USE OF 1-ANILINO-8-NAPHTALENE SULFONATE TO STUDY STRUCTURAL TRANSITIONS IN CELL MEMBRANE OF PMN LEUCOCYTES.

D.Romeo[+], R.Cramer and F.Rossi
Istituto di Chimica Biologica[+] and Istituto di Patologia Generale, Università di Trieste, 34127 Trieste, Italy

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Summary.

The interaction of 1-anilino-8-naphtalene sulfonate (ANS) with intact guinea pig PMN leucocytes results in a marked increase in fluorescence intensity, which is interpreted as binding of ANS to hydrophobic regions of cell membrane. When polystyrene spherules are added to ANS-labeled leucocytes there is an instantaneous rise in fluorescence of the bound dye. By increasing the polystyrene particles/leucocyte ratio a parallel enhancement of fluorescence and oxidation of 1-^{14}C-glucose is observed. This result suggests that a correlation exists between structural transitions in the cell membrane and metabolic stimulations within the leucocyte.

Concomitant with phagocytosis in polymorphonuclear (PMN) leucocytes there is a marked enhancement of respiration and of direct oxidation of glucose-6-phosphate via the hexose monophosphate pathway (HMP) (1-4). This metabolic stimulation appears not to be dependent on ingestion of particulate materials by the phagocyte (5). Similar effects can be observed after interaction of surfactants (6,7), anti-leucocyte antibodies (8) and phospholipase C (9) with the leucocyte surface. Rossi and Zatti (10) have suggested that an alteration of the cell membrane, produced either chemically or by contact with bacteria and inert matter, triggers the activation of a granule-bound NADPH oxidase. A higher rate of activity of this enzyme would be responsible for the increased oxygen uptake. Since the HMP is regulated by the availability of NADP, an adequate increase of the NADP/NADPH ratio would lead to the enhanced oxidation of glucose-6-phosphate.

In recent years 1-anilino-8-naphtalene sulfonate (ANS) has been commonly used as a fluorescent probe of changes of membrane structure (11-14). ANS interacts with membranes to give a typical enhancement and blue-shift of fluorescence. This result is interpreted as binding of ANS to hydrophobic regions of membranes, since similar changes in quantum yield and emission spectra are seen when the dye is dissolved in solvents of low polarity.(11).

In the present comunication we describe the *in vitro* interaction of

ANS with intact guinea pig leucocytes. The ANS-labeled leucocytes have been used to study changes in fluorescence efficiency upon interaction between the cells and polystyrene spherules. These changes have been correlated with the stimulation of HMP activity.

MATERIALS AND METHODS

PMN leucocytes were collected from guinea pig peritoneal exudates, washed once and suspended in calcium-free Krebs-Ringer phosphate, pH 7.4, as previously described (2). More than 85% of the cells in the exudate were mature polymorphonuclear leucocytes.

The Mg salt of ANS (Eastman) was recrystallyzed twice from hot water solutions. The purified compound gave a single spot on thin layer chromatography (14). The molar absorption coefficient used to calculate ANS concentrations was $4.95 \cdot 10^3$ $M^{-1}cm^{-1}$ at 350 nm (15).

Fluorescence measurements were made on an Eppendorf fluorimeter at an excitation wavelength of 366 nm, recording the emission between 420 and 3000 nm. Analysis of leucocytes with and without ANS by fluorescence microscopy was carried out with a Zeiss microscope (500 x).

Protein was determined according to the method of Lowry, Rosebrough, Farr and Randall (16), with bovine serum albumin as standard. $^{14}CO_2$ production from 1-^{14}C-glucose was followed as reported by Rossi and Zatti (3).

Polystyrene spherules (Difco, 0.81 μ diameter) were dialyzed against distilled water and the concentration of the suspension was determined by counting in the microscope.

RESULTS

Addition of PMN leucocytes to a solution of ANS resulted in a marked rise in fluorescence intensity of the dye, as illustrated in Fig.1. The ANS fluorescence was a linear function of protein concentration up to about 0.6 mg protein/ml, the slope of the curve being slightly increased above this concentration. The enhancement of fluorescence was very rapid, and occurred during the time spent to stir the content of the cuvette in the fluorimeter (about two seconds). Beyond this point, no change in fluorescence was observed for at least 3 minutes.

Fig. 1.B shows a titration curve of leucocytes with ANS. At the end

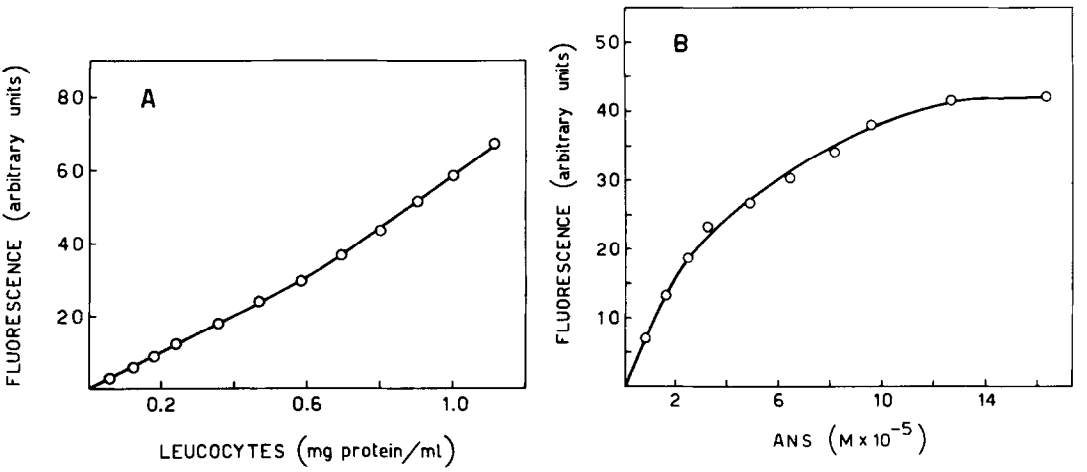

Figure 1. - Fluorescence of 1-anilino-8-naphtalene sulfonate (ANS) in the presence of guinea pig leucocytes.

A. - Measurements were made at 25° immediately after adding PMN leucocytes at protein concentrations indicated in the abscissa to a 3.2 x 10^{-5}M ANS solution in Krebs-Ringer phosphate, pH 7.4.

B. - Measurements were made at 25° on a suspension which contained PMN leucocytes (0.5 mg protein/ml) in Krebs-Ringer phosphate buffer, pH 7.4 and ANS at the final concentrations indicated in the abscissa. Fluorescence of ANS alone was subtracted.

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of the experiment the leucocytes were spun down and the concentration of ANS in the supernatant solution was measured spectrophotometrically at 350 nm. By using proper corrections for residual absorption originating from leucocytes, the amount of ANS recovered in the supernatant was 100 ± 5%, while the pellet retained about 70% of the original fluorescence. On the basis of the sensitivity of the spectrophotometric analysis, one may estimate that the amount of ANS bound to the leucocytes was at the most 0.1 nmoles/10^6 cells.

As shown in Fig.2, a marked increase of fluorescence intensity was observed when polystyrene spherules were added to leucocytes pre-treated with ANS. After the initial increase in fluorescence, no further rise or decay was noticed for at least 5 minutes. Control experiments showed that, when the spherules were added to ANS or unlabeled leucocytes, there was also a small rise in fluorescence emission (Fig.2). Since a similar increment was obtained by adding particles to buffer alone, this appears due to intrinsic fluorescence of the polystyrene.

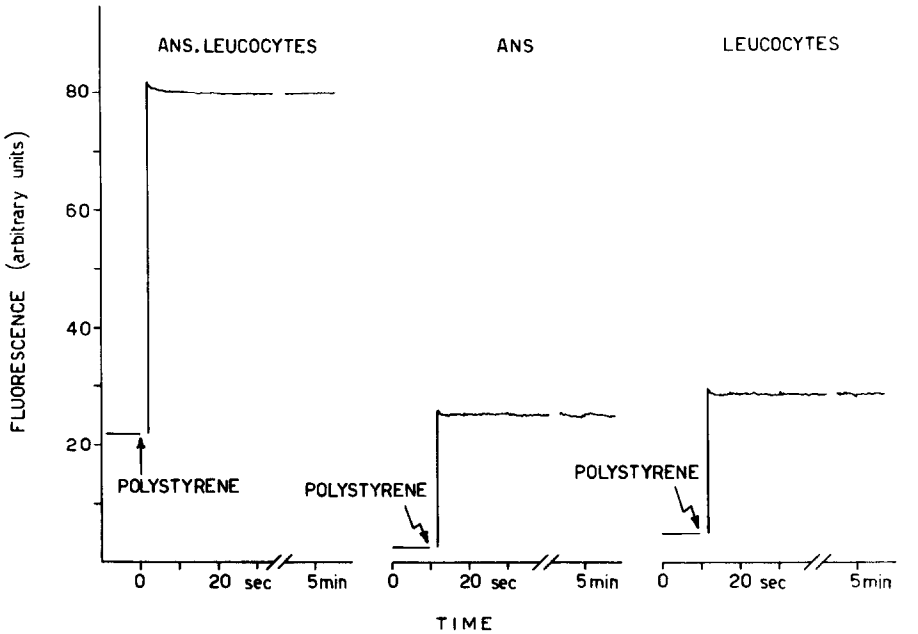

Figure 2. - Increase of fluorescence intensity of ANS bound to PMN leucocytes upon addition of polystyrene spherules.

A. - Leucocytes in Krebs-Ringer phosphate buffer pH 7.4 (1.1 mg protein/ml) were pipetted into a $6 \cdot 10^{-5}$M solution of ANS at 30°. After 5 minutes cells were spun down and resuspended to the original volume. To aliquots of cells brought to 37°, polystyrene particles (0.81 μ diameter, 150 spherules/leucocyte) were added and the fluorescence recorded within 1-2 seconds.

B. - Polystyrene spherules were added to $6 \cdot 10^{-5}$M ANS.

C. - Polystyrene spherules were added to unlabeled leucocytes (1.1 mg protein/ml).

In order to gain information about the relation of the enhancement of ANS fluorescence to the changes in HMP activity induced by the interaction between leucocytes and particles, the two events were followed at different polystyrene particles/leucocyte ratios. As shown in Fig.3, by increasing the amount of spherules available to the cells, a parallel enhancement of fluorescence intensity and production of $^{14}CO_2$ from 1-^{14}C-glucose was observed, suggesting that the two processes are somehow linked and dependent on the number of collisions between leucocytes and spherules.

DISCUSSION

In a system as complex as a leucocyte there are probably a large number of binding sites for a dye such as ANS, with different affinities and

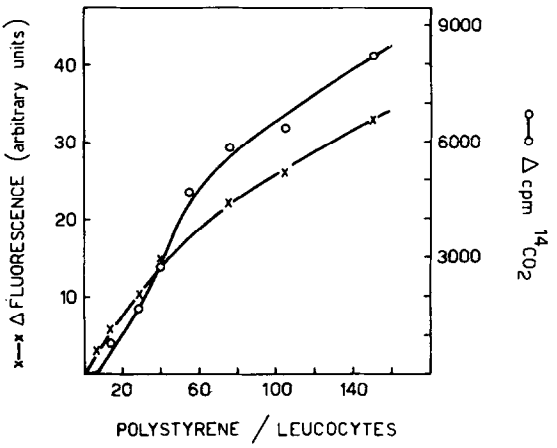

Figure 3. - Increase of fluorescence intensity of ANS bound to PMN leucocytes and of oxidation of 1-^{14}C-glucose upon addition of polystyrene spherules. Experimental conditions as indicated in Fig.2, the only modification being the varying of polystyrene particles/leucocyte ratio. $^{14}CO_2$ production from 1-^{14}C-glucose was followed by incubating the leucocytes at 37° for 10 minutes as reported by Rossi and Zatti (3).
Control experiments have shown that ANS binding to leucocytes did not impair the metabolic stimulation induced by phagocytosis. Background fluorescence due to the polystyrene particles was subtracted.

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fluorescence-enhancement properties. The simplest interpretation of the very fast enhancement seen when ANS is added to a leucocyte suspension is that it represents binding to easily accessible sites embedded in a nonpolar microenvironment. We assume that these sites in intact leucocytes are virtually confined to the cell surface. The following evidence supports this assumption. (i) Whenever there is "penetration" of ANS through a membrane system, the fluorescence increment follows a two-phase curve, while, if all the interaction sites are promptly available, only a fast phase is observed. In fact, Freedman and Radda (12) have shown that the rate of interaction of ANS with erythrocyte stroma is characterized by a fast and a slow phase. If the ghost are sonicated the slow phase disappears and the half-life of the fast phase is 5 mseconds (14). Furthermore, Azzi (personal comunication)has shown that the interaction of ANS with phospholipid vescicles delimited by a single membrane is characterized only by a fast phase, whereas it becomes biphasic when the vescicles are multilamellar. (ii) Disruption of the leucocytes by sonication in the presence of ANS resulted in a very marked increa-

se in fluorescence intensity, suggesting that by this procedure a great number of cytoplasmic binding sites were made available. (iii) A weak green fluorescence at the cell periphery was seen in the microscope after exposure of the leucocytes to ANS.

Dyckmann and Wetman (17) have reported that, after interaction of PMN leucocytes with ANS, the cytoplasm appears in the microscope as highly fluorescent. They have used however an ANS concentration which is about 100-fold higher than that employed in our experiments. In view of the fact that ANS has the general structure of a surface active agent, a partial damage to the cell under their conditions, followed by extensive penetration of the dye, cannot be excluded. Concentrations similar to those used in our experiments have been shown by Wallach et al.(18) not to lyse intact erythrocytes.

Rossi and Zatti (10) have postulated that the contact between leucocytes and particles promotes a structural rearrangement of plasma membrane. This event would be followed by the release of an unknown effector, which, by interaction with specific cellular granules, would trigger the activation of an NADPH oxidase. Recently, we have shown that this activation occurs within 5-10 seconds after the addition of particles to a leucocyte suspension (19). The apparent change in membrane conformation (*), as indicated by the increase in fluorescence, takes place within 2 seconds, thus preceding any measurable metabolic stimulation.

The oxidized pyridine nucleotide produced by the action of NADPH oxidase would stimulate the oxidation of glucose-6-phosphate via the HMP. The results reported in Fig.3 suggest that a correlation exists between alteration of cell membrane and increased production of $^{14}CO_2$ from 1-^{14}C-glucose. This is wholly consistent with the above hypothesis.

The precise nature of the conformational changes in the membrane is as yet obscure. Since the rise in fluorescence upon addition of polystyrene particles to the leucocyte suspension is instantaneous and no further chan-

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(*) A 'change in membrane conformation' would be an overall result of either a transition in the configuration of protein(s) or a molecular rearrangement of lipids. This change can be monitored by ANS fluorescence, if it leads to a variation of the polarity at the chromophore binding sites.

ge is thereafter observed, it is certainly not induced by the actual phagocytic process.

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