Radiation Damage to Lymphocyte Membrane. Changes of Binding and Fluorescent Parameters of 1-Anilino-8-naphthalene sulphonate.

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Abstract. The changes of binding and fluorescent parameters of 1-anilino-8-naphthalene sulphonate (ANS) bound to lymphocyte membrane after irradiation *in vitro* were investigated. No significant dose dependence was found for either fluorescence or its polarization within the dose range of 0.1 to 0.7 Gy from a gamma – neutron field ^{252}Cf source. Marked changes were however found for parameters of ANS binding to membrane after irradiation. The number of binding sites decreased by about 60% and the dissociation constant K decreased by about 70% as compared to control values. The quantum yield of fluorescence also decreased compared with control value. The experimental findings were interpreted as suggesting structural changes in the lipid environment of the lymphocyte membrane caused by irradiation.

Key words: Irradiation — Membrane — Lymphocyte — Fluorescence—1-anilino-8-naphthalene sulphonate binding

Introduction

More than 20 years ago membrane damage was considered to play a key role in cell killing by ionizing radiation (Alper 1968). Such a role has remained an open question despite many different experimental techniques that have been used to assess it. Studies on the loss of membrane functional properties induced by ionizing radiation have been performed on a variety of biological samples using different methods (Ashwell et al. 1986; Edwards et al. 1984; Ruifrok et al. 1985). The search for a sensitive technique to detect the production of damage, and the identification of its molecular nature, can be of relevance in the definiton of membrane components as targets for ionizing radiation, and in the understanding of the biological significance of such damage (Parasassi et al. 1991).

The revival of interest in fluorescent probes in recent years has resulted in nu-

merous studies dealing with the pertinence of selected fluorophores for monitoring the changes of membrane structure and dynamics (Radda 1975; Loew 1988). One commonly used fluorescent probe is 1-anilino-8-naphthalene sulphonate (ANS). Its fluorescence is extremely sensitive to changes in the probe environment, as the probe binds noncovalently to both membrane proteins and lipids.

The aim of our study was to evaluate the alterations of membrane dynamic parameters of peripheral lymphocytes after irradiation *in vitro*. Our attention was focused on radiation with high linear energy transfer (LET), the neutron field, which is characterised by marked relative biological efficiency especially in the low dose range. The technique of fluorescene spectrometry and ANS as the specific fluorescence probe were used.

Materials and Methods

Lymphocyte preparation: The lymphocytes were isolated from freshly withdrawn heparinized venous blood of healthy persons by standard verographine method. Two ml of blood was layered over with 2 ml of verographine solution (60% Verographine Spofa:aqua pro inj., 2:5) and centrufuged at 2500 rpm for 30 min. The ring of lymphocytes formed on desity interface was sucked off and washed 3 times with PBS (pH 7.3, osmolarity 290 mOsm) by centrifuging at 1500 rpm for 15 min. Isolated lymphocytes (usual recovery about $2 \cdot 10^6$ cells/ml blood) from a stock suspension of about $20 \cdot 10^6$ cells/ml in PBS were then used as control and experimental group. Subsequently, both groups were handled in the same way including time interval between cell isolation and fluorescence measurements.

Irradiation: The lymphocyte suspension was irradiated in a thin wall vessel in a volume of 1 ml at ambient temperature by gamma neutron field of ^{252}Cf (Amersham), at a dose rate of 0.3 Gy/hour. The mixed radiation field consisted of 60% neutrons and 40% gamma. As the relative biological efficiency of ^{252}Cf neutrons equals 3.7 for cell membrane damage (Tatara et al. 1989), the all doses were expressed as particle dose of neutrons. Detailed dosimetric description of the source was published elsewhere (Nikodémová et al. 1990). The irradiated samples were finally diluted with PBS to yield $2 \cdot 10^6$ cells/ml. Control samples were sham irradiated at the same conditions. The functional state of cells was checked at the time of the fluorescence measurement by the standard dye exclusion method (0.5% Trypan blue after 5 min). The viability of nonirradiated samples ranged between 95 and 98%, and that of the irradiated ones between 90 and 95%.

Sample preparation and fluorescence measurements: Amonium 1-anilino-8-naphthalene sulphonate (Sigma) 0.01 to 0.1 ml, 0.01 mol/l was added gradually to the cell suspension and immediatelly measured. The "blank" sample for each experiment was prepared by ANS titration of a mixture without cells. The interaction of ANS with the membrane is almost instantaneous. The time interval between the cell isolation and the fluorescence measurement was kept constant (3 hours) for all samples. The fluorescence intensity and its polarization was measured with a luminescent spectrometer LS-5 (Perkin Elmer) equipped with a thermostated cell holder and an automatic polarization accessory. ANS fluorescence was excited at 395 nm with a monochromator slit of 5 nm, and emission was observed at 495 nm. Quartz Perkin Elmer cuvettes 10 mm × 10 mm × 40 mm were used. For each ANS concentration, the measured fluorescence intensities were corrected

for the inner filter effect. Correction for the contribution of unbound ANS was done by substracting "blank" fluorescence intensities from the fluorescence of samples with cells. Each experimental point for both the dose and the concentration dependences was calculated from 5 parallel samples. The luminescent spectrometer was calibrated with ANS in spectrometric methanol. The value of $a_{395} = 5140\ \text{mol}^{-1}$ and $Q = 0.22$ were used for molar extinction coefficient and quantum yield, respectively. The absolute fluorescence value may be then expressed as

$$F = I_0 \cdot a_{395} \cdot Q \cdot c \tag{1}$$

where I_0 is the intensity of excitation radiation and c is the fluorophore concentration.

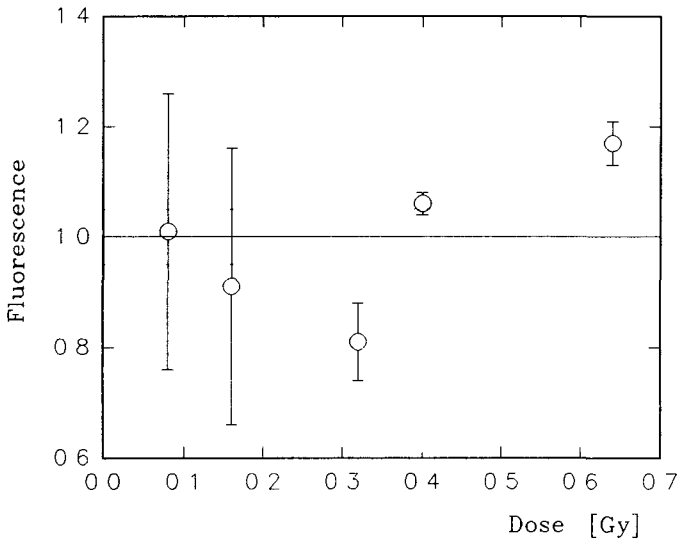

Figure 1. Changes of fluorescence intensity of ANS bound to membranes of lymphocytes after irradiation. The fluorescence is expressed in relative units (controls = 1.0) ($N = 5$).

Results

Irradiation dose-dependent changes of fluorescence intensity were expressed as mean values and the corresponding standard deviation for the individual doses and the value for controls (nonirradiated system) is represented by horizontal lines (means and S.D.) Fluorescence values are expressed as relative to control mean value (Fig. 1). For the irradiation doses used, the dose dependence of fluorescence is nonmonotonous and scattered. Fluorescence intensity, however, is mainly a measure of the probe environmental polarity. In a complex system such as intact

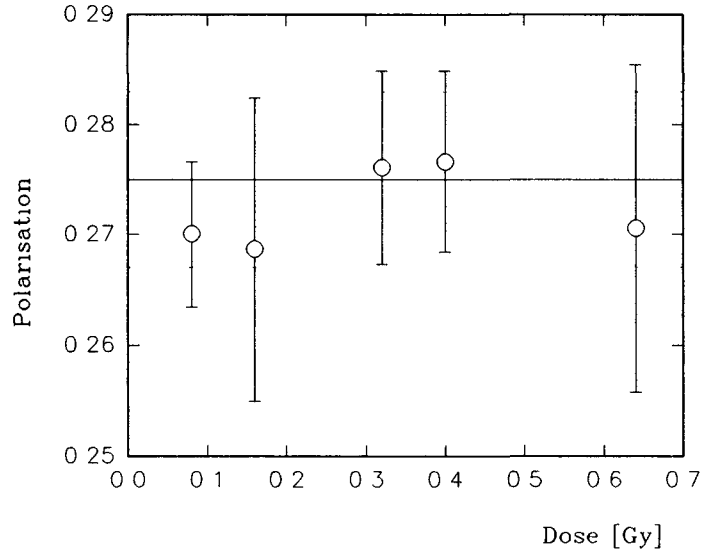

Figure 2. Changes of steady-state fluorescence emission polarization of ANS-lymphocyte membrane after irradiation. The solid line represents the mean value of polarization for control ± S.D. ($N = 5$).

living cell membrane, fluorescence intensity is influenced by a number of factors; an unambiguous interpretation of experimental data is thus difficult.

Fluorescence polarization as a measure of the probe molecule mobility can reveal changes in fluidity of the membrane core. Experimental values of steady-state fluorescence emission anisotropy after irradiation with low doses of neutron field are presented in Fig. 2. The values lie within the range of the mean ± S.D. for controls and thus suggest no significant changes in fluidity of the membrane core in the vicinity of the fluorophore group, for the dose interval studied. For further experiments the dose inducing maximum observed difference from controls was chosen.

A more detailed insight into the interaction of ANS with the cell membrane and into its irradiation-induced changes can be gained by studying the binding parameters of this system. The fluorescence quantum yield is significantly increased after the interaction of ANS with the binding site on the membrane. The overall fluorescence intensity is thus dependent on ANS binding level, and increases with the increasing ANS concentration in the system. The interaction of the fluorophore with the membrane can be described by the following model

$$F + M \overset{K}{\longleftrightarrow} FM \tag{2}$$

where K is the dissociation constant of the fluorophore F-membrane M complex. The changes of fluorescence intensity with the concentration of the fluorophore at $C_F \gg C_M$ can be described (Wang and Edelman, 1971) by the following equation

$$F = \frac{F_m \cdot C_F}{K + C_F} \tag{3}$$

where F_m is maximum fluorescence when ANS is bound to all accessible binding sites ($C_F - > \infty$), K is the dissociation constant of the ANS – membrane complex. The experimental values of fluorescence in dependence on the ANS concentration are illustrated in Fig. 3. The courses of the fitted curves clearly show saturation

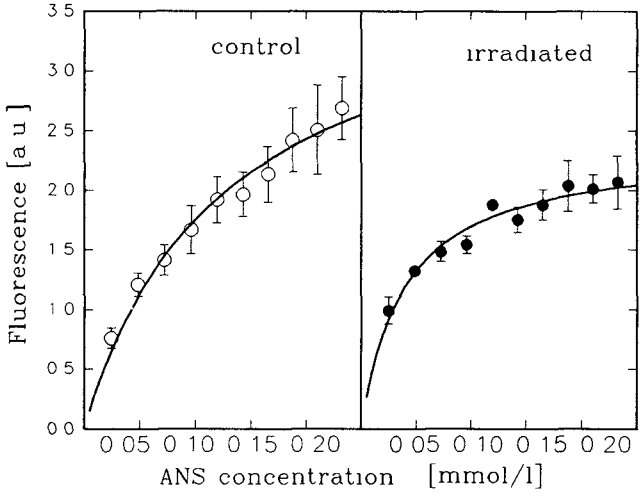

Figure 3. The dependence of ANS-lymphocyte membrane fluorescence on ANS concentration for nonirradiated system and after irradiation with 0.3 Gy from a ^{252}Cf neutron source.

relevant for the binding curves type described by expression [3]. The experimental values thus enable to calculate, employing the regression model of a rectangular hyperbole, the estimates of F_m and K for both the nonirradiated and the irradiated system. The values obtained are listed in Tab. 1. High values of the correlation coefficient confirm that the model can be used to fit the course of the experimental dependencies. The obtained values of the binding parameters F_m and K show a marked decrease after irradiation. This result seems to be controversial at first sight since a decrease of K suggests an increase of ANS interaction with the membrane binding sites, and F_m is determined, among others, by the number of molecules bound. Further analysis of binding requires a look at the stochiometry of the interaction.

To determine the stoichiometry of ANS binding to the lymphocyte membrane it is necessary to measure changes of fluorescence in dependence on the concentration of membranes (or cells) at a constant ANS concentration (Wang and Edelman, 1971). The binding isotherm can be expressed as

$$F = \frac{C_F \cdot q \cdot C_M}{K/n + C_M} \tag{4}$$

where C_M is the membrane concentration (or that of cells), q is the factor related to the quantum yield of the photoreaction, and n is the number of independent binding sites on the membrane.

The experimental values of fluorescence in dependence on the lymphocyte concentration are shown in Fig. 4. The courses of the fitted curves also clearly show saturation character relevant for the binding curves type described by expression (4).

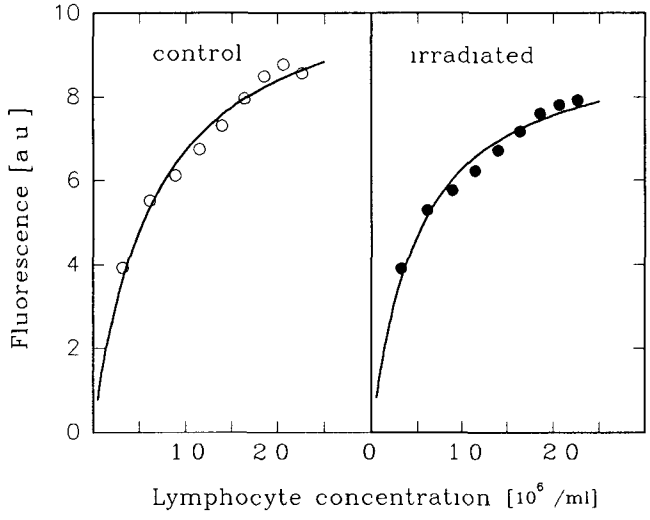

Figure 4. The dependence of ANS-lymphocyte membrane fluorescence on the concentration of lymphocytes for nonirradiated system and after irradiation with 0.3 Gy from a ^{252}Cf neutron source.

These dependencies and values of K obtained in previous experiments (Tab. 1) can be used to calculate, by the regression model of binding isotherm, the estimates of q and n for both nonirradiated and irradiated systems. The values of q substituted into equation [1] then give the quantum yield of fluorescence of a bound ANS molecule. The results are listed in Tab. 2.

Table 1. Parameters of ANS binding to lymphocyte membrane in control and irradiated systems.

Dose [Gy]	Correl. r	$F_m \pm$ S.D. [arb.unit]	K ± S.D. [mmol/l]
0	0.977	3.95 ± 0.28	0.125 ± 0.019
0.3	0.948	2.36 ± 0.09	0.039 ± 0.006

Table 2. Stoichiometry of ANS binding to lymphocyte membrane and quantum yield of fluorescence in control and irradiated systems.

Dose [Gy]	Correl. r	Q ± S.D.	n ± S.D. [mmol/l]
0	0.988	0.086 ± 0.003	0.184 ± 0.023
0.3	0.985	0.072 ± 0.003	0.075 ± 0.009

Also, in this experimental arrangement the high value of the correlation coefficient confirms that the model used is really appropriate. The marked decrease of both the binding site number and the quantum yield value is in agreement with the decrease of F_m and K values, and it may explain the above mentioned apparent discrepancy.

Discussion

The specific fluorescence probe 1-anilino-8-naphthalene sulphonate, ANS, is known to bind to polar - apolar interfaces, with the chromophoric group extending into the hydrocarbon core and the sulphonate group located in the plane of the membrane polar head groups (Slavík 1982). Therefore, spectral parameters of the bound probe could provide information about changes of its molecular environment caused by irradiation.

The interaction of 1-anilino-8-naphthalene sulphonate with the lymphocyte membrane after irradiation suggests the occurrence of significant alterations in the membrane structure as compared with nonirradiated cells.

Since the number of bound ANS molecules increases only with the increasing ANS concentration up to a certain saturation limit, specific binding sites are assumed to exist in membranes. In membranes composed of a mixture of different lipids, most ANS molecules are bound in lecithin – sphingomyelin pockets due to the strong interaction of ANS molecules with lecithin and sphingomyelin heads.

The value of polarization as a measure of mobility of the fluorophore group obtained in our experiments ($P = 0.275 \pm 0.008$ for controls) is in good agreement with values found for a mixture of lipids (0.25–0.3), and it confirms the expected predominatly lipid character of the binding sites. The number of ANS molecules bound to the membrane is also strongly influenced by the surface charge of the membrane. Quantum yield, on the other hand, is strongly influenced by the polarity of the environment but may also reflect other properties of the molecules surrounding ANS.

The value of quantum yield in our experiments lies within the range of values reported for various mixtures of lipids (0.015–0.2) (Slavík 1982). Also, the value of the free binding sites number is in good agreement with values found for erythrocyte membranes (0.15 mmol/10^6 cells) (Watala and Jozwiak 1989). The value of K, however, is markedly smaller as compared to that for erythrocytes, and it provides evidence for a stronger affinity of binding sites to ANS on the lymphocyte membrane.

The value of maximum fluorescence F_m for ANS – membrane after irradiation decreased by about 40% as compared to control value. This decrease closely corresponds with the postirradiation decrease of the dissociation constant of ANS – membrane complex, K, by about 70% and with the decrease of binding sites number, n, by about 60%. The number of accessible binding sites decreased after irradiation, the affinity of ANS molecules for the membrane however increased.

The number of protein binding sites for ANS in biological membranes is considerably smaller than the number of lipid binding sites, whereas their affinity is greater (the ratio of $ANS_{protein}$: ANS_{lipid} is 1 : 10 to 1 : 100) (Slavík 1982). Thus, as the ANS concentration increases the probe first binds to proteins and subsequently to lipids.

The changes of membrane lipid structure after low dose irradiation have already been studied in erythrocytes (Parasassi et al. 1991). Degradation of polyunsaturated fatty acids due to lipoxigenational processes were described also for lymphocyte membranes after irradiation (Kubasova et al. 1990). The free radical mediated chain reactions in particular play a considerable importance in processes of lipid structure damage (Niki et al. 1991) and thus influence their role in the fate of a cell (Horrobin 1991). Changes in structural arrangments of the membrane lipid bilayer, especially in the lipid polar head domain, may cause "burying" of the binding sites in the lipid domain of the membrane, and thus they may increase the proportion of ANS molecules bound in the protein domain. Although there are fewer binding sites in this domain, their affinity to ANS is higher (Slavík 1982). Our experimental findings concerning the binding parameters of ANS molecules and the lymphocyte membrane after irradiation support this interpretation. Also, the decrease of the quantum yield value by about 16% suggests an increased polarity of the binding site environment due to the access of H_2O molecules.

In conclusion, experimental evidence has been presented for alterations of lymphocyte membrane structure after irradiation with high LET neutron field in a low dose range The structural changes of the cell membrane, especially in the lipid domain, may significantly influence transport processes through the membrane Cells may be damaged rather than killed at these low dose ranges but membrane damage may be generally detrimental to the life of the cell Further studies are needed to elucidate the mechanism of membrane damage due to radical processes caused by ionizing radiation and/or other pollutants, and the role of such damage in cell killing

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