

## Report

# Phosphorylation of Histone H2AX on Ser 139 and Activation of ATM During Oxidative Burst in Phorbol Ester-Treated Human Leukocytes

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## KEY WORDS

DNA repair, DNA damage, DNA double-strand breaks, free radicals, ROIs, anti-oxidants, oxidative stress, granulocytes, monocytes

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## ABSTRACT

Oxidative burst is a defense mechanism used by specialized phagocytes such as granulocytes or monocytes to kill the invading microorganisms through generation of superoxide anions. Oxidative burst also results in DNA damage of the phagocytes. Phagocytes are terminally differentiated cells, some of very short life-span cells. We could find no reports whether oxidative burst-mediated DNA damage triggers in such cells histone H2AX-Ser139 phosphorylation and activation of Ataxia Telangiectasia Mutated (ATM), the signals otherwise used to activate DNA repair and checkpoint pathways in proliferating cells. We now present the evidence that induction of oxidative stress in human peripheral blood leukocytes by phorbol myristate acetate (PMA) was associated with intense phosphorylation of histone H2AX and with ATM activation, seen already 60 min after exposure to PMA. The modifications of H2AX and ATM in individual granulocytes, monocytes and lymphocytes were detected prior to caspases activation and thus were unrelated to induction of apoptosis. A large intercellular variation in response was observed, and only a fraction of cells in these subpopulations showed H2AX and ATM modifications. The data are compatible with the earlier observations of DNA damage during oxidative burst and suggest that even in terminally differentiated cells that have a short life-span, DNA damage triggers recruitment of the DNA repair machinery. The observed H2AX phosphorylation in lymphocytes may reflect their DNA damage by the superoxide ions propagating from the neighboring granulocytes and/or monocytes.

## INTRODUCTION

The innate immune system utilizes professional phagocytes (neutrophils, monocytes, macrophages and eosinophils) to rapidly neutralize invading pathogens. Activation of these cells during an inflammatory response prevents infections from spreading. One of the mechanisms used by phagocytes in combating pathogens involves rapid generation of microbicidal oxidants through a process often referred to as oxidative burst.<sup>1</sup> During this process the membrane-bound multiprotein enzyme complex defined as the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, by transferring electrons from NAHPH to O<sub>2</sub>, generates a superoxide anion (O<sub>2</sub><sup>-</sup>) that is capable of killing microorganisms.<sup>1-6</sup> In addition to functioning in immune defense, the NADPH oxidase system has been found in a variety of cell types other than phagocytes where it appears to play a role in inter- and intra-cellular signaling.<sup>1,7-9</sup>

Oxidative burst is often experimentally induced by exposure of cells to the tumor promoter phorbol myristate acetate (PMA). The level of oxidants generated during the burst can be conveniently estimated in individual cells by flow cytometry; when a cell oxidizes a probe such as dichlorofluorescein (DCFH) or one of its analogues, it becomes fluorescent.<sup>10-12</sup> Stimulation of phagocytes with PMA was shown to induce DNA damage including formation of DNA strand breaks, that can be detected not only in phagocytes themselves but also in the neighboring cells.<sup>13-16</sup> Apparently, the reactive oxygen species (ROS) generated by the phagocytes have the capability to induce DNA damage in the cells that are in their proximity.

Activation of Ataxia Telangiectasia Mutated (ATM) through its phosphorylation on Ser1981 (ATM-S1981<sup>P</sup>), and phosphorylation of one of the variants of histone H2A, histone H2AX on Ser139 ( $\gamma$ H2AX), are early markers of a cell's response to DNA damage, particularly if the damage involves formation of DNA double-strand breaks (DSBs).<sup>17-19</sup> These modifications of ATM and H2AX trigger pathways that are involved in DNA repair and in activating checkpoints that halt progression through the cell cycle.<sup>20-25</sup> The pause

in cell cycle progression is needed to allow for DNA repair to succeed prior to resumption of DNA replication or cell division.

Granulocytes and monocytes are non-replicating, terminally differentiated cells. The lifespan of peripheral blood granulocytes is short (hours to a few days) and they die by apoptosis.<sup>26</sup> One may expect, therefore, that damage to DNA in these cells may not necessarily trigger DNA repair because the cells do not proliferate and are programmed to die in a short time anyway. Likewise, since the cells do not progress through the cycle, activation of ATM or H2AX phosphorylation is unnecessary as a signal to activate the cell cycle checkpoints designed to halt cell progression through the cycle. It was interesting, therefore, to explore whether DNA damage induced in these cells by oxidative burst triggers ATM activation and H2AX phosphorylation, which as mentioned, activate pathways of DNA repair and cell cycle checkpoints. In the present study, oxidative burst in peripheral blood leukocytes was induced by PMA and activated ATM and  $\gamma$ H2AX in these cells were detected by microscopy, as the presence of characteristic immunofluorescent (IF) foci,<sup>17,18,27</sup> while the intensity of ATM-S1981<sup>P</sup> and H2AX IF was measured by flow- and laser-scanning cytometry, as described previously.<sup>28-32</sup>

## MATERIALS AND METHODS

**Cells isolation and treatment.** Human peripheral blood was collected from healthy volunteers by venipuncture into heparinized syringes which were then maintained upright for 1 hour at room temperature to allow erythrocytes to sediment at 1xg. The plasma containing white blood cells (WBCs) was collected and the cells were rinsed with RPMI-1640 medium (GIBCO/BRL Life Technologies, Inc., Grand Island, NY). Cells were suspended in RPMI-1640 supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin and 2 mM L-glutamine (all from GIBCO/BRL) in 25 ml FALCON flasks (Becton Dickinson Co., Franklin Lakes, NJ). In some experiments mononuclear cells were isolated by gradient centrifugation.<sup>32</sup> Phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St Louis, MO) was added at a concentration of 1  $\mu$ M to the suspension and cells were incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> in air. In some experiments cultures of WBCs were treated with 1  $\mu$ M of flavopiridol (obtained from Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD) for up to 24 h. See Figure legends for other details.

**Immunocytochemical detection of  $\gamma$ H2AX, activated ATM and caspase activation.** After 1 h of PMA stimulation, the cells were washed in phosphate-buffered saline (PBS) and cytocentrifuged onto microscope slides. Then, slides were submerged into 1% ice-cold methanol-free formaldehyde (Polysciences, Inc., Warrington, PA) dissolved in PBS for 15 min, followed by suspension in 80% ice-cold ethanol in which they were stored overnight at -20°C. Cells were washed twice in PBS and incubated first in 0.1% Triton X-100 (Sigma-Aldrich) for 15 min at room temperature, then in a 1% solution of bovine serum albumin (BSA) for 30 min at room temperature to suppress non-specific antibody (Ab) binding. Cells were then incubated with 50  $\mu$ l of 1% BSA in PBS

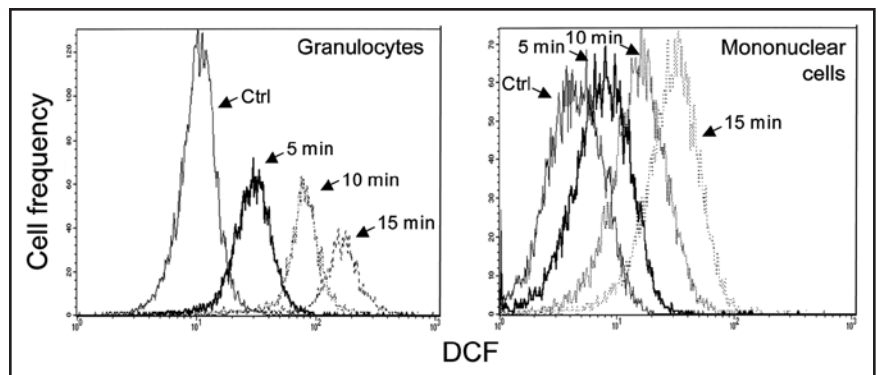


Figure 1. Effect of treatment of granulocytes and mononuclear cells with PMA on their ability to oxidize dichlorofluorescein diacetate (DCFH-DA). The WBCs isolated from buffy coat were suspended in culture medium, incubated with 10  $\mu$ M DCFH-DA for 30 min and their green fluorescence was then measured (Ctrl) by flow cytometry. The cell suspension was subsequently treated with 1  $\mu$ M PMA, the cell aliquots were sampled and cellular light scatter properties and fluorescence was measured 5, 10 and 15 min after addition of PMA. Based on differences in intensity of side- vs. forward- scatter of the laser beam light (SSC vs. FSC) granulocytes were distinguished from mononuclear cells<sup>35</sup> and intensity of fluorescence of cells in these subpopulations were plotted, as shown.

containing 1:100 diluted anti-phospho-histone H2A.X (*Ser-139*) anti-mouse Ab (Upstate Biotechnologies, Lake Placid, NY) or 1:100 diluted anti-phospho-ATM (*Ser-1981*) anti-mouse Ab (Upstate) or 1:100 cleaved caspase-3 (*Asp-175*) anti-rabbit Ab (Cell Signaling Technology, Inc., Danvers, MA), and incubated for 2 h at room temperature. Cells were rinsed with PBS and incubated with 50  $\mu$ l of 1% BSA in PBS containing 1:100 Alexa Fluor 488 F(ab')<sub>2</sub> fragment of goat anti-mouse IgG (H+L) (Invitrogen, Carlsbad, CA) or 1:100 Alexa Fluor 488 F(ab')<sub>2</sub> fragment of goat anti-rabbit IgG (H + L) (Invitrogen) for 40 min at room temperature in the dark. After washing with PBS, cells were counter-stained with 1  $\mu$ g/ml 4,6-diamidino-2-phenylindole (DAPI; Invitrogen) for 10 min at room temperature in the dark. Activation of caspases was also measured using FAM-VAD-FMK, the fluorochrome-labeled inhibitor of caspases (FLICA; Immunochemistry Technologies LLC, Bloomington, MN), as described before.<sup>33,34</sup>

**Fluorescence measurements.** Cellular blue (DAPI) and green (Alexa Fluor 488) fluorescence emission was measured concurrently in the same cells using an iCys laser scanning cytometer (LSC; CompuCyte, Cambridge, MA) utilizing standard filter settings; fluorescence was excited with the violet diode and 488-nm argon ion lasers, respectively. The intensities of integrated fluorescence and maximal pixel were measured and recorded for each cell. At least 3,000 cells were measured per sample. Because large number of cells were measured per sample, the SEM of the means of  $\gamma$ H2AX IF or ATM-S1981<sup>P</sup> IF are relatively small. Each sample was run in duplicate or triplicate and repeated at least three times.

**Reactive oxygen species (ROS) detection.** The 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (C-DCFH-DA; Invitrogen) is converted to the highly fluorescent derivative DCF following oxidation by ROS and peroxides. Cells suspended in culture medium were preincubated with 10  $\mu$ M C-DCFH-DA for 30 min at 37°C. A 1 ml volume of cell suspension were taken from the flask before (Ctrl) and 5, 10 and 15 min after adding 1  $\mu$ M PMA and the fluorescence was measured using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA). The green fluorescence was quantified using the standard optics of the FACScan. CellQuest software (Becton-Dickinson) was used to analyze the data.

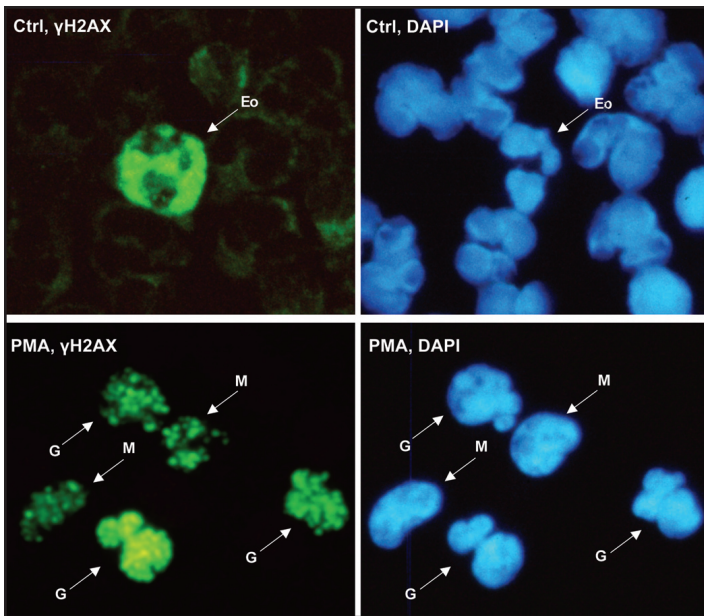


Figure 2. The presence of phosphorylated H2AX in granulocytes and monocytes treated with PMA. WBCs were untreated (Ctrl; upper panels) or incubated in the presence of 1  $\mu$ M PMA for 1 h (bottom panels), then deposited on microscope slides by cytocentrifugation and fixed. The presence of  $\gamma$ H2AX was detected with Ab that specifically reacts with the H2AX-Ser139 phosphorylated epitope<sup>18,36</sup> (left panels) and DNA was counterstained with DAPI (right panels). Among untreated (Ctrl) cells only the eosinophil (Eo) shows strong cytoplasmic labeling with the Ab, which is an artifact resulting from the nonspecific binding of the fluorochrome to eosinophil granules,<sup>37</sup> while nuclei of other cells are negative. Among the PMA-treated cells the distinct foci of  $\gamma$ H2AX immunofluorescence are present in nuclei of both granulocytes (G) and monocytes (M).

Granulocytes and mononuclear cells were distinguished by their characteristic properties to scatter laser light in the forward and right angle (side) direction.<sup>35</sup> Each sample was run in duplicate or triplicate and experiments were repeated at least three times.

## RESULTS

Oxidation of DCFH to DCF is a sensitive assay commonly used to measure oxidative stress.<sup>10-12</sup> This assay has been used to detect the generation of superoxide anions in leukocytes treated with PMA (Fig. 1). As it is evident, even short exposure of cells to PMA led to a marked increase in oxidation of DCFH (note logarithmic scale of DCF fluorescence). Granulocytes were distinctly more responsive in terms of DCFH oxidation compared to mononuclear cells. In the experiment shown in (Fig. 1), a suspension of all white blood cells (WBC) obtained from buffy coat was exposed to PMA, mononuclear cells were distinguished from granulocytes based on differences in their properties to scatter laser-beam light<sup>35</sup> and DCF fluorescence of mononuclear cells and granulocytes was plotted separately. The mean value of DCF fluorescence ( $\pm$  SEM) estimated for the subpopulation of untreated granulocytes was  $9.8 \pm 0.1$  (arbitrary units) as compared with  $31.8 \pm 0.3$ ,  $86.3 \pm 0.7$  and  $178.9 \pm 1.5$  for the cells treated with PMA for 5, 10 and 15 min respectively. In the case of lymphocytes, the mean DCF fluorescence of the untreated cells was 3.9, and for the cells treated with PMA for 5, 10 and 15 min the mean fluorescence was  $8.3 \pm 0.1$ ,  $18.0 \pm 0.2$  and  $33.7 \pm 0.2$ , respectively. A similar pattern of response of mononuclear cells to PMA was observed when these cells were isolated by density gradient sedimentation<sup>32</sup> and were treated

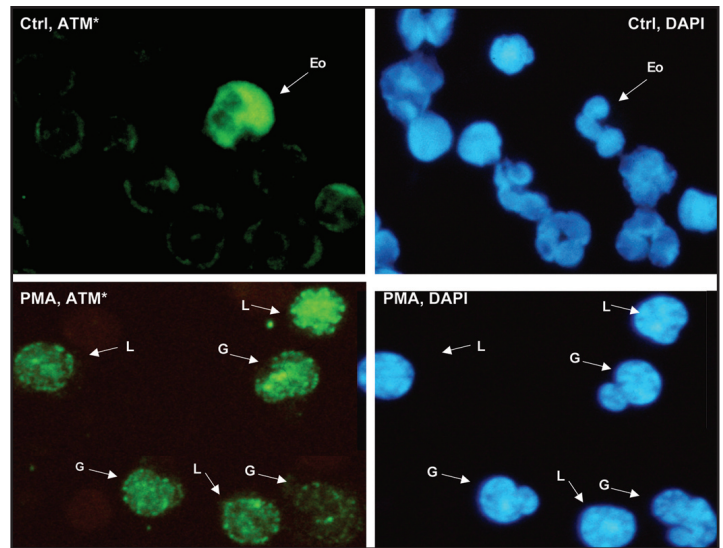


Figure 3. The presence of activated ATM in cells treated with PMA. The WBCs, untreated (Ctrl; top panels) or treated with 1  $\mu$ M PMA for 1 h (bottom panels), were immunostained with Ab specific to ATM-S1981<sup>P</sup>.<sup>27</sup> Among untreated cells only the eosinophil (Eo) is nonspecifically stained, as explained in the legend to Figure 2. Focal immunofluorescence of ATM-S1981 is evident in nuclei of PMA treated granulocytes (G) and of lymphocytes (L).

with PMA while separated from granulocytes (data not shown).

Histone H2AX phosphorylation can be detected as the formation of characteristic immunofluorescent foci of  $\gamma$ H2AX in nuclear chromatin.<sup>18,29,36</sup> As shown in (Fig. 2) no such foci were apparent in the nuclei of untreated cells (Ctrl; top panels), and these nuclei were essentially H2AX IF negative. The eosinophil (Eo) is included in this figure to demonstrate intense green fluorescence of the fluorochrome used to stain these cells: the cytoplasmic labeling of eosinophils with fluorochrome-tagged antibodies is due to nonspecific fluorochrome binding to proteins in the eosinophils' granules.<sup>37</sup> In contrast to the untreated cells,  $\gamma$ H2AX IF foci were distinctly visible in nuclei of both granulocytes (G) and monocytes (M) treated with PMA (bottom panels). In fact, the nuclei of a fraction of lymphocytes also had distinct  $\gamma$ H2AX foci (not shown). Significant intercellular variation was apparent, with some granulocytes and monocytes having a multiplicity of  $\gamma$ H2AX foci and other with only a few.

Figure 3 illustrates the pattern of immunofluorescence of cells stained with Ab to activated (Ser1981 phosphorylated) ATM.<sup>27</sup> While untreated cells were ATM-S1981<sup>P</sup> negative, the treatment with PMA led to formation of immunofluorescent foci of ATM-S1981<sup>P</sup>, similar in appearance to  $\gamma$ H2AX labeling. Also, in analogy to  $\gamma$ H2AX labeling, PMA-treated granulocytes, monocytes as well as lymphocytes showed the presence of ATM-S1981<sup>P</sup> foci with a large intercellular variability in the number of foci per cell.

Laser scanning cytometry<sup>38</sup> was used to quantify immunofluorescence of  $\gamma$ H2AX and of activated ATM in untreated and PMA treated leukocytes (Fig. 4). This instrument allows one to quantify both the intensity of fluorescence integrated over the whole cell (IF) and also the intensity of the maximal pixel (MP).<sup>38</sup> In the case of  $\gamma$ H2AX expression or ATM activation, when fluorescence of these proteins is primarily localized in individual foci (Figs. 2 and 3), the intensity of fluorescence represented by MP rather than by the integrated value is a more sensitive marker of their modification, since it also reflects their high local density within such foci.<sup>38</sup> The data shown in Figure 4 reveal that PMA treatment distinctly increased the percentage

of cells in which the level of  $\gamma$ H2AX and ATM-S1981<sup>P</sup> IF was above that of the untreated cells. The effect of PMA in stimulating H2AX phosphorylation vs. ATM activation was more pronounced in the case of  $\gamma$ H2AX (27% positive cells) than of ATM-S1981<sup>P</sup> (14%). The experiment illustrated in Figure 4 was repeated several times yielding essentially identical results (data not shown).

Spontaneously, as well as in response to a variety of agents, granulocytes undergo apoptosis during which caspase-3 is activated and DNA undergoes fragmentation.<sup>39-42</sup> Experiments were designed, therefore, to test whether the observed H2AX phosphorylation and ATM activation were solely in response to the primary DNA damage induced by oxidative burst and not in response to DNA fragmentation occurring during the course of the cell undergoing apoptosis. Toward this end we have probed caspase activation in PMA-treated cells using FAM-VAD-FMK, the fluorochrome-labeled inhibitor and marker of activation of all caspases.<sup>33,34</sup> No evidence of caspase activation was detected in cells treated for up to 3 h with PMA (Fig. 5). In parallel, we also attempted to detect the presence of activated caspase-3 immunocytochemically.<sup>34</sup> With this approach we also were unable to detect caspase-3 activation for up to 3 h after administration of PMA (data not shown). In contrast, ATM activation and H2AX phosphorylation were seen already within 60 min of the start of treatment (Figs. 2–4).

## DISCUSSION

The present data demonstrate that induction of oxidative stress in peripheral blood leukocytes by PMA triggers H2AX phosphorylation and ATM activation in a large proportion of cells, including granulocytes, monocytes and lymphocytes. The observed modifications of these proteins do not seem to be due to induction of cell apoptosis, which is known to cause extensive DNA fragmentation followed by ATM activation and H2AX phosphorylation.<sup>30,43</sup> Indeed, neither the pan-caspase FLICA activation marker FAM-VAD-FMK,<sup>33</sup> (Fig. 5) nor the Ab against the cleaved (active) caspase-3,<sup>34</sup> (data not shown) detected caspase activation (the event that precedes DNA fragmentation) at the time of ATM activation and H2AX phosphorylation. On the other hand caspase-3 activation was observed in granulocytes undergoing spontaneous apoptosis during prolonged growth in cultures or after treatment with flavopiridol (Fig. 5), the cyclin-dependent kinases inhibitor, under conditions reported to accelerate apoptosis of granulocytes.<sup>44</sup> Given the above, it is unlikely that apoptosis-generated DNA fragmentation contributed in the present study to the observed modifications of these proteins. In all probability, the PMA-induced activation of ATM and phosphorylation of H2AX in granulocytes, monocytes and lymphocytes treated with PMA, were triggered by the DNA lesions induced by the superoxide anion released during oxidative burst. ATM and H2AX modifications seen in lymphocytes could be caused by highly mobile superoxide anions released from granulocytes and/or monocytes that penetrated into lymphocytes and damaged their DNA. As mentioned, DNA damage was observed in different types of cells neighboring granulocytes during oxidative burst.<sup>15,16</sup>

Significant inter-individual cell variability was observed in the extent of ATM activation and H2AX phosphorylation, with some cells showing minimal-, or nearly no-response, while other cells demonstrated very strong  $\gamma$ H2AX or ATM-S1981<sup>P</sup> immunolabeling

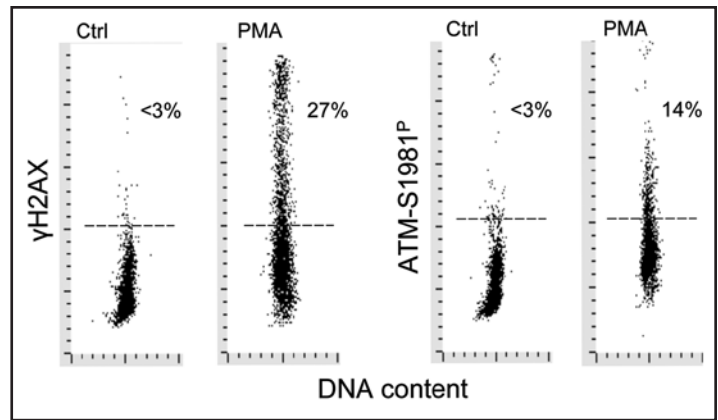


Figure 4. Effect of PMA on H2AX phosphorylation and ATM activation in leukocytes as measured by intensity of  $\gamma$ H2AX and ATM-S1981<sup>P</sup> IF by LSC. The WBCs were untreated (Ctrl) or treated with 1  $\mu$ M PMA for 1 h, then deposited on slides by cytocentrifugation and immunostained for  $\gamma$ H2AX and ATM-S1981<sup>P</sup>, their DNA counterstained with DAPI, and intensity of cellular green (maximal pixel) and blue fluorescence (integrated value) measured by LSC.<sup>38</sup> The gating threshold (dashed lines) marks the upper level of immunofluorescence of 97% cells from the untreated samples; the untreated cells with fluorescence above this level most likely are eosinophils that are nonspecifically labeled with the fluorochrome (see Figs. 2 and 3).<sup>35</sup>

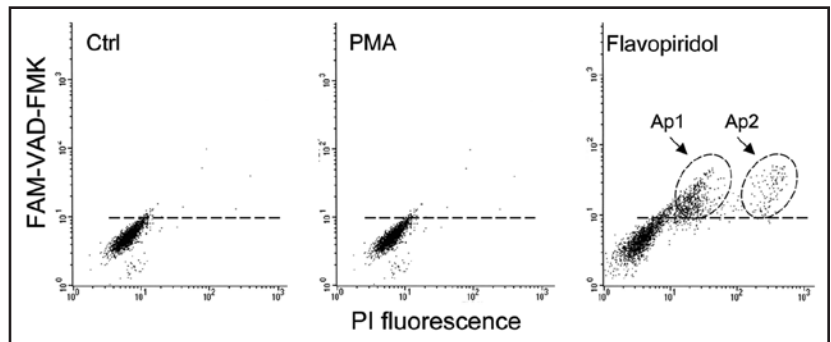


Figure 5. Lack of caspase activation in granulocytes treated with PMA. WBCs were untreated (Ctrl), treated in culture with 1  $\mu$ M PMA for 3 h (PMA), or with 1  $\mu$ M flavopiridol for 22 h, and then incubated for 30 min in the presence of 10  $\mu$ M pan-caspase ligand/marker FAM-VAD-FMK and 1  $\mu$ g/ml of propidium iodide (PI).<sup>33,34</sup> Cellular green (FAM-VAD-FMK) and red (PI) fluorescence was measured by flow cytometry; granulocytes were identified based on their side vs. forward laser light scattering properties.<sup>35</sup> Early apoptotic cells (Ap1) are characterized by increased FAM-VAD-FMK fluorescence and moderate increase in PI fluorescence while late apoptotic cells (Ap2) show increased FAM-VAD-FMK and a marked rise in PI-fluorescence.<sup>33</sup> Note lack of apoptotic cells in the PMA-treated culture and their presence in the flavopiridol-treated culture, which was used as a positive control.<sup>44</sup>

(Fig. 4). In the case of granulocytes or monocytes this variability may be a reflection of intercellular variability in the extent of the oxidant produced, which, as is evident from the degree of oxidation of DCFH-DA (Fig. 1), was quite significant. In the case of lymphocytes, it may be a dependent on their distance from the phagocytes in cell suspension during the burst.

As mentioned earlier, there is extensive evidence in the literature that oxidative burst in phagocytes by PMA causes damage to their DNA.<sup>13,14,44,45</sup> In most investigations DNA damage induced during oxidative burst was demonstrated either by detecting the oxidation products such as 8-oxo-7,8-dihydro-2'-deoxyguanosine (9-oxodG) or single-strand DNA breaks by using alkaline single cell gel electrophoresis.<sup>13-16,46</sup> The presence of DSBs, however, also has been detected in cells subjected to oxidative stress.<sup>47,48</sup> One would expect,

however, that oxidative DNA damage generates many fewer DSBs as primary lesions, compared to the multiplicity of SSLs or base damage. In proliferating cells, the SSLs or base adducts induced by oxidative stress or by radiation become converted into DSBs during DNA replication as a consequence of collisions between DNA replication forks and the lesions.<sup>49</sup> Granulocytes, monocytes or peripheral blood lymphocytes do not replicate DNA and therefore they lack this mechanism of DSBs formation. The oxidative burst-induced H2AX phosphorylation and ATM activation as presently observed, thus, may be in response to SSLs or oxidative base damage rather than to the DSBs generated as primary lesions. Based on the observation of H2AX phosphorylation in G<sub>1</sub> cells following UV light irradiation it has been proposed that this event may be mechanistically associated with nucleotide excision repair (NER) rather than with the presence of DSBs.<sup>50</sup> Such mechanism, thus, may also be primarily responsible for the H2AX phosphorylation observed during oxidative burst, or for constitutive H2AX phosphorylation in untreated cells.<sup>30-32</sup>

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