ACRIDINE ORANGE INDUCES TRANSLOCATION OF PHOSPHATIDYLSERINE TO RED BLOOD CELL SURFACE.

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ABSTRACT:

Clustering of band-3 on red blood cells (RBC) surface has been assumed to catalyze RBC phagocytosis. In studying this subject, acridine orange (AO) has commonly been employed, on the assumption that it specifically induces band-3 clustering. In the present study we show that AO strongly induces translocation of phosphatidylserine (PS) to RBC surface. Since surface PS is well known to induce RBC intercellular interaction, these findings suggest that the use of AO as a specific inducer of band-3 clustering is questionable. It is possible that band-3 clustering and PS translocation are interdependent, and this interrelationship has yet to be explored.
The removal of senescent or damaged red blood cells (RBC) is an essential process for the homeostasis of the circulatory system. One of the mechanisms of the phagocytic removal of senescent RBC from the blood circulation by spleen macrophages is antibody-dependent phagocytosis (Kay 1975).

It has been proposed that RBC aging is associated with clustering of band-3 at the RBC surface, and the clustered band-3 binds to IgG antibodies, leading to RBC phagocytosis (Low et al. 1985). It has also been proposed that band-3 clustering is responsible for the enhanced adherence of Plasmodium falciparum-infected RBC to endothelial cells (EC) (Thevinin et al. 1997). Other studies have shown that RBC recognition by macrophages, and adherence to EC, are induced also by phosphatidylserine (PS) at the RBC surface, which is known to be translocated to the outer leaflet of the membrane in hemoglobinopathies and other pathological conditions (Kuypers 1998). Both band-3 clustering and PS exposure are known to increase with RBC aging (Kannan et al. 1991; Connor et al. 1994).

In studying band-3 clustering and its implications to RBC intercellular interactions, a widely used procedure has been the treatment of RBC with acridine orange (AO), as it has been assumed to be a specific inducer of band-3 clustering (Low et al. 1985; Ando et al. 1997; Turrini et al. 1991). However, although RBC intercellular interactions are also induced by PS, the effect of AO treatment on RBC membrane phospholipids has not been examined. The present study was undertaken to examine the influence of AO treatment on PS level at the RBC surface.
METHODS

Blood was drawn from five healthy donors, upon their consent according to the Helsinki Committee Regulations (Permit No 20-30/03/01, Israel). RBC were isolated from plasma by centrifugation for 10 min at 1000 g, washed three times in Ca^{2+}/Mg^{2+} free phosphate buffered saline (PBS: 140 mM NaCl, 10 mM Na^{7}/K^{7}-phosphate, pH 7.4), and suspended in PBS (at 10% hematocrit). The suspension was supplemented with increasing AO concentrations ranging from 0.5 to 4.0 mM (final concentration), and incubated in covered tubes (to keep them in darkness) at 25°C in a shaking bath for 15 min, as in the previous studies that used AO for band-3 clustering (Low et al. 1985). AO-treated RBC were washed 3 times with PBS by centrifugation. As previous described (Eda and Sherman, 1998), this treatment induced the formation of echinocytes but no hemolysis was observed.

To detect PS at the RBC surface, in the present study we used allophycocyanin-conjugated recombinant human annexin V (rh Annexin V-APC; Bender Medsystems, Vienna, Austria) as the PS ligand. APC was used here instead of a fluorescein 5-isothiocyanate (FITC), which is commonly used for PS determination, since the FITC fluorescence spectrum overlaps with that of AO; FITC is exited at 488 nm and emits at 520 nm, at which AO exhibits high emission too. In contrast, APC can be exited at 630 nm and emits at 660 nm, away from the AO fluorescence spectrum. The fluorescence of the AO-treated RBC was compared to that of control RBC, which were incubated for 15 min in AO-free PBS.

For positive control, PS translocation was induced by treatment of RBC with calcium and Ca-ionophore. RBCs at 16% hematocrit were equilibrated in PBS supplemented with 1 mM CaCl\textsubscript{2} for 3 minutes at 37°C. Subsequently, the Ca-ionophore A23187 (Sigma Chemical Co., St. Louis, MO) was added to the RBC suspension to a final concentration of 4 µM and incubated for 1 h at 37°C. To terminate the process, calcium was removed by RBC washing.
with PBS containing 1 mM EDTA. The cells were then washed three times in Ca\(^{2+}\)/Mg\(^{2+}\) free PBS containing 1% bovine serum albumin to remove the ionophore (Kuypers et al. 1996).

For annexin V-APC binding, 2x10^6 RBC were suspended in 200 µl HEPES-buffered saline supplemented with 2.5 mM CaCl\(_2\) and incubated with 5 µl annexin V-APC for 15 min at 37°C in the dark. The fluorescently-labeled cells were then washed once, suspended in 500 µl of the same buffer and determined in fluorescence-activated cell sorter (FACS). Data acquisitions were performed on a Becton Dickinson FACScan (Becton Dickinson, San Jose, CA) and analysis was done with CellQuest software (Becton Dickinson). The fluorescence of annexin V-APC-labeled erythrocytes was recorded at the emission 660 nm (channel FL4) with the excitation at 630 nm, by a red diode laser. A total of 10,000 events were acquired for each sample. The percentage of annexin V-APC positive erythrocytes was determined from the fluorescence signal in excess over that obtained with a negative (unlabeled) control RBC for each sample.

To determine the AO incorporation into RBC membrane, 2x10^6 AO-treated RBC (as above) were suspended in 500 µl of HEPES-buffered saline (10 mM HEPES-Na, 140 mM NaCl, 2.5 mM CaCl\(_2\), pH 7.4) and analyzed in the flow cytometer for AO fluorescence (excitation wavelength 488 nm, emission wavelength 505 to 545 nm). The percentage of AO-labeled erythrocytes was determined from the autofluorescence signal in excess over that obtained with a negative control (not treated with AO) RBC.

**RESULTS AND DISCUSSION**

Representative results of flow cytometric analysis are shown in Figure 1, demonstrating AO-induced PS translocation to the RBC surface (as expressed by the binding of annexin V-
APC), compared to untreated RBC (negative control) and Ca-ionophore treated RBC (positive control).

Fig. 2A shows that treatment of RBC with AO strongly increased the binding of annexin to the cell surface in a dose-dependent manner, up to about 30% annexin-V labeled cells. This effect is not due to possible ATP depletion (which is unlikely to be significant in such short-time treatment), since 1. PS translocation was not detected in negative control RBC, subjected to the same duration of incubation. 2. It has previously been shown that inhibition of aminophospholipid translocase (flipase), or rapid ATP depletion, does not lead to cell surface exposure of PS in normal RBC, even after overnight incubation (de Jong K et al. 1999).

Determination of AO incorporation (by the AO fluorescence emission of AO-treated RBC, depicted in Fig. 2B), shows that at 1 mM AO 95% of the RBC are already labeled with AO (Fig 2B-I). However, this figure also shows that the incorporation of AO to RBC was monotonously increased with its buffer concentration, as determined by the mean intensity of AO fluorescence in the cells (Fig 2B-II). Comparing Figs. 2A and 2B-II reveals a clear correlation between the increase in the mean AO fluorescence and the percent of Annexin V-labeled RBC, although the Annexin V-labeled RBC does not exceed 30%, while all the cells are labeled with AO. This discrepancy can be explained in two ways: 1. The AO incorporation into the cell has to exceed a certain level in order to induce PS translocation. 2. The PS at the cells surface has to exceed a certain threshold level in order to produce a detectable signal of the Annexin V-APC fluorescence.

The results of this study demonstrate that treatment of RBC with AO is very effective in inducing translocation of PS to the cell surface. It is thus plausible to conclude that the use of AO as a specific inducer of band-3 clustering for studying RBC intercellular interaction is
questionable. Since PS is well known to facilitate RBC adhesion and recognition by other cells, the AO-induced RBC intracellular interaction is not necessarily due to band-3 clustering. It is possible that band-3 clustering and PS translocation are interdependent, as previously suggested (Hassoun et al. 1998), but this interrelation has yet to be explored.

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Figure 1. Dot-plot analysis of AO-treated RBCs labeled with annexin V-APC: The panels depict: untreated (negative control) RBCs (A), Calcium/Ionophore treated (positive control) (B), treated with 1 (C) and 4 mM (D) AO. The fluorescence of annexin V-APC labeled RBCs was detected at the emission 660 nm (channel FL4) with the excitation at 630 nm by a red diode laser. The percentage of annexin V-APC positive RBCs was determined from the fluorescence signal in excess of that obtained with a negative (unlabeled) control for each sample. The figure depicts data obtained from one blood sample but representative of 5 blood samples.

Figure 2. FACS analysis of acridine-orange (AO)-treated RBC with/without labeling with Annexin V-APC: (A) Effect of AO concentration on translocation of PS to RBC surface: The figure shows the percentage of RBC labeled with annexin V-APC as a function of AO concentration. The curve represents a sigmoid fit ($r = 0.999$). (B) Dose dependent AO incorporation to RBC, determined by AO autofluorescence: (I) - percentage of AO-labeled RBC. (II) – mean intensity of AO fluorescence in RBC population. Each datum is the mean ± SEM for 5 blood samples, each determined in triplicates.
Koshkaryev et al., Figure 1.

A

0.4%

B

66.8%

C

12.5%

D

24.7%