# Mechanisms of Phosphatidylserine Exposure, A Phagocyte Recognition Signal, on Apoptotic T Lymphocytes

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## Summary

The appearance of phosphatidylserine (PS) on the cell surface during apoptosis in thymocytes and cytotoxic T lymphocyte cell lines provokes PS-dependent recognition by activated macrophages. Flow cytometric analysis of transbilayer lipid movements in T lymphocytes undergoing apoptosis reveals that downregulation of the adenosine triphosphate–dependent aminophospholipid translocase and activation of a nonspecific lipid scramblase are responsible for PS reaching the surface from its intracellular location. Both mechanisms are expressed at the same time, and precede DNA degradation, zeiosis, and cell lysis in the apoptotic pathway.

The ability of apoptotic cells to trigger their own engulfment by phagocytic cells prior to cell lysis is crucial to the avoidance of the tissue damage and inflammation associated with necrosis (1). Timely generation of recognition signals on the surface of apoptosing cells is therefore a key event in the apoptotic program. In those cells where macrophage recognition has been studied, two such signals have been identified. The first is activation or exposure of a ligand for the vitronectin receptor (2–4). The second is the appearance of phosphatidylserine (PS) in the external leaflet of the plasma membrane lipid bilayer (3–5). The ability of macrophages to recognize exposed PS is indicated by the ability of PS vesicles to specifically inhibit the phagocytosis of erythrocytes (6) and apoptotic lymphocytes (5) with PS exposed on their surface.

The transbilayer distribution of phospholipids, including PS, in the plasma membrane of lymphocytes is not as well characterized as it is in erythrocytes and platelets, where PS is normally restricted to the inner leaflet of the bilayer (7–9). When platelets are activated or erythrocytes are loaded with $Ca^{2+}$, PS moves transversely across the bilayer to the outer leaflet of the plasma membrane, generating an active surface for assembly of the coagulation factors Va and Xa into the prothrombinase complex (8, 9) and increasing binding of the fluorescent membrane probe merocyanine 540 (MC540) (10). Similarily, the surface of apoptotic T lymphocytes in vivo (11) or in vitro (5) becomes a better substrate for assembly of prothrombinase and for binding of MC540, suggesting that PS is exposed during apoptosis.

Because continuous T lymphocyte lines exhibit behavior similar to primary T lymphocytes in regard to PS exposure (5), we have used the DO11.10 hybridoma cell line as a model system in which to study the mechanisms of transbilayer lipid movements which bring PS to the cell surface

during apoptosis. In this report, we also show when PS is translocated to the external lipid bilayer in the course of apoptotic events.

## Materials and Methods

*Cell Culture and Activation.* The DO11.10 cell line, a gift of Barbara Osborne (University of Massachusetts, Amherst, MA), was passaged in Eagle's Medium with glutamine (GIBCO BRL, Gaithersburg, MD) and supplemented with 10% fetal bovine serum (GIBCO BRL), at 37°C in a humidified atmosphere of 8% $CO_2$. Apoptosis was induced by adding anti-CD3 mAb (145-2C11; hamster anti–mouse; Boehringer Mannheim Corp., Indianapolis, IN) directly to culture flasks at a concentration of 0.5 μg/ml when cells were in log phase at a density of $10^6$/ml.

*Cell Death Assays.* Cell lysis and DNA degradation were measured using the membrane-impermeable dye YO-PRO-3 iodide (YOPO-PI; Molecular Probes Inc., Eugene, OR) or the membrane permeable DNA chromophore acridine orange (Sigma Immunochemicals, St. Louis, MO), respectively. At various times after addition of antibody 1-ml aliquots of cell culture were removed and stained directly with either 0.5 μM YOPO-PI or 50 μM of the DNA chromophore acridine orange (AO), and samples were kept at room temperature and analyzed by flow cytometry within 10 min. Electron microscopy was described previously (12).

*Cytometric Analysis of Lipid Movements.* Internalization of fluorescent 12-[(7-nitro-2-1,3-benzoxadiazol-4-yl) amino]dodecanoyl (NBD) phospholipid analogues by untreated DO11.10 cells was monitored as described previously (13).

Transbilayer movements of NBD-PS and NBD-phosphatidylcholine (PC) in activated DO11.10 cells were measured during the course of apoptosis. At the specified times after the induction of apoptosis, 1-ml aliquots of culture were pelleted and resuspended in 0.5 ml Hanks' buffer containing 1 mM $Mg^{2+}$, 2 mM $Ca^{2+}$, and NBD-PS or NBD-PC (Avanti Polar Lipids Inc., Ala-

baster, AL) at 3 µM and incubated for 4 min at room temperature. Cells were pelleted for 1 min and resuspended in 0.5 ml Hanks' buffer containing 5 mg/ml of fatty acid–free BSA (Sigma Immunochemicals). Samples were left at room temperature, YOPO-PI was added, and flow cytometric analysis was performed within 2 min from extraction. Intact cells only were analyzed for NBD fluorescence by gating out those unable to exclude YOPO-PI.

Flow Cytometry. All samples were analyzed on an Epics-Profile II flow cytometer (Coulter Electronics, Hialeah, FL) tuned to 488 nm. YOPO-PI and AO emissions were measured through 635- and 525-nm bandpass filters, respectively. NBD-PS, -phosphatidylethanolamine (PE), and -PC fluoresence emissions were detected using a 525-nm bandpass filter. Statistics were performed with the Epics II workstation software.

## Results and Discussion

Activation of DO11.10 cells via the TCR–CD3 complex with anti-CD3 antibody induces the morphological

changes characteristic of lymphocyte apoptosis (14–16): dilation of the endoplasmic reticulum and cytoplasmic vacuolization (Fig. 1 E), plus perinuclear chromatin condensation and cell surface blebbing or zeiosis (Fig. 1 F). The first step in defining where in this cascade of events PS escapes to the surface is establishing the timing of chromatin degradation, change in morphology and loss of membrane integrity in activated DO11.10 cells.

As shown in Fig. 1 A, cultures treated with anti-CD3 develop a subpopulation of cells that have lost membrane integrity and can be identified by staining with the membrane-impermeable dye YOPO-PI (gate A). They also develop a subpopulation of cells with lower forward light scatter and increased side scatter (Fig. 1 B), that have shrunk and become more condensed (17, 18). At the same time, cell fragments appear in the light scatter profile (decreased forward and side scatter), indicating the onset of zeiosis. Because 98% of the subpopulation of cells with normal scatter characteristics (gate B) excluded YOPO-PI while the
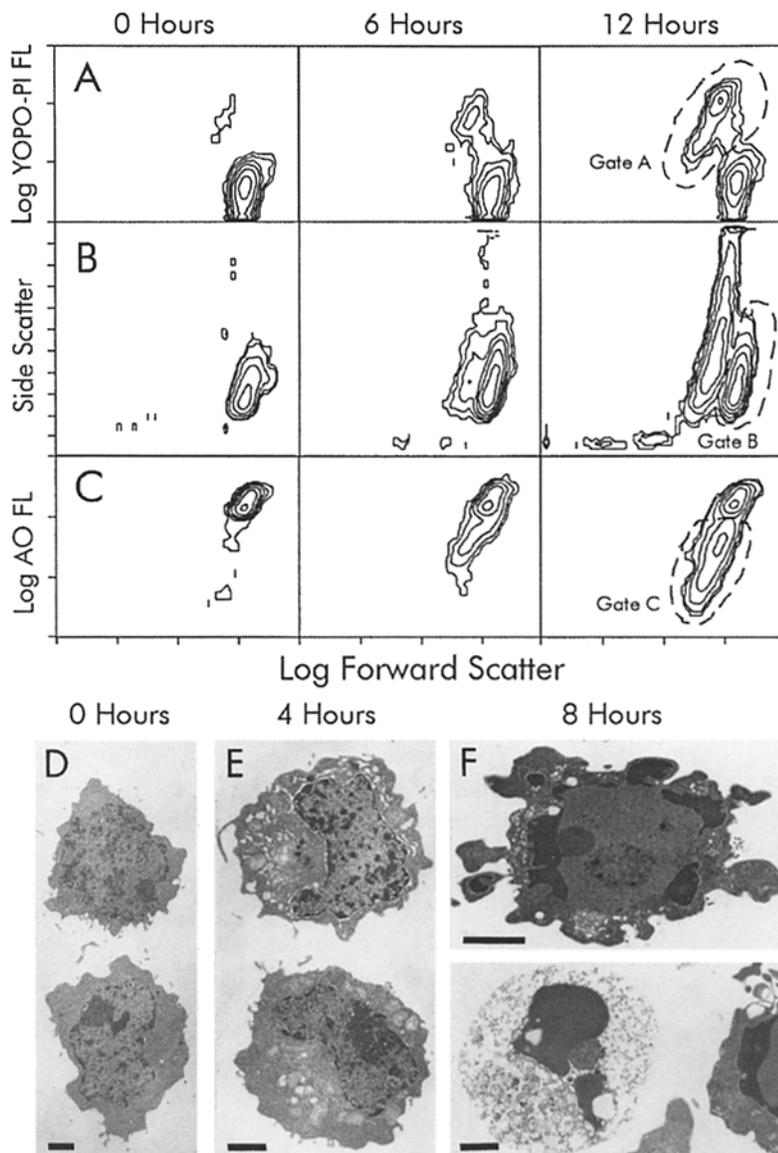


Figure 1. Apoptotic events induced in DO11.10 hybridoma cells by anti-CD3. Shown is flow cytometric analysis of cells at various times following induction of apoptosis. Loss of membrane integrity was determined using YOPO-PI (A), cell size, and density of all the cells was monitored via forward and side scatter (B) and degradation of nuclear chromatin was assayed using AO (C). Apoptotic morphology was directly observed using electron microscopy (D–F). Dead cells persist without disintegrating for >24 h (F, lower frame). Bars, 2 µm.

same fraction of cells outside of gate B did not, loss of membrane integrity must be roughly coincident in time with cell shrinkage and zeiosis. By 12 h, mortality, as defined by these criteria, reached ~55% of the cell population and did not increase substantially with additional incubation up to 18 h.

After staining with AO (Fig. 1 C), cells with degraded chromatin were identified as a discrete subpopulation with reduced fluorescence (gate C) compared to the major population of cells in $G_1/G_0$ of the cell cycle. Plotting the fraction of cells in gates A (lysed cells) and C (cells with degraded DNA) as a function of time after induction of apoptosis (Fig. 2 A) reveals that the onset of substantial DNA degradation occurs ~1 h before loss of membrane integrity.

Because chromatin degradation occurs before cell shrinkage and loss of membrane integrity, intact cells with reduced DNA should be identifiable and quantifiable. The cells in gate C of the AO profile that fall in gate B of the scatter profile (YOPO-PI–negative cells) are plotted in Fig. 2 B as a function of time after induction of apoptosis. This population reaches a peak in frequency between 6 and 8 h after induction of apoptosis. The subsequent decrease in frequency implies that these cells pass into the population of lysed cells against the constant background of cells which fail to enter the apoptotic pathway.

In any simple model, the appearance of PS as a signal for phagocytosis requires that this lipid not ordinarily be present on the plasma membrane surface. In platelets and erythrocytes, PS is kept from the surface by ATP-dependent internalization mediated by the aminophospholipid translocase
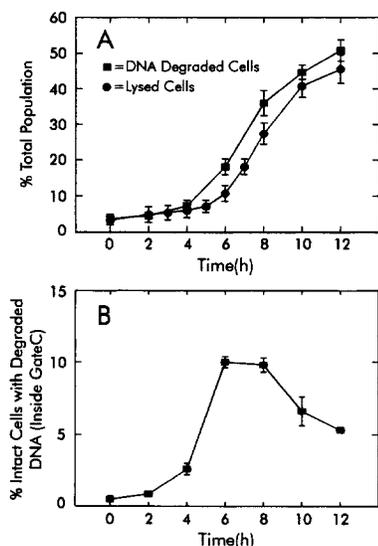


**Figure 3.** Internalization of fluorescent phospholipid analogues by untreated DO11.10 cells. After the specified times at room temperature NBD-PC, -PE or -PS–labeled cells were back extracted with BSA to remove any probe remaining in the outer leaflet, then analyzed by flow cytometry. Values represent the relative fluorescence intensity of the extracted cell populations.

(19–21). The characteristic signature of this enzyme is its ability to move PS and, with less efficiency, PE, but not PC, to the cell interior. Although direct measurement of endogenous phospholipid distribution and movement is not possible, the presence of this enzyme in the plasma membrane of DO11.10 cells can be assessed by examining the movement of fluorescent phospholipid analogues inserted into the outer leaflet of the plasma membrane; their internalization is measured as fluorescence that is not extractable from the outer membrane leaflet by BSA. As shown in Fig. 3, NBD-PS and NBD-PE, but not NBD-PC, are rapidly transported to the interior of DO11.10 cells, implying that the aminophospholipid translocase is present and active in DO11.10 cells before the onset of apoptosis.

The presence of an active translocase in apoptotic DO11.10 cells would result in reinternalization of any PS that might reach the cell surface. Indeed, the estimates of translocase activity implied by the data in Fig. 3 may underestimate the effectiveness of the enzyme for endogenous phospholipids, since the fluorescent probes may be moved more slowly than their natural counterparts (22). Accordingly, internalization of NBD-PS was measured as a function of time after induction of apoptosis. Immediately after activation, NBD-PS internalization by the translocase produced a tight peak of fluorescent cells (Fig. 4 A, top inset). By 6 h after activation (Fig. 4 A, lower inset), however, a substantial subpopulation of cells, identified by reduced staining, were no longer able to internalize NBD-PS, implying the translocase is no longer active in these cells. It should be noted that the cells in this subpopulation are all intact, since cells unable to exclude YOPO-PI were gated out and do not appear in the profile. As expected, these cells display the same scatter characteristics as the bulk of the population, confirming that downregulation of the translocase takes place in cells before cell shrinkage, which is coincident with lysis. When the fraction of intact cells unable to internalize NBD-PS (gate D) was plotted as a function of time after induction of apoptosis, the curve shown in Fig. 4 A was generated, indicating that such cells begin to appear at 4 h and reach a peak in frequency between 5 and 7 h after induction of apoptosis.



**Figure 2.** Timing of apoptotic events. (A) Cell viability and chromatin degradation as a function of time after induction of apoptosis. Values plotted are from the analyses in Fig. 1 for cells with degraded chromatin (Fig. 1, gate C) and lysed cells (Fig. 1, gate A). (B) Chromatin degradation in YOPO-PI–negative cells as a function of time after induction of apoptosis. Cells with reduced DNA fluorescence (Fig. 1, gate C) were examined with respect to light scatter, and those cells falling within of Fig. 1 (gate B) were plotted as a percent of intact cells. Error bars represent the standard deviation of three independent experiments; some error bars are hidden by the data symbols.
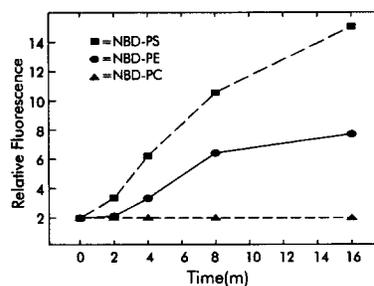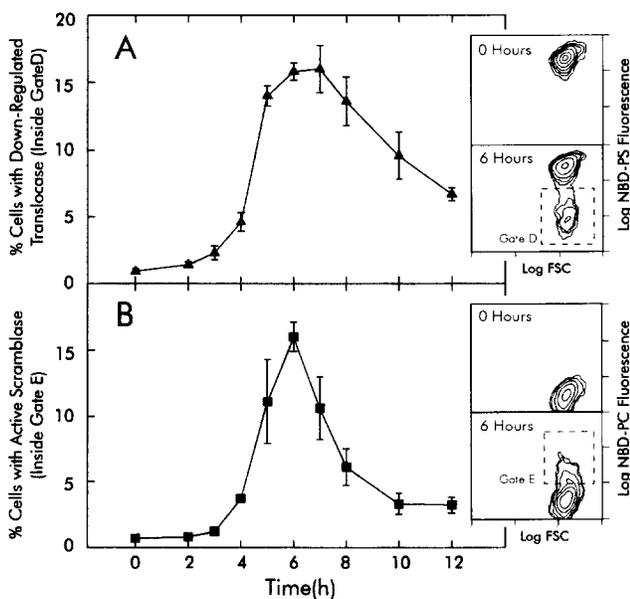
**Figure 4.** Internalization of the fluorescent phospholipid analogues NBD-PS (*A*) and NBD-PC (*B*) by DO11.10 cells induced to undergo apoptosis. The percentage of viable cells inside gate D (reduced NBD-PS fluorescence) and gate E (increased NBD-PC fluorescence) were plotted as a function of time after induction of apoptosis. Inserts are representative 0- and 6-h samples. Standard deviations are for three independent experiments; some error bars are hidden by the data symbols.

If passive diffusion of phospholipids, including PS, across the bilayer occurs at the low rate observed with NBD-PC in uninduced cells (Fig. 3), the rapid exposure of PS on apoptotic cells in which the translocase has been downregulated must occur by some other mechanism. In erythrocytes and platelets, a "scramblase" activity facilitates bidirectional transbilayer movement of all phospholipids, including the otherwise inert PC (21–25). To test for such an activity in apoptotic lymphocytes, activated cells were analyzed for elevated transbilayer movement of NBD-PC. As shown in Fig. 4 *B* (*top insert*), cells at 0 h fail to internalize NBD-PC during a 5-min incubation, as evidenced by the tight peak of cells with low fluorescence. At 6 h, however, a substantial subpopulation of cells appeared in which elevated NBD-PC internalization was discernable (Fig. 4 *B, lower inset*). The frequency of these cells (*gate E*) reached a maximum at 6 h after induction of apoptosis (Fig. 4 *B*), followed by a decrease in frequency, implying, as above, that cells in which this movement occurs subsequently enter the lysed cell fraction.

Together, these data argue that the program of apoptosis in T lymphocytes includes a coordinated alteration of the mechanisms that regulate transbilayer plasma membrane

lipid distribution, including downregulation of the amino-phospholipid translocase that normally maintains asymmetry, and activation of nonspecific transbilayer lipid movement. Neither process is observed in that fraction of the cell population that does not enter the apoptotic pathway, even though these cells are exposed to the same stimuli and culture conditions experienced by their apoptotic brethren. In preliminary experiments, similar alterations in lipid movements have been observed in DO11.10 cells treated with dexamethasone or PMA plus ionophore to induce apoptosis (data not shown), suggesting that this process is independent of the stimulus to apoptose. This alteration in lipid movements occurs at 5–6 h after exposure to anti-CD3 antibody, preceding the degradation of nuclear chromatin by $\sim1$ h and preceding cell membrane permeation and zeiosis by $\sim2$ h. Recent studies of activation induced apoptosis in T cells suggests that the pathway proceeds by induction of the Fas/Fas-ligand system for cell death (26–28). During anti-CD3–induced apoptosis in the A1.1 T cell hybridoma line (26), Fas mRNA levels rise $\sim1$–2 h after induction, with Fas protein appearing in the membrane by 4 h; Fas ligand mRNA is induced somewhat later, with at least some protein reaching the cell surface by 4.5 h after induction. If the time course for these events is similar in anti-CD3–induced DO11.10 cells, the results presented here would indicate that the alteration in PS distribution occurs almost immediately after the development of these autocrine signals for apoptosis.

While this evidence suggests that loss of lipid asymmetry is closely linked to expression of the Fas/Fas-ligand signalling system, the nature of the internal signal that modulates these alterations in lipid movements is still unclear. Both inhibition of the translocase and activation of the scramblase, however, are observed in erythrocytes and platelets upon elevation of cytoplasmic $Ca^{2+}$ (22, 24), suggesting that cytoplasmic $Ca^{2+}$ levels may be a second messenger for PS presentation on apoptotic lymphocytes. The activation of the $Ca^{2+}$-induced intracellular protease calpain during steroid hormone–induced apoptosis (29) is consistent with this sequence of events. In fact, downregulation of the translocase and activation of the scramblase is blocked when DO11.10 cells are activated in the presence of 2 mM EGTA (data not shown). It is therefore likely that the influx of extracellular $Ca^{2+}$ observed during T cell hybridoma apoptosis (16) plays a role in coordinating the processes that bring PS to the surface. The relationship between Fas-mediated and $Ca^{2+}$-mediated intracellular signalling and the pathways leading to DNA degradation, morphological changes, and cell lysis (30, 31) will be logical targets of further investigations.

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