression mediated by PAF (clear region), whereas cytochalasin B did not effect on PAF's ability to maintain sIgM expression (shaded region). C: Graphical representation of sIgM expression (sIgM mean channel fluorescence was expressed as a percentage of the control sIgM mean channel fluorescence) Following incubation with αIgM (2 μg/ml) Ramos cells demonstrate a significant decrease in sIgM expression whether untreated or preincubated with cycloheximide or cytochalasin B. sIgM expression was maintained following co-incubation with PAF (10^{-7} M) in the untreated and cytochalasin B preincubated condition, while cycloheximide pretreatment inhibited the protective effects of PAF. The PAF antagonist WEB 2170 (10^{-6} M) also inhibited the effects of PAF. Results are expressed as mean ± SEM. N=7 (★ $p < 0.001$ as compared to Control).*

DNA content analysis was performed to directly visualize the appearance of non random 180-200 bp breaks which hall-mark apoptosis (55, 100). In this cell population, αIgM induced DNA fragmentation in an average of 20.3% ± 2.3% of Ramos cells, compared to 9.0% ± 1.5% of control cells ($p<0.05$ compared to control) (Figure 17A). Co-incubation of PAF with αIgM diminished the population of apoptotic cells, with only 9.8% ± 1.1% of the cells with <2n DNA content (ns compared to control). There was a significant decrease of the action of PAF when cells were pre-incubated with the specific PAF antagonist Web 2170 (Figure 17B). Preincubation with 7.5 μg/ml cycloheximide did not change the induction of apoptosis following αIgM incubation in the untreated cells 22.4% ± 2.3% (ns compared to αIgM alone), nor did it affect the ability of PAF to decrease the number of apoptotic cells (10.4% ± 1.1%, ns compared to control) (Figure 17B). Preincubation with

cytochalasin B also did not affect the induction of apoptosis by αIgM crosslinking or PAF's ability to decrease the induction of apoptosis (data not shown).

Figure 17

A



B

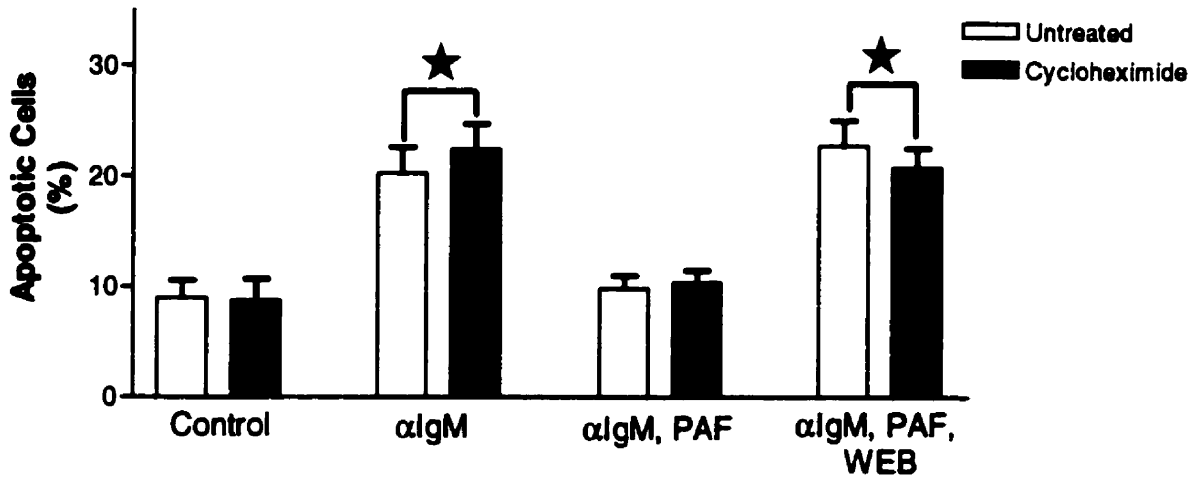


Figure 17 *A: Representative histogram of 6 experiments demonstrating cells with <2n DNA content as representative of apoptotic cells. The x axis represents FL2 area and the y axis cell number.* ***Top panel:*** *Demonstrates that incubation with αIgM (2 μg/ml) resulted in a significant increase in the number of cells with <2n DNA content, which is reversed by the simultaneous addition of PAF (10^{-7} M). PAF's effects were blocked by WEB 2170 (10^{-6} M).* ***Middle panel:*** *Demonstrates that cycloheximide did not alter the induction of apoptosis by αIgM (2 μg/ml) or the ability of PAF (10^{-7} M) to abrogate the effects of αIgM. B: Graphical representation of percent apoptotic Ramos cells (<2n DNA content). Cells incubated with αIgM (2 μg/ml) whether untreated or preincubated with cycloheximide resulted in a significant increase in the number of cells with <2n DNA content, which is reversed by the simultaneous addition of PAF (10^{-7} M). PAF's effects were blocked by WEB 2170 (10^{-6} M). N=6 (★ $p < 0.01$ as compared to Control).*

Discussion

B lymphocyte differentiation from a non-committed precursor cell into an immunoglobulin secreting plasma cell is accompanied by the deletion of most precursor B lymphocytes via apoptosis (13). As a result, only B lymphocytes which are appropriately mature and are in the proper environment will become memory B cells or plasma cells. Immune selection of B lymphocytes also takes place following antigenic stimulation of immature B lymphocytes (101). This process occurs mainly in lymph nodes where B cells, following engagement of an antigen on their B cell receptor, enter the germinal center and divide rapidly, and subsequently produce cells with immunoglobulin receptors exhibiting differential affinities for the antigen (66, 102). Clones of B lymphocytes with high antigen affinity will be maintained while cells with low affinity will undergo programmed cell death.

We have demonstrated that PAF abrogated apoptosis in Ramos B lymphocytes following incubation with αIgM (37). In addition, PAF maintained the ability of the Ramos cells to secrete IgM which was diminished following αIgM incubation. Very little is known about the progression from αIgM crosslinking to PCD, and how phenotypic changes on the surface of B lymphocytes may reflect the progression of B cells through growth arrest to PCD. In the current studies, we have attempted to characterize changes in cellular integrity following αIgM incubation, and to ascertain if these effects were abrogated by PAF.

We assessed if sIgM crosslinking affected the immune competence of the remaining, non-apoptotic lymphocytes by measuring cell surface markers crucial for immune responses,

such as VLA-4 and BCR expression. Immature B lymphocytes cap their BCR rapidly following sIgM crosslinking and re-express it slowly, unlike more mature B cells (103). PAF not only protected B lymphocytes from undergoing apoptosis but allowed for sIgM expression to be equal to control and therefore retain their immune competence. In the cells that had not undergone apoptosis following sIgM crosslinking there was a significant decrease in the expression of sIgM at 24 hours; co-incubation with PAF led to a significant increase in BCR expression at 24 hours. Engagement of VLA-4 in germinal center B cells may contribute to B lymphocyte maturation or selection, and inhibits apoptotic signals (66, 87). There was a marked decrease in VLA-4 expression in the population of cells that had excluded PI and were Annexin V$^-$ following αIgM stimulation. PAF not only restored the total number of viable cells, but augmented VLA-4 to control levels, restoring the cells to a more functional state.

Apoptosis is often defined morphologically by the presence of nuclear destruction, and cleavage of DNA into 180-200 bp fragments (100). Nuclear changes begin 6 or more hours after the initial trigger for cell death and many changes can be detected prior to the appearance of DNA cleavage. The earliest change in PCD is loss of mitochondrial transmembrane potential ($\Delta\Psi m$), which appears within one hour of cell surface receptor cross linking, or other apoptotic stimuli. This is closely followed by the generation of superoxide (O_2^-), and 60-120 minutes later, there is turnover and exposure of PS from the inner cell membrane to the outer plasma membrane (104-107). Mitochondrial NADPH oxidase, which may be a source of O_2^- in B lymphocytes, is stimulated by changes in $\Delta\Psi m$. Superoxide is metabolised to H_2O_2 via superoxide dismutase,

and then H_2O_2 is cleaved to toxic $OH^{\cdot}$ as the cell undergoes further apoptotic changes. Alterations of $\Delta\Psi m$ precede the nuclear changes in BCR mediated apoptosis. If $\Delta\Psi m$ is stabilized within the first 1-2 hours following BCR stimulation, by cyclosporin for example (107), superoxide is not produced, PS turnover does not ensue, and further apoptotic changes are blocked. Once PS turnover has proceeded, cyclosporin can no longer inhibit apoptosis.

It is therefore of importance to understand the coexistent membrane changes that accompany the PS turnover. It is in fact not surprising that a surface molecule such as VLA 4 is diminished in the apoptotic process. In order for PS residues to be expressed, the disruption of the surface membrane is widespread and the membrane integrity is compromised. In this rearrangement of the bimolecular leaflet, normal surface expression is therefore probably impossible. However, the effect of PAF on VLA-4 expression is not simply an effect on membrane disruption. VLA-4 was diminished even in the population that had no PS surface expression implying that PAF has a direct action on the adhesion receptor expression in association with its effect on apoptosis.

In contrast, the ability of PAF to affect the expression of sIgM implies a different set of functions for PAF. Apoptosis in these studies is initiated by the cross linking of sIgM on Ramos cells, which results in the internalization of the BCR into the cell by a process known as capping (108). BCR re-expression is a balance between the capping and endocytosis of sIgM that have been crosslinked, a process that usually requires cytoskeleton activity, and new protein synthesis in order to generate a new BCR. This is an active operation, and the expression of the BCR is also therefore an active process. In order to differentiate these effects,

preincubation with cytochalasin B and cycloheximide was utilized. We found that downregulation of the BCR following sIgM crosslinking was not dependent on cytoskeletal activity as it was not inhibited by adequate doses of cytochalasin B (109). Cycloheximide at 7.5 μg/ml, has been previously shown to neither induce nor inhibit apoptosis yet has an effect on protein synthesis (109). In fact, this dose of cycloheximide blocked the expression of the BCR without affecting apoptosis. The increased sIgM expression induced by PAF would allow B lymphocytes to engage in further interactions with antigen, and drive the B cells to mature to antibody secreting cells. Since cytochalasin B preincubation did not affect sIgM downregulation, Ramos B cells may not require cytoskeleton activity to form caps but may internalize the sIgM complex by patches (98) and internalize the complex via membrane fluidity changes. More investigation into this phenomenon is currently in progress.

Our previous studies have shown that PAF and αIgM can act in parallel in B cell lines, suggesting that they neither interact with the same receptor, nor do they cause heterologous desensitization (18, 73). We have also demonstrated that PAF can act if added one hour following stimulation with αIgM; these data are not supportive of any steric interaction (37). Moreover, re-expression of the BCR is inhibited by treatment of the cells with the protein synthesis inhibitor cycloheximide, using doses that have been clearly demonstrated to block de novo protein synthesis but do not affect apoptosis (109). The same dose of cycloheximide that inhibits PAF's effect on the expression of the BCR has no effect on the rescue of Ramos cells from apoptosis. This adds strength to our previous findings, which suggested that PAF's

maximal effect on abrogation of apoptosis was 1-2 hours prior to or following BCR stimulation. This time course and lack of requirement for protein synthesis argues that the probable explanations for PAF's ability to inhibit apoptosis in Ramos cells includes a stabilising effect on mitochondria which blocks changes in $\Delta\Psi m$ and superoxide production (110). Although this could be through upregulation of bcl2, which localizes to mitochondria, other mechanisms are possible (14). PAF clearly is capable of inducing other profound changes in its action on Ramos cells, as is witnessed by a requirement for protein synthesis to permit expression of the BCR.

B lymphocyte differentiation from a non-committed precursor cell into an immunoglobulin secreting plasma cell is accompanied by the deletion of most precursor B lymphocytes via apoptosis. Immune selection of B lymphocytes also takes place following antigenic stimulation of immature B lymphocytes. Because PAF is secreted as part of the immune response for cell recruitment in infections (14, 27) as well in inflammatory states (84), B lymphocytes can be profoundly affected by this mediator under many conditions. We have demonstrated that PAF's ability to maintain immune competence in αIgM stimulated Ramos B cells is mediated via at least two pathways, a protein synthesis sensitive pathway, that results in preservation of sIgM expression and one that does not require protein synthesis, abrogation of αIgM mediated apoptosis of B cells.

CONCLUSION

B lymphocytes play a crucial role in the immune system, as the sole producers of antibodies, and as antigen presenting cells (111). B lymphocytes recognize and respond to antigens via surface receptors. These surface immunoglobulin receptors ideally recognize only foreign antigens, and produce high affinity antibodies (111). Errors or disruptions in this process can lead to the production of B cell clones that make nonsense immune globulin or autoantibodies. B cell development is therefore very tightly regulated (40, 111).

Once released from the bone marrow, circulating immature B lymphocytes are recruited to lymphoid tissues where they are exposed to antigen which initiates their differentiation (17). As they process antigen via the BCR, B cells can differentiate into antibody secreting cells where they multiply rapidly in contact with follicular dendritic cells and T cells to form germinal centers (112). Interaction of B cells with follicular dendritic cells and the antigens they present stimulates further maturation into either plasma cells or memory B cells (112).

Apoptosis is a process of non-necrotic, programmed cell death that is employed by organisms to remodel, or recycle cells that are defective or no longer useful. During maturation, stimulation of the BCR frequently leads to deletion of B cells by apoptosis, rather than causing proliferation and cell differentiation. Ligation of the sIgM receptor on immature B cells or on germinal centre B cells leads to growth arrest and death of the B cells by apoptosis (38, 113). Immortalised cell lines which undergo apoptosis following BCR ligation serve as important models of B cell

development; these include the immature murine cell line WEHI-231 (57), and the immature human B cell line Ramos (85). A key feature of the death of these cells by apoptosis is that it can be arrested if the cells are stimulated by the combination of antigen receptor ligation in addition to a second signal (57). This may be a protective mechanism, ensuring that a B lymphocyte is in the appropriate environment in order to respond to antigen. These second signals include adhesion molecules (66, 114, 115), cytokines (41) and T cell help via the CD40 ligand (39). After the cell has been appropriately stimulated by the two signals, cell growth, maturation, and antibody production occurs (57).

In our model of PCD, PAF fulfills the role of second signal. The lipid mediator is able to preserve the viability of αIgM stimulated Ramos cells, and abrogate the signals for apoptosis. These actions were specifically inhibited by the PAF receptor antagonist Web 2170. This is significant as many of the other signals that modulate B cell apoptosis are T cell dependant such as CD40 ligand and IL-4. Tonsils are rich in monocytes, follicular dendritic cells and vascular endothelium; all potential sites of PAF production (44). Therefore, a T cell independent signal may be the initial signal that leads to further interaction of B cells within the germinal centre and allows their maturation to plasma or memory B cells.

Apoptosis was associated with production of oxidants such as $OH^{\cdot}$ radicals. Intracellular antioxidants such as catalase are effective in scavenging H_2O_2 and inhibiting further toxic radical production. We have shown in the Ramos cell line that catalase effectively inhibits αIgM induced PCD. This not only suggests that production of reactive oxygen substances is important

in PCD associated with BCR ligation, but also implicates H_2O_2 as a key molecule in the initiation of apoptosis in B lymphocytes. PAF was also able to inhibit apoptosis induced by H_2O_2. In cells simultaneously incubated with PAF and either αIgM or H_2O_2, the rate of DCFH oxidation, and therefore the accumulation of $OH^{\cdot}$, was greatly diminished. Not unexpectedly, depletion of GSH is a characteristic of Ramos cells that undergo apoptosis. In further support of PAF's role in maintaining the balance of oxidant and reactive oxygen substances, cells treated with PAF do not show any depletion of GSH.

We assessed if sIgM crosslinking affected the immune competence of the remaining, non-apoptotic lymphocytes by measuring cell surface markers crucial for immune responses, such as VLA-4 and BCR expression. PAF not only protected B lymphocytes from undergoing apoptosis but allowed for sIgM expression to be equal to control and therefore retain their immune competence. In the cells that had not undergone apoptosis following sIgM crosslinking there was a significant decrease in the expression of sIgM at 24 hours; co-incubation with PAF led to a significant increase in BCR expression at 24 hours. There was a marked decrease in VLA-4 expression in the population of cells that had excluded PI and were Annexin V^- following αIgM stimulation. PAF not only restored the total number of viable cells, but augmented VLA-4 to control levels, restoring the cells to a more functional state.

Cycloheximide at 7.5 μg/ml, has been previously shown to neither induce nor inhibit apoptosis yet blocks protein synthesis (109). In fact, this dose of cycloheximide blocked the

re-expression of the BCR without affecting apoptosis. The effect of PAF on sIgM expression would allow B lymphocytes to engage in further interactions with antigen, and drive the B cells to mature to antibody secreting cells. The time course and lack of requirement for protein synthesis argues that the probable explanations for PAF's ability to inhibit apoptosis in Ramos cells include a stabilising effect on mitochondria which blocks changes in $\Delta\Psi m$ and superoxide production (110). PAF clearly is capable of inducing other profound changes in its action on Ramos cells, as is witnessed by a requirement for protein synthesis to permit expression of the BCR. Taken together, we have demonstrated that PAF's ability to maintain immune competence in αIgM stimulated Ramos B cells is mediated via at least two pathways, a protein synthesis sensitive pathway, that results in preservation of sIgM expression and one that does not require protein synthesis, abrogation of αIgM mediated apoptosis of B cells.

These data indicate that PAF potentially provides important signals to B cells, allowing them to progress to antibody secreting cells. In future studies, we must verify if these observations in immortalised cell lines are applicable to human peripheral B cells. We have determined that a B cell line that undergoes apoptosis following BCR ligation is rescued by addition of PAF. The next step is to determine if PAF will also be able to reverse the action of BCR stimulation and inhibit apoptosis in fresh B cells. We will attempt to identify how PAF rescues B lymphocytes from programmed cell death, and whether this is related to the inhibition of reactive oxygen substances produced following BCR stimulation. Both the time course and the proposed mechanism of action suggest that PAF alters events that are detectable in the first few hours of

apoptosis, prior to nuclear changes. These events include changes in mitochondrial transmembrane potential ($\Delta\Psi m$) (105) and caspases (116). Another group of studies will define the early apoptotic changes in αIgM stimulated Ramos cells, and indicate where PAF alters these destructive events. We will initially establish the link between mitochondrial permeability changes and reactive oxygen substance production by αIgM stimulated Ramos cells. We will assess whether PAF decreases superoxide by blocking changes in mitochondrial permeability, or by direct action within the superoxide pathway. With this, we will be able to determine the sequence of events following αIgM stimulation that explains the critical time (within 2 hours) that PAF can inhibit BCR induced apoptosis. We will also further explore how PAF alters $\Delta\Psi m$ and production of reactive oxygen substances.

We have determined an important T cell independent event in B lymphocyte maturation and antibody synthesis mediated by PAF. B cells have significant contact with monocytes, follicular dendritic cells and vascular endothelium in lymph nodes such as tonsils all of which are rich in PAF. Therefore, defining the role of PAF is crucial for understanding B cell growth and development. We have learned about the early events in B cell apoptosis, specifically the links between reactive oxygen substances and the induction of apoptosis. These regulatory events will contribute to the understanding of antibody regulation and dysregulation which is a cardinal feature of allergic and autoimmune disease. Because PAF can be modulated pharmacologically only careful dissection of the action of this mediator will allow for proper use of these potential therapeutic modalities and modulation of B cell responses to inflammation.

References

1. **Cohen, J. J.** 1994. Apoptosis: physiologic cell death. *Journal of Laboratory & Clinical Medicine 124:761.*

2. **Wyllie, A. H.** 1993. Apoptosis (the 1992 Frank Rose Memorial Lecture). *British Journal of Cancer 67:205.*

3. **Bar, R. P.** 1996. Apoptosis- The cells silent exit. *Life Sciences 59:369.*

4. **Cohen, J. J.** 1991. Programmed cell death in the immune system. [Review] [159 refs]. *Advances in Immunology 50:55.*

5. **Cohen, J. J.** 1993. Apoptosis. [Review] [54 refs]. *Immunology Today 14:126.*

6. **Osmond, D. G., S. Rico-Vargas, H. Valenzona, L. Fauteux, L. Liu, R. Janani, Lu, L, and K. Jacobsen.** 1994. Apoptosis and macrophage-mediated cell deletion in the regulation of B lymphopoiesis in mouse bone marrow. [Review] [72 refs]. *Immunological Reviews 142:209.*

7. **Cummings, M. C., C. M. Winterford, and N. I. Walker.** 1997. Apoptosis. *American Journal of Surgical Pathology 21:88.*

8. **Abastado, J. P.** 1996. Apoptosis: function and regulation of cell death. *Research in Immunology 147:443.*

9. **Williams, G. T.** 1994. Apoptosis in the immune system. [Review] [29 refs]. *Journal of Pathology 173:1.*

10. **Solary, E., L. Dubrez, and B. Eymin.** 1996. The role of apoptosis in the pathogenesis and treatment of diseases. *European Respiratory Journal 9:1293.*

11. **Griffiths, G.** 1995. The cell biology of CTL killing. *Current Opinion in Immunology 7:343.*

12. **Gustavo, M., W. C. Liles, F. Radella, K. P. Steinberg, J. T. Ruzinski, M. Jonas, E. Y. Chi, L. D. Hudson, and T. R. Martin.** 1997. Neutrophil apoptosis in the acute respiratory distress syndrome. *American Journal of Respiratory and Critical Care Medicine 156:1969.*

13. **Cohen, J. J., R. C. Duke, V. A. Fadok, and K. S. Sellins.** 1992. Apoptosis and programmed cell death in immunity. [Review] [174 refs]. *Annual Review of Immunology 10:267.*

14. **Izumi, T., and T. Shimizu.** 1995. Platelet-activating factor receptor: gene expression and signal transduction. [Review] [231 refs]. *Biochimica et Biophysica Acta 1259:317.*

15. **McManus, L. M., D. S. Woodard, S. I. Deavers, and R. N. Pinckard.** 1993. PAF molecular heterogeneity: pathobiological implications. [Review] [88 refs]. *Laboratory Investigation 69:639.*

16. **Carlson, S. A., T. K. Chatterjee, and R. A. Fisher.** 1996. The third intracellular domain of the platelet-activating factor receptor is a critical determinant in receptor coupling to phosphoinositide phospholipase C-activating G proteins. Studies using intracellular domain minigenes and receptor chimeras. *Journal of Biological Chemistry 271:23146.*

17. **Lederman, S., M. J. Yellin, G. Inghirami, J. J. Lee, D. M. Knowles, and L. Chess.** 1992. Molecular interactions mediating T-B lymphocyte collaboration in

human lymphoid follicles. Roles of T cell-B-cell-activating molecule (5c8 antigen) and CD40 in contact-dependent help. *Journal of Immunology 149:3817.*

18. **Mazer, B., J. Domenico, H. Sawami, and E. W. Gelfand.** 1991. Platelet-activating factor induces an increase in intracellular calcium and expression of regulatory genes in human B lymphoblastoid cells. *Journal of Immunology 146:1914.*

19. **Franklin, R. A., B. Mazer, H. Sawami, G. B. Mills, N. Terada, J. J. Lucas, and E. W. Gelfand.** 1993. Platelet-activating factor triggers the phosphorylation and activation of MAP-2 kinase and S6 peptide kinase activity in human B cell lines. *Journal of Immunology 151:1802.*

20. **Mazer, B. D., H. Sawami, A. Tordai, and E. W. Gelfand.** 1992. Platelet-activating factor-mediated transmembrane signaling in human B lymphocytes is regulated through a pertussis- and cholera toxin-sensitive pathway. *Journal of Clinical Investigation 90:759.*

21. **Schulam, P. G., A. Kuruvilla, G. Putcha, L. Mangus, J. Franklin-Johnson, and W. T. Shearer.** 1991. Platelet-activating factor induces phospholipid turnover, calcium flux, arachidonic acid liberation, eicosanoid generation, and oncogene expression in a human B cell line. *Journal of Immunology 146:1642.*

22. **Kuruvilla, A., C. Pielop, and W. T. Shearer.** 1994. Platelet-activating factor induces the tyrosine phosphorylation and activation of phospholipase C-gamma 1, Fyn and Lyn kinases, and phosphatidylinositol 3-kinase in a human B cell line. *Journal of Immunology 153:5433.*

23. **Smith, C. S., L. Parker, and W. T. Shearer.** 1994. Cytokine regulation by platelet-activating factor in a human B cell line. *Journal of Immunology 153:3997.*

24. **Kishimoto, S., W. Shimadzu, T. Izumi, T. Shimizu, T. Fukuda, S. Makino, T. Sugiura, and K. Waku.** 1996. Regulation by IL-5 of expression of functional platelet- activating factor receptors on human eosinophils. *Journal of Immunology 157:4126.*

25. **Parent, J. L., and J. Stankova.** 1993. Transforming growth factor-beta up-regulates the gene expression of the human platelet-activating factor receptor in monocytic and B cell lines. *Biochemical & Biophysical Research Communications 197:1443.*

26. **Parent, J. L., C. L. Gouill, E. Escher, M. Rola-Pleszczynski, and J. Stakova.** 1996. Identification of transmembrane domain residues determinant in the structure-function relationship of the human platelet- activating factor receptor by site-directed mutagenesis. *Journal of Biological Chemistry 271:23298.*

27. **Barnes, P. J.** 1991. *Research and Clinical Forums 13:61.*

28. **Koltai, M., D. Hosford, and P. Braquet.** 1993. *Journal of Lipid Mediators 6:183.*

29. **Cundell, D. R., N. P. Gerard, C. Gerard, I. Idanpaan-Heikkila, and E. I. Tuomanen.** 1995. Streptococcus pneumoniae anchor to activated human cells by the receptor for platelet-activating factor. *Nature 377:435.*

30. **Fairbairn, S. M., K. A. Marr, P. Lees, F. M. Cunningham, and C. P. Page.** 1996. Effects of platelet activating factor on the distribution of radiolabelled

leucocytes and platelets in normal horses and asymptomatic horses with chronic obstructive pulmonary disease. *Research in Veterinary Science 61:107.*

31. **Condliffe, A. M., E. R. Chilvers, C. Haslett, and I. Dransfield.** 1996. Priming differentially regulates neutrophil adhesion molecule expression/function. *Immunology 89:105.*

32. **Packham, M. A., D. H. Ruben, and M. L. Rand.** 1996. Exogenous diacylglycerols synergize with PAF with human platelets, but inhibit PAF-induced responses of rabbit platelets. *Journal of Lipid Mediators & Cell Signalling 15:69.*

33. **Lacasse, C., S. Turcotte, D. Gingras, J. Stankova, and M. Rola-Pleszczynski.** 1997. Platelet-activating factor stimulates interleukin-6 production by human endothelial cells and synergizes with tumor necrosis factor for enhanced production of granulocyte-macrophage colony stimulating factor. *Inflammation 21:145.*

34. **Weyrich, A. S., T. M. McIntyre, R. P. McEver, S. M. Prescott, and G. A. Zimmerman.** 1995. Monocyte tethering by P-selectin regulates monocyte chemotactic protein-1 and tumor necrosis factor-alpha secretion. Signal integration and NF-kappa B translocation [see comments]. *Journal of Clinical Investigation 95:2297.*

35. **Izumi, T., Z. Honda, H. Mutoh, K. Kume, and T. Shimizu.** 1995. Regulation and signal transduction of PAF receptor. *Advances in Prostaglandin, Thromboxane, & Leukotriene Research 23:461.*

36. **Elstad, M. R., C. J. Parker, F. S. Cowley, L. A. Wilcox, T. M. McIntyre, S. M. Prescott, and G. A. Zimmerman.** 1994. CD11b/CD18 integrin and a beta-glucan

receptor act in concert to induce the synthesis of platelet-activating factor by monocytes. *Journal of Immunology 152:220.*

37. **Toledano, B. J., Y. Bastien, F. Noya, S. Baruchel, and B. Mazer.** 1997. Platelet-activating factor abrogates apoptosis induced by cross- linking of the surface IgM receptor in a human B lymphoblastoid cell line. *Journal of Immunology 158:3705.*

38. **Norvell, A., L. Mandik, and J. G. Monroe.** 1995. Engagement of the antigen-receptor on immature murine B lymphocytes results in death by apoptosis. *Journal of Immunology 154:4404.*

39. **Hasbold, J., and G. G. Klaus.** 1990. Anti-immunoglobulin antibodies induce apoptosis in immature B cell lymphomas. *European Journal of Immunology 20:1685.*

40. **Baixeras, E., L. Bosca, C. Stauber, A. Gonzalez, A. C. Carrera, J. A. Gonzalo, and C. Martinez.** 1994. From apoptosis to autoimmunity: insights from the signaling pathways leading to proliferation or to programmed cell death. [Review] [147 refs]. *Immunological Reviews 142:53.*

41. **Parry, S. L., J. Hasbold, M. Holman, and G. G. Klaus.** 1994. Hypercross-linking surface IgM or IgD receptors on mature B cells induces apoptosis that is reversed by costimulation with IL- 4 and anti-CD40. *Journal of Immunology 152:2821.*

42. **Hockenbery, D. M., Z. N. Oltvai, X. M. Yin, C. L. Milliman, Korsmeyer, and SJ.** 1993. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell 75:241.*

43. **Denizot, Y., F. Rougier, F. Dupuis, C. Verger, C. Dulery, M. Laskar, J. C. Aldigier, and V. Praloran.** 1996. PAF and hematopoiesis. VIII. Biosynthesis and metabolism of PAF by human bone marrow stromal cells. *Experimental Hematology 24:1327.*

44. **Blank, M. L., Z. L. Smith, V. Fitzgerald, and F. Snyder.** 1995. The CoA-independent transacylase in PAF biosynthesis: tissue distribution and molecular species selectivity. *Biochimica et Biophysica Acta 1254:295.*

45. **Mazer, B., K. L. Clay, H. Renz, and E. W. Gelfand.** 1990. Platelet-activating factor enhances Ig production in B lymphoblastoid cell lines. *Journal of Immunology 145:2602.*

46. **Kovar, J., and F. Franek.** 1986. Serum-free medium for hybridoma and parental myeloma cell cultivation. *Methods in Enzymology 121:277.*

47. **Perandones, C. E., V. A. Illera, D. Peckham, L. L. Stunz, Ashman, and RF.** 1993. Regulation of apoptosis in vitro in mature murine spleen T cells. *Journal of Immunology 151:3521.*

48. **Bass, D. A., J. W. Parce, L. R. Dechatelet, P. Szejda, M. C. Seeds, and M. Thomas.** 1983. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. *Journal of Immunology 130:1910.*

49. **Mazer, B. D., H. Renz, and E. W. Gelfand.** 1991. An ELISA spot assay for quantitation of human immunoglobulin-secreting cells. *Journal of Allergy & Clinical Immunology 88:235.*

50. **Tietze, F.** 1969. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Analytical Biochemistry 27:502.*

51. **Valentine, M. A., S. L. Bursten, W. E. Harris, K. E. Draves, Pollok, BA, J. Ostrowski, K. Bomsztyk, and E. A. Clark.** 1992. Generation of phosphatidic acid and diacylglycerols following ligation of surface immunoglobulin in human B lymphocytes: potential role in PKC activation. *Cellular Immunology 141:373.*

52. **Bastien, Y., and B. D. Mazer.** 1994. Detection of the human platelet-activating factor receptor mRNA in human B lymphocytes by polymerase chain reaction on reverse transcripts (RT-PCR) [published erratum appears in Biochem Biophys Res Commun 1994 Oct 14;204(1):436]. *Biochemical & Biophysical Research Communications 202:1373.*

53. **Darzynkiewicz, Z., S. Bruno, G. Del Bino, W. Gorczyca, Hotz, MA, P. Lassota, and F. Traganos.** 1992. Features of apoptotic cells measured by flow cytometry. [Review] [63 refs]. *Cytometry 13:795.*

54. **Gold, R., M. Schmied, G. Rothe, H. Zischler, H. Breitschopf, H. Wekerle, and H. Lassmann.** 1993. Detection of DNA fragmentation in apoptosis: application of in situ nick translation to cell culture systems and tissue sections. *Journal of Histochemistry & Cytochemistry 41:1023.*

55. **Nicoletti, I., G. Migliorati, M. C. Pagliacci, F. Grignani, and C. Riccardi.** 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *Journal of Immunological Methods 139:271.*

56. **Darzynkiewicz, Z., X. Li, and J. Gong.** 1994. Assays of cell viability: discrimination of cells dying by apoptosis. [Review] [42 refs]. *Methods in Cell Biology 41:15.*

57. **Fang, W., J. J. Rivard, J. A. Ganser, T. W. LeBien, K. A. Nath, D. L. Mueller, and T. W. Behrens.** 1995. Bcl-xL rescues WEHI 231 B lymphocytes from oxidant-mediated death following diverse apoptotic stimuli. *Journal of Immunology 155:66.*

58. **Buttke, T. M., and P. A. Sandstrom.** 1994. Oxidative stress as a mediator of apoptosis [see comments]. [Review] [59 refs]. *Immunology Today 15:7.*

59. **Slater, A. F., C. S. Nobel, and S. Orrenius.** 1995. The role of intracellular oxidants in apoptosis. [Review] [52 refs]. *Biochimica et Biophysica Acta 1271:59.*

60. **Korsmeyer, S. J., X. M. Yin, Z. N. Oltvai, D. J. Veis-Novack, Linette, and GP.** 1995. Reactive oxygen species and the regulation of cell death by the Bcl-2 gene family. [Review] [32 refs]. *Biochimica et Biophysica Acta 1271:63.*

61. **Bensasson, R. V., E. J. Land, and T. G. Truscott.** 1993. Activated forms of oxygen. In *Excited states and free radicals in biology and medicine.* Oxford Science Publication, New York, p. 102.

62. **Zhu, H., G. L. Bannenberg, P. Moldeus, and H. G. Shertzer.** 1994. Oxidation pathways for the intracellular probe 2',7'- dichlorofluorescein. *Archives of Toxicology 68:582.*

63. **Aukrust, P., A. M. Svardal, F. Muller, B. Lunden, R. K. Berge, and S. S. Froland.** 1995. Decreased levels of total and reduced glutathione in CD4+ lymphocytes in common variable immunodeficiency are associated with activation of

the tumor necrosis factor system: possible immunopathogenic role of oxidative stress. *Blood 86:1383.*

64. **Franklin, R. A., A. Tordai, B. Mazer, N. Terada, J. Lucas, and E. W. Gelfand.** 1995. Platelet activating factor activates MAPK and increases in intracellular calcium via independent pathways in B lymphocytes. *Biochemical & Biophysical Research Communications 209:1111.*

65. **Ishida, T., N. Kobayashi, T. Tojo, S. Ishida, T. Yamamoto, and J. Inoue.** 1995. CD40 signaling-mediated induction of Bcl-XL, Cdk4, and Cdk6. Implication of their cooperation in selective B cell growth. *Journal of Immunology 155:5527.*

66. **Koopman, G., R. M. Keehnen, E. Lindhout, W. Newman, Y. Shimizu, G. A. van Seventer, C. de Groot, and S. T. Pals.** 1994. Adhesion through the LFA-1 (CD11a/CD18)-ICAM-1 (CD54) and the VLA-4 (CD49d)-VCAM-1 (CD106) pathways prevents apoptosis of germinal center B cells. *Journal of Immunology 152:3760.*

67. **Cohen, G.** 1994. Enzymatic/nonenzymatic sources of oxyradicals and regulation of antioxidant defenses. [Review] [16 refs]. *Annals of the New York Academy of Sciences 738:8.*

68. **Nunez, G., R. Merino, D. Grillot, and M. Gonzalez-Garcia.** 1994. Bcl-2 and Bcl-x: regulatory switches for lymphoid death and survival. [Review] [76 refs]. *Immunology Today 15:582.*

69. **Sundaresan, M., Z. X. Yu, V. J. Ferrans, K. Irani, and T. Finkel.** 1995. Requirement for generation of H2O2 for platelet-derived growth factor signal transduction. *Science 270:296.*

70. **Heffetz, D., W. J. Rutter, and Y. Zick.** 1992. The insulinomimetic agents H2O2 and vanadate stimulate tyrosine phosphorylation of potential target proteins for the insulin receptor kinase in intact cells. *Biochemical Journal 288:631.*

71. **Schieven, G. L., J. M. Kirihara, D. L. Burg, R. L. Geahlen, Ledbetter, and JA.** 1993. p72syk tyrosine kinase is activated by oxidizing conditions that induce lymphocyte tyrosine phosphorylation and Ca2+ signals. *Journal of Biological Chemistry 268:16688.*

72. **Qin, S., T. Inazu, M. Takata, T. Kurosaki, Y. Homma, Yamamura, and H.** 1996. Cooperation of tyrosine kinases p72syk and p53/56lyn regulates calcium mobilization in chicken B cell oxidant stress signaling. *European Journal of Biochemistry 236:443.*

73. **Rozsnyay, Z., G. Sarmay, and J. Gergely.** 1995. Rapid desensitization of B-cell receptor by a dithiol-reactive protein tyrosine phosphatase inhibitor: uncoupling of membrane IgM from syk inhibits signals leading to Ca2+ mobilization. *Immunology Letters 44:149.*

74. **Powell, D. W.** 1992. Epithelial secretory responses to inflammation. Platelet activating factor and reactive oxygen metabolites. [Review] [30 refs]. *Annals of the New York Academy of Sciences 664:232.*

75. **Fedyk, E. R., and R. P. Phipps.** 1994. Reactive oxygen species and not lipoxygenase products are required for mouse B-lymphocyte activation and differentiation. *International Journal of Immunopharmacology 16:533.*

76. **Yao, X. R., H. Flaswinkel, M. Reth, and D. W. Scott.** 1995. Immunoreceptor tyrosine-based activation motif is required to signal pathways of receptor-mediated growth arrest and apoptosis in murine B lymphoma cells. *Journal of Immunology 155:652.*

77. **Minshall, C., S. Arkins, G. G. Freund, and K. W. Kelley.** 1996. Requirement for phosphatidylinositol 3'-kinase to protect hemopoietic progenitors against apoptosis depends upon the extracellular survival factor. *Journal of Immunology 156:939.*

78. **Beckwith, M., R. G. Fenton, I. M. Katona, and D. L. Longo.** 1996. Phosphatidylinositol-3-kinase activity is required for the anti- ig-mediated growth inhibition of a human B-lymphoma cell line. *Blood 87:202.*

79. **Gjertsen, B. T., and S. O. Doskeland.** 1995. Protein phosphorylation in apoptosis. [Review] [309 refs]. *Biochimica et Biophysica Acta 1269:187.*

80. **Sakata, N., H. R. Patel, N. Terada, A. Aruffo, G. L. Johnson, and E. W. Gelfand.** 1995. Selective activation of c-Jun kinase mitogen-activated protein kinase by CD40 on human B cells. *Journal of Biological Chemistry 270:30823.*

81. **Thivierge, M., J. L. Parent, J. Stankova, and M. Rola-Pleszczynski.** 1996. Modulation of human platelet-activating factor receptor gene expression by protein kinase C activation. *Journal of Immunology 157:4681.*

82. **Giembycz, M. A., C. Kroegel, and P. J. Barnes.** 1990. Platelet activating factor stimulates cyclo-oxygenase activity in guinea pig eosinophils. Concerted biosynthesis of thromboxane A2 and E-series prostaglandins. *Journal of Immunology 144:3489.*

83. **Cleary, A. M., S. M. Fortune, M. J. Yellin, L. Chess, and S. Lederman.** 1995. Opposing roles of CD95 (Fas/APO-1) and CD40 in the death and rescue of human low density tonsillar B cells. *Journal of Immunology 155:3329.*

84. **Koltai, M., D. Hosford, and P. Braquet.** 1993. PAF-induced amplification of mediator release in septic shock: prevention or downregulation by PAF antagonists. [Review] [64 refs]. *Journal of Lipid Mediators 6:183.*

85. **Valentine, M. A., and K. A. Licciardi.** 1992. Rescue from anti-IgM-induced programmed cell death by the B cell surface proteins CD20 and CD40. *European Journal of Immunology 22:3141.*

86. **Norvell, A., M. L. Birkeland, J. Carman, A. L. Sillman, R. Wechsler-Reva, and J. G. Monroe.** 1996. Use of isolated immature-stage B cells to understand negative selection and tolerance induction at the molecular level. [Review] [71 refs]. *Immunologic Research 15:191.*

87. **Roldan, E., A. Garcia-Pardo, and J. A. Brieva.** 1992. VLA-4-fibronectin interaction is required for the terminal differentiation of human bone marrow cells capable of spontaneous and high rate immunoglobulin secretion. *Journal of Experimental Medicine 175:1739.*

88. **Warner, G. L., and D. W. Scott.** 1991. A polyclonal model for B cell tolerance. I. Fc-dependent and Fc- independent induction of nonresponsiveness by pretreatment of normal splenic B cells with anti-Ig. *Journal of Immunology 146:2185.*

89. **Fadok, V. A., D. R. Voelker, P. A. Campbell, J. J. Cohen, D. L. Bratton, and P. M. Henson.** 1992. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *Journal of Immunology 148:2207.*

90. **Vermes, I., C. Haanen, H. Steffens-Nakken, Reutelingsperger, and C.** 1995. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *Journal of Immunological Methods 184:39.*

91. **Koopman, G., C. P. Reutelingsperger, G. A. Kuijten, R. M. Keehnen, S. T. Pals, and M. H. van Oers.** 1994. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood 84:1415.*

92. **Manabe, A., K. G. Murti, E. Coustan-Smith, M. Kumagai, F. G. Behm, S. C. Raimondi, and D. Campana.** 1994. Adhesion-dependent survival of normal and leukemic human B lymphoblasts on bone marrow stromal cells. *Blood 83:758.*

93. **Ryan, D. H.** 1993. Adherence of normal and neoplastic human B cell precursors to the bone marrow microenvironment. *Blood Cells 19:225.*

94. **Clark, E. A., K. H. Grabstein, and G. L. Shu.** 1992. Cultured human follicular dendritic cells: Growyh characteristics and interactions with B lymphocytes. *Journal of Immunology 148:3327.*

95. **Issekutz, T. B.** 1990. Effects of six different cytokines on lymphocyte adherence to microvascular endothelium and in vivo lymphocyte migration in the rat. *Journal of Immunology 144:2140.*

96. **Taylor, R. B., W. Dufus, M. C. Raff, and D. P. S.** 1971. Redistribution and pinocytosis of lymphocyte surface immunoglobulin molecules induced by anti-immunoglobulin antibody. *Nature 233:225.*

97. **Loor, F., L. Forni, and B. Pernis.** 1972. The dynamic state of the lymphocyte membrane. Factors affecting the distribution and turnover of surface immunoglobulins. *European Journal of Immunology 2:203.*

98. **de Groot, C., J. Wormmeester, and C. Mangnus-Smet.** 1981. Capping of surface immunoglobulin on rabbit and mouse lymphocytes. II. Cytoskeletal involvement in different subpopulations. *European Journal of Cell Biology 25:202.*

99. **de Groot, C., and J. Wormmeester.** 1981. Capping of surface immunoglobulin on rabbit and mouse lymphocytes. I. Kinetics. *European Journal of Cell Biology 24:9.*

100. **Darzynkiewicz, Z., G. Juan, X. Li, W. Gorczyca, T. Murakami, and F. Traganos.** 1997. Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). [Review] [108 refs]. *Cytometry 27:1.*

101. **Norvell, A., and J. G. Monroe.** 1996. Acquisition of surface IgD fails to protect from tolerance- induction. Both surface IgM- and surface IgD-mediated signals induce apoptosis of immature murine B lymphocytes. *Journal of Immunology 156:1328.*

102. **Bonnefoy, J. Y., S. Henchoz, D. Hardie, M. J. Holder, and J. Gordon.** 1993. A subset of anti-CD21 antibodies promote the rescue of germinal center B cells from apoptosis. *European Journal of Immunology 23:969.*

103. **Pernis, B., and P. Roth.** 1982. Expression and dynamics of membrane immunoglobulins. [Review] [162 refs]. *Pharmacological Reviews 34:65.*

104. **Castedo, M., A. Macho, N. Zamzami, T. Hirsch, P. Marchetti, J. Uriel, and G. Kroemer.** 1995. Mitochondrial perturbations define lymphocytes undergoing apoptotic depletion in vivo. *European Journal of Immunology 25:3277.*

105. **Castedo, M., T. Hirsch, S. A. Susin, N. Zamzami, P. Marchetti, A. Macho, and G. Kroemer.** 1996. Sequential acquisition of mitochondrial and plasma membrane alterations during early lymphocyte apoptosis. *Journal of Immunology 157:512.*

106. **Zamzami, N., P. Marchetti, M. Castedo, C. Zanin, Vayssiere, JL, P. X. Petit, and G. Kroemer.** 1995. Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. *Journal of Experimental Medicine 181:1661.*

107. **Kroemer, G., N. Zamzami, and S. A. Susin.** 1997. Mitochondrial control of apoptosis. [Review] [71 refs]. *Immunology Today 18:44.*

108. **Klingenstein, R. J., and H. B. Dickler.** 1979. The effects of pharmacologic agents on immune-complex-induced redistribution of B-lymphocyte Fc receptors. *Scandinavian Journal of Immunology 10:145.*

109. **Ashman, R. F., D. Peckham, and L. L. Stunz.** 1996. Fc receptor off-signal in the B cell involves apoptosis. *Journal of Immunology 157:5.*

110. **Wang, X., and G. P. Studzinski.** 1997. Antiapoptotic action of 1,25-dihydroxyvitamin D3 is associated with increased mitochondrial MCL-1 and RAF-1 proteins and reduced release of cytochrome c. *Experimental Cell Research 235:210.*

111. **Janeway, C. A., and R. Travers.** 1996. *Immunobiology*. Garland Publishing.

112. **Pulendran, B., R. van Driel, and G. J. Nossal.** 1997. Immunological tolerance in germinal centres. [Review] [65 refs]. *Immunology Today 18:27.*

113. **Galibert, L., N. Burdin, C. Barthelemy, G. Meffre, I. Durand, E. Garcia, P. Garrone, F. Rousset, J. Banchereau, and Y. J. Liu.** 1996. Negative selection of human germinal center B cells by prolonged BCR cross-linking. *Journal of Experimental Medicine 183:2075.*

114. **Koopman, G., R. M. Keehnen, and S. T. Pals.** 1993. Interaction through the LFA-1/ICAM-1 pathway prevents programmed cell death of germinal center B cells. *Advances in Experimental Medicine & Biology 329:387.*

115. **Koopman, G., R. M. Keehnen, E. Lindhout, D. F. Zhou, C. de Groot, and S. T. Pals.** 1997. Germinal center B cells rescued from apoptosis by CD40 ligation or attachment to follicular dendritic cells, but not by engagement of surface immunoglobulin or adhesion receptors, become resistant to CD95-induced apoptosis. *European Journal of Immunology 27:1.*

116. **Susin, S. A., N. Zamzami, M. Castedo, E. Daugas, H. G. Wang, S. Geley, F. Fassy, J. C. Reed, and G. Kroemer.** 1997. The central executioner of apoptosis: multiple connections between protease activation and mitochondria in Fas/APO-

1/CD95- and ceramide-induced apoptosis. *Journal of Experimental Medicine 186:25.*