Vol. 80, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 30, 1978 Pages 451-457

MEMBRANE MICROVISCOSITY DIFFERENCES IN NORMAL AND LEUKAEMIC HUMAN LYMPHOCYTES

*Sheena M. Johnson and +Mary Kramers

*Division of Immunological Medicine
Clinical Research Centre
Watford Road
Harrow, U.K.

+MRC Leukaemia Unit
Royal Postgraduate Medical School
Hammersmith Hospital
Du Cane Road
London W12

Received December 1, 1977

SUMMARY A study of the fluorescence polarization and fluorescent lifetimes of 1,6 diphenyl hexatriene in human normal and leukaemic lymphocytes, lymphocyte plasma membranes and liposomes from the plasma membranes failed to reveal any fluidity differences which could be attributed to the leukaemic transformation. The plasma membranes were more viscous than the whole cells, and on average the liposomes were only 57% as viscous as the plasma membranes from which they were prepared. The average fluorescent lifetime of DPH in the liposomes was 7.9 nanoseconds as opposed to 9.7 in the plasma membrane. The polarization degree of DPH in the lymphocytes was much lower and more variable than that of DPH in platelets, polymorphonuclear leucocytes or erythrocyte membranes.

INTRODUCTION

Using the fluorescent probe 1,6 diphenyl 1,3,5-hexatriene (DPH) Inbar and Shinitzky and co-workers (1,2,3) have reported a marked increase in the fluidity of the plasma membrane lipid of malignant human lymphocyte cells compared to normal lymphocytes. They attribute this to a lower cholesterol/phospholipid ratio in the plasma membranes of malignant cells. We have studied normal human tonsil lymphocytes, circulating blood mononuclear cells, human chronic lymphocytic and acute lymphoblastic leukaemic cells and find that there is no evidence for an increase in the fluidity of the leukaemic cell plasma membranes compared to those of normal lymphocytes.

Polarisation values for intact platelets, granulocytes and erythrocyte

Abbreviation used is DPH, 1,6 diphenyl 1,3,5 hexatriene

0006-291X/78/0802-0451$01.00/0

ghosts were also measured. All were considerably higher than the values for lymphocytes and we attribute the discrepancy between Inbar and Shinitzky's findings and our own principally to the presence of platelets in their heparinised blood samples.

Recent work has shown that the probe is not restricted to the plasma membrane but is distributed uniformly among the phospholipids of the lymphocytes (5). Plasma membranes were therefore isolated and liposomes prepared from them. The fluidities of both were studied but again no differences attributable to the leukaemic cell transformation could be discovered.

MATERIALS AND METHODS

Cells: Tonsil lymphocytes were obtained from enlarged but not inflamed tonsils freshly removed at operation from children and young adults by the method described in reference (5). The presence of monocytes was detected by incubating about 2 x 10^5 cells for 1 hour at 37° in 0.1 ml of a solution containing RPMI 1640 buffer at pH 7.2 with Hepes (Gibco-Biocult), 10% inactivated foetal calf serum and 0.0005% w/v neutral red. Cells which had ingested the dye showed scarlet patches and were counted as monocytes. Polymorphonuclear leucocytes were identified by the shape of their nuclei on staining with 0.01% crystal violet in 2% acetic acid. In the tonsil cell preparations polymorphonuclear leucocytes and monocytes were <1% and the preparations consisted of >90% viable lymphocytes, as judged by trypan blue exclusion. Circulating blood lymphocytes were obtained from two sources. 20 ml of blood from healthy adult donors was defibrinated to remove platelets, then layered on a Ficoll-Triosil gradient as described by Boyum (6) to remove polymorphonuclear leucocytes and erythrocytes. After centrifuging at 1000g for 25 minutes the cells at the interface were collected and washed free of Ficoll-Triosil by centrifugation in Dulbecco's phosphate buffered saline, without calcium or magnesium (7). Monocytes and any contaminating polymorphonuclear leucocytes were estimated as described previously. Cell viability was >90% but the preparations contained 0-10% polymorphonuclear leucocytes and 5-30% monocytes. If membranes were required, 'buffy coat cells' were obtained from the blood bank. These consisted of the lymphocyte rich fraction of the blood of 5 donors which had been centrifuged to obtain the platelet rich plasma. The buffy coat cell residues in acid citrate dextrose buffer were defibrinated by adding 0.05g $CaCl_2$ and 0.05g $MgCl_2$ $6H_2O$ in 2.5 ml H_2O per 100 ml cells and stirring gently until the blood clot formed and retracted. The very thick cell suspension was then diluted with an equal volume of Dextran 110 in 0.9% sodium chloride and the red cells allowed to settle for 1 hour at room temperature. The supernatant was centrifuged as described previously, and the cells resuspended in phosphate buffered saline, pH 7.2. They were then centrifuged on a Ficoll-Triosil gradient and treated as described for the circulating blood samples. In this case there was virtually no polymorphonuclear leucocyte contamination, but monocytes were about 20%. 20 ml blood samples from patients with chronic lymphocytic leukaemia and acute lymphoblastic leukaemia were treated as described for the blood samples from normal individuals.

Abbreviated used is RPMI, Roswell Park Memorial Institute.

T cell separation: T cells were separated from B cells by rosetting with neuraminidase treated sheep erythrocytes, essentially as described by Janossy et al (9,10).

Polymorphonuclear leucocytes: Blood from normal donors was defibrinated and diluted 1:1 with Dextran in 0.9% saline. The red cells were allowed to settle for 1 hour at room temperature, then the supernatant was layered onto Ficoll-Triosil as described previously. The pellet was retained and washed and erythrocytes removed by ammonium chloride lysis as described previously. After washing, the polymorphonuclear leucocytes were suspended in phosphate buffered saline.

Platelets: Buffy coat cells from the blood bank were not defibrinated, but diluted with an equal volume of Dulbecco's phosphate buffered saline. They were centrifuged at 300g and the white cells collected from the top of the pellet. These were layered on the Ficoll-Triosil gradient and the cells at the interface collected. These were diluted with phosphate buffered saline and centrifuged at 200g for 10 minutes to remove the lymphocytes. The supernatant was then centrifuged at 1000g for 15 minutes. The pellet was washed once in phosphate buffered saline.

Erythrocyte ghosts: Erythrocytes were obtained from normal venous blood. The cells were collected from the bottom of a Ficoll-Triosil gradient and, after washing and labelling, were lysed by suspending in 10 mM phosphate buffer, pH 7.2 at 0°. They were centrifuged once at 300g for 15 minutes to remove polymorphonuclear leucocytes, then the supernatant was centrifuged at 20,000g for 30 minutes at $4^{\circ}C$. The pellet was washed with buffer at $4^{\circ}C$ until it and the supernatant were colourless.

Plasma membrane preparation: The plasma membranes of the lymphocytes were isolated as described in reference (5). Their lipid composition, enzyme characterization and purity are more extensively discussed in Johnson and Robinson (manuscript in preparation). No difference was found between the cholesterol/phospholipid ratio in the plasma membranes of the tonsil and leukaemic lymphocytes.

Preparation of liposomes: The plasma membrane suspensions in tris chloride buffered saline was extracted with 2:1 v/v chloroform methanol as described by Folch et al (12). The organic phase was evaporated to dryness under reduced pressure in a 100 ml round bottomed flask and liposomes prepared by mechanically shaking the lipid extract in tris buffered saline as described by Papahadjopoulos and Watkins (14) except that the glass spheres were omitted.

Microviscosity measurements

Cell labelling: The cell suspensions were labelled by incubating with a colloidal solution of 2×10^{6}M, 1,6 diphenyl 1,3,5 hexatriene in phosphate or tris buffered saline for 30 minutes at $37^{\circ}C$. Cells were centrifuged at 200g for 10 minutes, then resuspended in phosphate or tris buffered saline without diphenyl hexatriene. All cells were labelled whole and alive and no further DPH was added to the membrane preparations or liposomes.

Polarisation values: Polarisation values were read at $37.0 \pm 0.3^{\circ}C$ using an Elscint MV-1 microviscosimeter. To correct for depolarisation due to light scattering polarisation values were plotted against the optical density of the suspension at 450 nm and the extrapolated value of the polarisation for zero optical density was taken, reference (5). As reported by Inbar and Shinitzky (1) the light scattering depolarisation for whole cells was very small, but it was considerably greater for the much smaller plasma membrane fragments and liposomes.

Fluorescent lifetime measurements: The fluorescent lifetime of the probe was measured directly at 37 ± 0.5°C using an applied photophysics nanosecond spectrometer and Ortec electronics. When deconvoluted, the fluorescent decay was exponential within the limits of experimental error.

RESULTS AND DISCUSSION

Polarisation values for cells, plasma membranes and liposomes are shown in Table 1. From this table it can be seen that lymphocytes have the lowest and most variable polarisation values of all the blood cells. The standard deviations for the blood bank lymphocytes and monocytes are smaller because each value is the mean of pooled lymphocytes from 5 or 10 donors. At the 95% confidence level the DPH polarisation results for the normal tonsil and acute lymphoblastic leukaemic cells shown in Table 1 do not differ significantly. However, both are just significantly less that the chronic lymphocytic leukaemia cells which are again just significantly less than the circulating mononuclear cells, a result which seems impossible to correlate with normal to leukaemic cell transformation. Normal circulating heparinised ficoll separated mononuclear cells containing platelets had a DPH polarisation value of 0.251 ± 0.003 in good agreement with the value for 'normal lymphocytes' quoted by Inbar and Shinitzky. However, this figure became less as the lymphocytes were successively freed from contaminating cells. If the blood was defibrinated to remove platelets the mononuclear cells (lymphocytes and monocytes) from five individual donors had a polarisation value of 0.237 ± 0.006, similar to cells obtained from the blood bank. Purified T cells had a still lower polarisation value of 0.224, identical to that of the chronic lymphocytic leukaemic cells and not significantly different from the value of 0.218 of the tonsil T cells. Since the proportion of lymphocytes in the blood is markedly raised in chronic lymphocytic leukaemia, the effect of platelets and monocytes on the DPH polarisation of heparinised leukaemic blood is relatively less merely because the lymphocyte preparation is purer. We believe that this was the cause of the differences noted in references (1,2). The just significant differences between the chronic lymphocytic leukaemia cells and

Table 1. DPH Membrane Polarisation Values at 37°

cell	whole cell	plasma membrane	plasma membrane liposomes
Normal cells:			
Circulating lymphocytes + platelets + monocytes	0.251±0.003 (3)	ND	ND
Circulating lymphocytes + 20% monocytes	0.234±0.002 (6)	0.257±0.003(3)	0.229±0.010(3)
Circulating T lymphocytes	0.224±0.010 (4)	ND	ND
Tonsil lymphocytes	0.214±0.011(19)	0.244±0.009(7)	0.214±0.012(6)
Tonsil T lymphocytes	0.218±0.006 (5)	ND	ND
Polymorphonuclear leucocytes	0.261±0.004 (5)	ND	ND
Platelets	0.266±0.003 (4)	ND	ND
Erythrocytes	ND	0.288±0.004(6)	ND
Pathological cells:			
Chronic lymphocytic leukaemia	0.224±0.008(17)	0.248±0.013(7)	0.205±0.010(5)
Acute lymphoblastic leukaemia	0.212±0.009 (3)	0.234±0.010(3)	0.190±0.001(2)
Lymphosarcoma	0.227±0.007 (2)	0.236±0.009(2)	0.216 (1)

the tonsil lymphocytes may be due to the small proportion of monocytes still present in the leukaemic cells or to rather more blast cells in the tonsil preparation. Blast cells would be expected to be more fluid as they have a lower ratio of external/internal membranes than small lymphocytes, and from the Table it will be seen that the external plasma membranes have considerably higher polarisation values than the average value for all the membranes in the whole cell. This is in agreement with Shinitzky and Inbar's observations on the effect of cholesterol on liposome and cell fluidity (3).

Table 2 shows the lifetimes and microviscosities of the lymphocytes, plasma membranes and plasma membrane lipid extracts. The lifetimes of DPH are larger by an average of 1.8 nanoseconds in the plasma membranes compared to the liposomes made from them, showing the dependence of DPH lifetime on

Table 2. DPH fluorescent lifetimes, τ and lymphocyte membrane microviscosities, η at 37°

	Whole Cell		Plasma Membrane		Plasma Membrane Liposome	
	τ nano-seconds	η poise	τ nano-seconds	η poise	τ nano-seconds	η poise
Tonsil lymphocytes	8.7	1.7	9.6	2.5	7.6	1.4
Chronic lymphocytic leukaemic lymphocytes	8.6	1.8	10.0	2.7	8.0	1.4
Circulating lymphocytes + 20% monocytes	9.0	2.1	9.5	2.8	8.2	1.8

membrane environment. Microviscosities were calculated as described by Shinitzky and Barenholz (13) and Shinitzky and Inbar (14) except that directly measured fluorescent lifetimes were used. The validity of this procedure is discussed in reference (15).

From Table 2 it will be seen that any differences in the microviscosities between the three lymphocyte preparations are very small, and do not suggest that leukaemic cells are more fluid than normal lymphocytes. As might be expected, the plasma membranes are more rigid than the whole cells because their cholesterol/phospholipid ratio is higher. The plasma membrane liposomes are more fluid than the unextracted membranes by almost a factor of 2. It is not known whether this change is due only to the loss of the membrane proteins, or whether it reflects a marked anisotropy in the lipids of the plasma membrane which is lost when they are randomised in the liposomes. Incubation with cytochalasin B or colchicine produced no polarisation change in the cells, suggesting that microfilaments and microtubules are not involved in membrane microviscosity when measured with DPH.

ACKNOWLEDGEMENTS The authors would like to thank Martin Buckland for the measurement of the fluorescent lifetimes and Dr Godfrey Beddard for his help in the convolution calculations. Ray Robinson gave valuable technical assistance.

REFERENCES

1. Inbar, M., Shinitzky, M. and Sachs, L. (1974) Febs.Lett. 38, 268-270.
2. Inbar, M. and Shinitzky, M. (1974) Proc.Nat.Acad.Sci.USA. 71, 4229-4231.
3. Shinitzky, M. and Inbar, M. (1976) Biochim.Biophys.Acta. 433, 133-149.
4. Pagano, R.E., Ozato, K. and Ruysschaert, J.-M. (1977) Biochim.Biophys. Acta. 465, 661-666.
5. Johnson, S.M. and Nicolau, C. (1977) Biochem.Biophys.Res.Commun. 76, 869-874.
6. Boyum, A. (1968) Scand.J.Clin.Lab.Invest. 21, Suppl. 97, 77-89.
7. Dulbecco, R. and Vogt, M. (1954) J.exp.Med. 99, 167-182.
8. Boyle, W. (1968) Transplantation 6, 761-764.
9. Janossy, G., Gomez de la Concha, E., Waxdal, M.J., Platts-Mills, T.A.E. (1976) Clin.exp.Immunol. 26, 108-117.
10. Janossy, G., Gomez de la Concha, E., Luquetti, A., Snajdr, M.J., Waxdal, M.J. and Platts-Mills, T.A.E. (1977) Scand.J.Immunol. 6, 109-123.
11. Folch, J., Lees, M. and Sloane Stanley, G.M. (1956) J.Biol.Chem. 226, 497-509.
12. Papahadjopoulos, D. and Watkins, J.C. (1967) Biochim.Biophys.Acta. 135, 639-652.
13. Shinitzky, M. and Barenholz, Y. (1974) J.Biol.Chem. 249, 2652-2657.
14. Shinitzky, M. and Inbar, M. (1974) J.Mol.Biol. 85, 603-615.
15. Nicolau, C., Hildenbrand, K. and Johnson, S.M. (1978) in Virus Transformed Cell Membranes (Nicolau, C., ed.), Academic Press (in press).