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PHYTOHAEMAGGLUTIN-INDUCED CHANGES IN SPIN LABEL REDUCTION IN LYMPHOCYTES FROM TUMOR-BEARING RATS

W.R. Hedrick, J.D. Zimbrick, and A. Mathew

Radiation Biophysics Program, University of Kansas

Lawrence, KS 66045

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Spin labeling techniques were utilized to investigate the rate of reduction of spin probes introduced into lymphocytes from normal and tumor-bearing animals. The response of the lymphocytes to phytohaemagglutin (PHA) stimulation was monitored by the spin labels Tempone (2,2,6,6-tetramethyl-4-oxo-piperidinooxy), PCA (2,2,5,5,-tetramethyl-1-pyrrolidinyl-oxy-3-carboxylic acid), and TMPN (2,2,6,6-tetramethyl-piperidinooxy). The EPR signal intensity of the nitroxide spin labels decreased according to first-order kinetics. For phytohaemagglutin challenged lymphocytes from tumor-bearing animals the Tempone signal loss was a factor of two less than the corresponding controls.

Free radical probes which are detectable by electron paramagnetic resonance (EPR) spectroscopy have been employed to determine the internal viscosity of barnacle muscles (1), yeast (2,3), and bacteria (4), red blood cells (5, 6), and lymphocytes (7). In addition, monitoring the kinetics of the decrease in EPR signal intensity from the spin labels can indicate the rate of reduction of the nitroxide moiety, which provides another parameter to characterize the environment of the spin probe.

The water-soluble spin label is generally incorporated into the cells via passive diffusion by incubating them in a solution containing the spin label. Unfortunately, freely diffusible spin labels can reside in the buffer surrounding the cells as well as in the protoplasm of the cells. The EPR signal originating from spin labels outside the cell is very similar in line shape and more intense than the intracellular signal and effectively masks the latter signal. The extracellular signal can be eliminated or, at least, broadened and greatly reduced in magnitude by the

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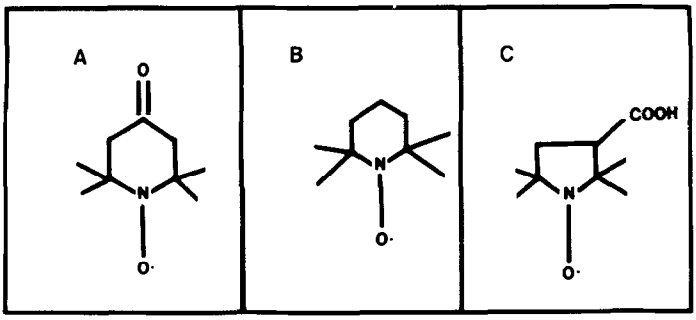

Figure 1. Structures of the spin labels. (A) Tempone. (B) TMPN. (C) PCA.

addition of nickel ions to the cell suspension (8), and thus the EPR signals from the respective environments can be differentiated.

In the present study, spin probe techniques were applied to lymphocytes isolated from Holtzman rats in which Walker 256 carcinosarcoma tumors were induced (9). The decay constants of the spin labels Tempone, TMPN, and PCA (shown in Figure 1) in lymphocytes from tumor-bearing animals were compared with those from control cells determine if their response to PHA stimulation depends on the presence or absence of tumors in the donor animals.

MATERIALS AND METHODS

Stock cultures of Walker 256 cells were maintained in 25 cm^2 tissue culture flasks with canted necks. The culture medium was McCoy 5A containing 10% calf serum with penicillin (200 IU/ml) and streptomycin (20 mg/ml). The flasks were incubated at 37°C in a humidified atmosphere maintained with 5% CO_2. Cells were harvested by treatment with a trypsin solution (0.05%) then resuspended in medium (4 x 10^6 cells/ml). Inocula of 2 x 10^6 cells were injected into the gastrocnemius muscles of adult female Holtzman rats (225-275 gm). Control animals received injections of medium only.

Spleens were dissected from animals in which tumors were palpable and then minced in a petri dish containing buffered saline at 0°C. The suspended cells were pipetted into a culture tube and centrifuged at 100 g for 10 min. The cells were washed once in a buffered saline before treating with NH_4Cl and Tris buffer solution to lyse the red blood cells (10). The lymphocytes were finally washed three times in buffered saline which contained 2mM calcium ion. A typical yield was 80 to 300 x 10^6 cells with greater than 90% viability determined by the exclusion of trypan blue stain. Control animals produced lower yields in the range of 50 to 150 x 10^6 cells.

The lymphocytes were divided into two or three fractions depending on the yield. Each fraction of 40 x 10^6 or more cells was suspended in 10 ml. of buffered saline. The suspensions were incubated at 37°C with 30 ug PHA per 10^6 cells for 60 minutes. After incubation with PHA the cells were

washed once and then treated with spin label. The control cell fraction was incubated with the spin label without being challenged with PHA.

After the cells were incubated for 15 minutes in buffered saline which contained 1 mM spin label, they were washed and resuspended in 150 ul of an isotonic solution of 1 mM spin label, 0.07 M $NiCl_2$ and Tris buffer (pH 7.4). The cell suspension was placed in a capillary tube (inside diameter 1.2 mm.) which was centrifuged at 100 g. for two minutes, so that a cell pellet was formed. The EPR spectrum was recorded as a function of time on a Varian Century E-109 spectrometer inferfaced with a Nicolet Instrument Corporation Model 1180 Data System. The EPR spectrum of the supernatant was also obtained. The temperature in the EPR cavity was regulated at 23°C by a Varian Variable Temperature Controller (Model E-4540).

RESULTS

The EPR signal intensity of the nitroxide spin labels decreased with time of exposure to the cells. Regression analysis indicated the loss of the signal followed first-order kinetics and thus, could be characterized by a decay constant. The decay constant for unstimulated lymphocytes from control animals were found to be 0.26 ± 0.12 min^{-1}, 0.16 ± 0.07 min^{-1}, and 0.01 ± 0.01 min^{-1} for Tempone, TMPN, and PCA, respectively. The biological reduction of the nitroxide moieties appears to be the major component of the decay.

A comparison of the decay constant obtained from lymphocytes for samples of varying cell number from the same animal demonstrated that the rate of decay was independent of the number of cells in the sample. However, fractions with higher cell content had increased signal intensity as would be expected.

TABLE 1

ANALYSIS OF THE DECAY CONSTANT MEASUREMENTS FOR TMPN IN LYMPHOCYTES USING THE STUENT-NEWMAN-KEULS TEST[a]

STIMULATION	SOURCE	RANK	DECAY CONSTANT (min^{-1}) (±S.D.)	NO.	DIFFERENCES 1	2	3	4
PHA	Walker 256	1	0.10 (±0.05)	15	--			
PHA	Control	2	0.21 (±0.10)	33	0.11[b]	--		
NONE	Control	3	0.26 (±0.12)	16	0.16[b]	0.02	--	
NONE	Walker 256	4	0.30 (±0.09)	17	0.20[b]	0.08	0.04	--

[a]The differences are computed as the mean of the row minus the mean of the column. Unmarked differences were tested and found to be not significant at P = 0.05.

[b]Tested and found to be significant at P = 0.05.

TABLE 2

ANALYSIS OF THE DECAY CONSTANT MEASUREMENTS FOR TMPN IN LYMPHOCYTES USING THE STUDENT-NEWMAN-KEULS TEST[a]

STIMULATION	SOURCE	RANK	DECAY CONSTANT (min^{-1}) (±S.D.)	NO.	DIFFERENCES 1	2	3	4
PHA	Walker 256	1	0.081 (±0.029)	15	--			
PHA	Control	2	0.107 (±0.024)	15	0.026[b]	--		
NONE	Walker 256	3	0.133 (±0.045)	15	0.052[b]	0.026[b]	--	
NONE	Control	4	0.156 (±0.066)	12	0.075[b]	0.049[b]	0.023	--

[a]The differences are computed as the mean of the row minus the mean of the column. Unmarked differences were tested and found to be not significant at P = 0.05.

[b]Tested and found to be significant at P = 0.05.

The means of the decay constant for Tempone as well as TMPN show statistically significnt differences between the stimulated and unstimulated groups (see Tables 1 and 2). Both Tempone and TMPN in PHA challenged lymphocytes from tumor animals consistently demonstrate markedly slower rates of decay compared with the unstimulated lymphocytes. Further, the mean of the decay constants for the tumor group is also statistically different (lower than) from that of similarly treated controls, while spin labels in the unstimulated lymphocytes from both types of animals exhibit no difference in the rate of signal loss.

The reduction of these rapidly decaying spin labels in lymphocytes was further characterized by pre-treating the cells with N-ethylmaleimide and by measuring the decay constant as a function of temperature. N-ethylmaleimide decreased the rate of decay by at least four-fold, indicating that sulfhydryl groups in the protoplasm make a substantial contribution to the loss of signal intensity. A 10°C decrease in temperature lowered the decay constant by a factor of 2.7 for Tempone and 3.8 for TMPN.

DISCUSSION

The spin probe, depending on the functional groups attached to the molecule, can reside in various environments. For example, the lipid-water partition coefficients for PCA, Tempone, and TMPN are, respectively,

0.05:1, 1.7:1 (2), and 5.3:1 (11) which are, as expected, in order of decreasing polarity. PCA partitions most favorably into aqueous regions, while Tempone and TMPN would sample not only aqueous regions but membrane structures as well.

The reduction of Tempone and TMPN in lymphocytes appears enzymatic and dependent on sulfhydryl groups. If these enzymes and sufhydryl-containing proteins are associated with membranes, then biochemical reduction of the nitroxide would more likely occur for these spin labels than the PCA, which displayed a relatively slow loss of EPR signal intensity. The partition coefficients of these respective probes indicate PCA is 35-100 times less likely to reside in lipid regions. Stier and Reitz (12) have shown that TMPN is a substrate for a membrane-bound enzyme system using NADPH as a reducing agent.

Many organic materials in biological systems have the potential to undergo free radical or oxidation-reduction reactions with the nitroxide moiety, which results in the loss of the probe's paramagnetic property (13). Kinetic analysis of the signal decay for the respective spin labels in lymphocytes yields decay constants which can be used to characterize the stimulated lymphocytes. The PHA challenged lymphocytes from subjects with malignant disease exhibit rates of EPR signal decay for Tempone and TMPN which are significantly less than those of the stimulated controls. Since the unstimulated lymphocytes from both normal and tumor animals show no difference in the decay constant, the effect of PHA on signal decay is dependent on the source of the lymphocytes. The implication is that the intracellular metabolism of lymphocytes from a tumor animal is selectively altered by exposure to PHA.

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