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CHANGES IN MEMBRANE FLUIDITY ASSOCIATED WITH LYMPHOCYTE STIMULATION BY SUCCINYL-CONCANAVALIN A

S.N. CHERENKEVICH [a], J.M. VANDERKOOI [b,*] and C. DEUTSCH [c]

[a] *Department of Biophysics, Byelorussian State University, Minsk (U.S.S.R.) and* [b] *Department of Biochemistry and Biophysics,* [c] *Department of Physiology, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104 (U.S.A.)*

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Time-resolved fluorescence anisotropy studies of human peripheral blood lymphocytes labeled with 1,6-diphenyl-1,3,5-hexatriene were carried out at temperatures between 4 and 38°C. For unstimulated freshly-isolated lymphocytes the calculated order parameters were found to be 0.62 at 4°C and 0.44 at 37°C. Mitogen-induced alterations in the order parameter were evident within minutes after addition of succinyl-concanavalin A to the cells, increasing to a value of 0.56 after 2 h at 37°C. Both stimulated and unstimulated cells show a decrease in fluorescence anisotropy over the next 2–3 days of culture and after the third day the time-resolved fluorescence anisotropy decay profiles of the two populations of cells were indistinguishable. Our results indicate that there are both short- and long-term changes in the membranes of the cell upon stimulation by mitogen.

Introduction

Resting (G_0) human peripheral blood lymphocytes may be stimulated to undergo proliferation in vitro with a variety of plant lectins [1]. Within minutes after mitogen addition increased fluxes of K, Na, and Ca occur [2], and within the first few hours there is receptor aggregation and removal. Other initial events include increased transport of sugars, nucleosides, and elevation in the levels of cellular cyclic nucleotides [1,3]. The mitogens need not enter the lymphocyte in order to initiate proliferation [4] and therefore, the critical early changes may indeed be membrane events.

Our studies are an attempt to obtain information regarding the role of the lymphocyte membrane lipids in mitogen-induced alterations in the cell metabolism and function. 1,6-Diphenyl-1,3,5-hexatriene was used as a probe of the hydrophobic region of the membrane. This probe, developed by Shinitzky and Inbar [5] has two features which make it a useful tool to study membranes. The long axis of the molecule and the absorption and emission dipole coincide, thereby simplifying analysis of anisotropy [6–8]. In addition, the motion of diphenylhexatriene is very sensitive to its lipid milieu, since it preferentially aligns parallel to the fatty acid side chains of the phospholipids [9]. Succinyl-concanavalin A was used in these studies because it produces little or no agglutination or cap formation [10]. Therefore, any effect of mitogen on diphenylhexatriene anisotropy can be interpreted without regard to any such effects.

Methods and Materials

Chemicals. Hank's balanced salt solution, (Ca^{2+}, Mg^{2+})-free, was obtained from Grand Island Biological Company. Modified Eagle medium with Earle's salts was made up from its individual

* To whom correspondence should be addressed.

components. Normal, unsubstituted Earle's medium contained 116 mM NaCl, 5.2 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 1 mM NaH_2PO_4, 6 mM glucose and 26 mM $NaHCO_3$. Hypaque (Sodium salt, 50% solution) was obtained from Winthrop Laboratories and succinyl-concanavalin A was obtained from Polysciences. [^{3}H]Thymidine was obtained from New England Nuclear Co. Sigma Chemical Co. provided Ficoll and diphenylhexatriene.

Lymphocyte Preparation. Heparinized human venous blood was collected from healthy adults. The lymphocytes were separated by gradient centrifugation using a modified Ficoll-Hypaque technique as previously described [11]. The resulting lymphocyte preparation generally contained less than 10–20% monocytes, as determined by the nonspecific esterase stain [12]. Viability of freshly prepared lymphocytes, as tested by Trypan blue exclusion, was greater than 98%.

Cultures were prepared by adding $5 \cdot 10^5$ cells to a 1-ml solution containing minimum Eagle's medium plus 10% of either pooled human AB serum or fetal calf serum, 1% L-glutamine and 1% penicillin-streptomycin. These were incubated in 5% CO_2 in humidified air at 37°C. Each day, 30 1-ml control cultures and 30 1-ml stimulated cultures (50 μg succinyl-concanavalin A per ml) were collected separately, centrifuged at $600 \times g$ for 3 min, and resuspended in (Ca^{2+}, Mg^{2+})-free Hank's balanced salt solution to final concentrations of $(2–8) \cdot 10^6$ cells/cm^3. This cell suspension was then used for the preparation of the samples for the fluorescence time-resolved anisotropy.

DNA synthesis in the cell cultures in the absence and presence of succinyl-concanavalin A (50 μg/ml) was assayed by addition of 0.25 μCi of [^{3}H]thymidine 8 h before the cultures were collected, and washed using a Cell Harvester (Model M12 from Brandel). The incorporation of [^{3}H]thymidine was determined using scintillation counting.

Fluorescence anisotropy measurements. The instrument used to measure fluorescence anisotropy was a single-photon-counting (Ortec, Inc.) nanosecond lifetime instrument under computer control. An air spark-gap was used for excitation. A 344 nm UV interference filter was used to isolate the exciting wavelengths, and a Schott GG 420 filter used for emission. Fluorescence anisotropy, $r(t)$ is defined as

$$r(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)} \tag{1}$$

where $I_{\parallel}$ is the intensity of the vertical component and $I_{\perp}$ is the intensity of the horizontal component of the emitted light. The denominator is proportional to the total fluorescence emitted and is therefore independent of anisotropy.

The fluorescence anisotropy, r, of a chromophore solubilized in systems with anisotropic ordering is a complex function containing a time-dependent part, $r(t)$, and a time-independent part, r. Nordio and Bresolin [13] and Luckhurst et al. [14] predicted that the time-dependent part of $r(t)$ is composed of an infinite number of exponentials. However, in our study, following Kawato et al. [7] and Lakowicz et al. [8] we have used the phenomenological approach that $r(t)$ at short times (in the nanosecond range) can be fit by a single-exponential curve. This approach gives useful information about the rotational motion of the fluorescence probe in anisotropic systems such as liposomes and intact cell membranes [15–17]. In this case, $r(t)$ can be written as:

$$r(t) = (r_0 - r_\infty)e^{-t/\phi} + r_\infty \tag{2}$$

where ϕ characterizes the relaxation time of the fluorescence probe that undergoes anisotropic rotational motion. The value of r_0 in this equation refers to the anisotropy of the probe with fully restricted motion; r_∞ is the value of anisotropy at long times after the flash. The ratio r_∞/r_0 was shown to be related to the order parameter S_v of a chromophore solubilized in a spherical aggregate of an isotropic solution [7,15,16,18]. In the case of diphenylhexatriene, for which the absorption and emission transition dipole moments coincide with the long axis of the molecule, the following equation can be written [7] for the order parameter, S_v:

$$\frac{r}{r_o} = S_v^2 \tag{3}$$

We have used the order parameter, S_v, to characterize the lipid order in cultured lymphocytes

which have been stimulated with succinyl-concanavalin A. The value of r_∞ was determined from $r(t)$. Estimates of the limiting anisotropy, r_0, given in the literature are divergent, but close to the theoretical value of 0.4 [6–8]. Therefore we used the value of $r_0 = 0.4$ in this work.

Labelling the cells with diphenylhexatriene. Diphenylhexatriene (Sigma Chemical Co.) was solubilized in tetrahydrofuran at 10^{-3} M and then diluted 500-fold in modified Hank's balanced salt solution, Ca^{2+}-free, followed by vigorous stirring for 1 h at 20 C. Longer incubation times, up to 2 h, had no effect on the value of anisotropy.

The final concentration of diphenylhexatriene in cell suspensions ($5 \cdot 10^5$ cells/ml) was $5 \cdot 10^{-7}$ M. Under these conditions, the effect of light scattering on the observed $r(t)$ values was negligible. No change in the $r(t)$ profile could be detected during the time needed to accumulate a decay curve (around 3 min). After selected experiments viability of the cells was measured by Trypan blue experiments and was greater than 90%.

Results

The fluorescence decay of diphenylhexatriene in human peripheral blood lymphocytes is composed of more than one component as illustrated by the time-dependence of the total fluorescence intensity ($I_\parallel + 2I_\perp$) at 7°C (Fig. 1). In this case the decay curve was fit by two exponentials with lifetimes of 3 and 10 ns with 30 and 70 percent contribution, respectively. The decay profile is typical for that found in biological and synthetic membranes, and is a consequence of heterogeneity of sites and/or excited-state reactions [9,19,20].

There is a significant difference in the decay profiles for the parallel and perpendicular components of light as shown in the decay profile of the difference emission ($I_\parallel - I_\perp$) (Fig. 1). Using Eqn. 1, we calculated fluorescence anisotropy, $r(t)$; typical anisotropy profiles are shown in Fig. 2 for two temperatures, 7 and 35°C. The diphenylhexatriene fluorescence anisotropy decayed rapidly within the first 15–20 ns after excitation to a constant value, r_∞. The non-zero value of r_∞, which indicates an apparent infinite rotational relaxation time, is a function of the degree of restriction on molecular rotational motion of the diphenylhexatriene imposed by the membrane interior. The value of r_∞ decreased from 0.14 to 0.08 as the temperature was increased from 7 to 35°C, indicating increased disorder. Using Eqn. 2, the order parameter is calculated to be 0.55 and 0.31, respectively, for these temperatures. The addition of succinyl-concanavalin A alters the anisotropy decay profile at 7 and 35°C by changing both r_∞ and the rate of decay of $r(t)$. Fig. 2 shows that at 7°C the mitogen decreased the rate of decay of anisotropy such that $r(t)$ reached a plateau about 10–12 ns later than in untreated cells. In addition, the values of r_∞ increased to 0.17 and 0.11 at 7 and 35°C, respectively, upon stimulation.

In order to correlate the change in the membrane structure with other physiological changes

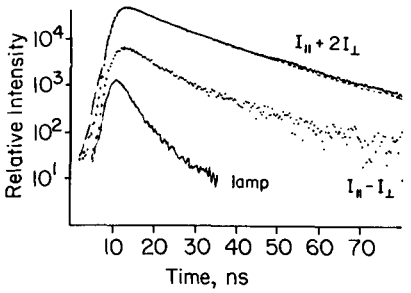

Fig. 1. Fluorescence decay profile of diphenylhexatriene in human lymphocytes. Total fluorescence decay profile ($I_\parallel + 2I_\perp$), difference ($I_\parallel - I_\perp$) and lamp function are indicated on the figure. Conditions are given in Materials and Methods. Temperature: 7°C. Dotted line represents the computer best fit to $I_\parallel + 2I_\perp$ curve with a double exponential decay function with lifetimes of 3 and 10 ns.

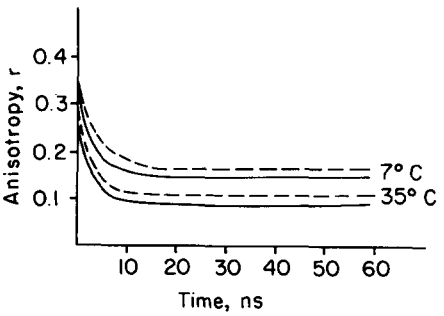

Fig. 2. Fluorescence anisotropy profile of diphenylhexatriene in human lymphocytes. The conditions are given in Materials and Methods and the temperatures are indicated on the figure. ————, no additions; — — —, 50 μg succinyl-concanavalin A per ml; Measurements were made 20 min after addition.

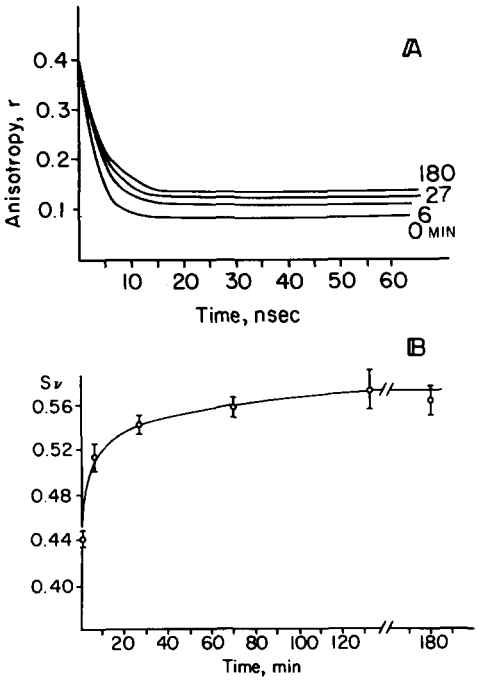

Fig. 3. Anisotropy decay profiles and order parameter of diphenylhexatriene in human lymphocytes after addition of succinyl-concanavalin A. Temperature: 37°C. (A) Decay profiles after addition of 50 μg succinyl-concanavalin A per ml. Each decay curve was obtained at the time (in min) specified in the figure. (B) Order parameter as a function of time after addition of succinyl-concanavalin A.

induced by succinyl-concanavalin A, the time-dependence of the response was examined. The effect of mitogen on diphenylhexatriene anisotropy occurred within minutes of mitogen addition, as shown in Fig 3. The order parameter, S_v, was calculated from the decay curves and is plotted as a function of incubation time with mitogen (Fig. 3b). It can be seen that the change was complete within 20–30 min after succinyl-concanavalin A addition.

Because the lipid order parameter, S_v, increases during the process of the succinyl-concanavalin A stimulation of lymphocytes, it was of interest to see whether S_v is also a function of culture time; we therefore determined the value of S_v throughout a 4-day culture period for untreated and stimulated lymphocytes (Fig. 4). During the first two days of cultivation the order parameter for diphenylhexatriene in lymphocytes decreased both

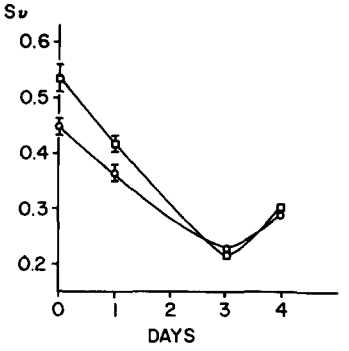

Fig. 4. Change in the order parameter, S_v, of diphenylhexatriene in lymphocytes as a function of cell cycle. Conditions are given in Materials and Methods. Temperature: 37°C. ○, no addition; □, 50 μg succinyl-concanavalin A per ml.

for control cells and stimulated cells. By the time of the third and fourth days of growth the values of S_v were the same for control and stimulated cells (Fig 4). In order to show that succinyl-concanavalin A stimulated the cells under culture conditions, DNA synthesis was assayed; the cells which were treated with concanavalin A showed

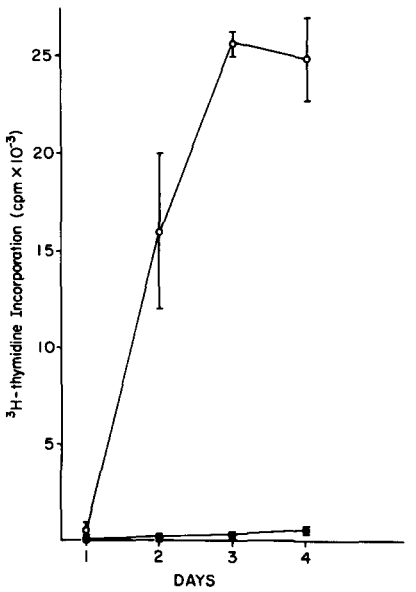

Fig. 5. [^{3}H]Thymidine incorporation in succinyl-concanavalin A-treated lymphocytes. Cells were cultured at $5 \cdot 10^5$ cells/ml in minimum essential medium in the presence (○) and absence (●) of succinyl-concanavalin A (50 μg/ml). 10% mixed human serum was used in all cultures and triplicate cultures were used for each data point.

enhanced incorporation of [^{3}H]thymidine (Fig. 5), indicating that stimulation had indeed occurred.

The values of S_v for diphenylhexatriene in lymphocytes were derived from the temperature dependence of $r(t)$ in the range of 4–40°C (Fig. 6). The dependence of S_v upon the temperature is a complex function for all conditions tested. For freshly prepared unstimulated lymphocytes the temperature profile is biphasic; at temperatures below 15°C the order parameter is high, 0.5 to 0.6, indicating restricted motion for diphenylhexatriene, while the low value for the order parameter above 32°C indicates that the membrane imposes little constraint on the rotation of diphenylhexatriene, and that diphenylhexatriene no longer has a preferential orientation in the membrane. The change in slope of the temperature profile in the range of 15–40°C is similar to that observed in lipid dispersions undergoing 'phase transition' in which the sharp transition temperature is broadened by the presence of a mixture of phospholipids or by the addition of protein [15,16]. The temperature profile of S_v for diphenylhexatriene in 4-day cultured cells was also complex, and there appeared to be a broadening of the temperature range over which the slope changes occur. The value of S_v increased after succinyl-concanavalin A addition to freshly isolated cells and to the cells cultured for four days over the entire temperature range.

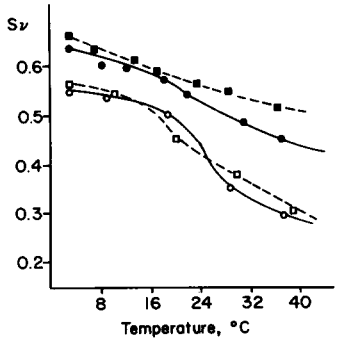

Fig. 6. The temperature dependence of the order parameter for diphenylhexatriene in lymphocytes. Conditions are given in Materials and Methods. Open and closed symbols refer to day zero and day four of cultivation, respectively. ○, ●, no additions; □, ■, 50 μg succinyl-concanavalin A per ml.

Discussion

Peripheral blood lymphocytes are a cell population that is uniformly in the resting state and that can be activated by the addition of a mitogen such as concanavalin A. This synchrony provides an opportunity to monitor the immediate metabolic and morphologic changes that accompany activation, and the longer-lasting changes occurring over subsequent days of culture. The former category includes increased fluxes of ions and small molecules and patching/capping, while the latter includes blast formation, increased RNA synthesis and DNA synthesis.

The cell membrane is implicated in the process of stimulation by mitogen. First, it contains the receptors for mitogen. Second, the alterations in transport properties appear to be a primary effect of the lymphocyte stimulant, since increased rates of entry of small molecules have been demonstrated within minutes of the addition of the stimulant and often occur independently of protein and RNA synthesis.

In this regard, it is especially interesting to observe that after succinyl-concanavalin A was added, the order parameter for diphenylhexatriene anisotropy increased (from 0.44 to 0.56), indicating that the motion of diphenylhexatriene became more restricted (Fig. 3). More than 50 percent of the change occurred within 6 min after concanavalin A addition; therefore the changes in the order parameter correlate with the observed rapid mitogen-induced changes in transport and metabolism.

The significance of the magnitude of the change in order parameter upon succinyl-concanavalin A addition is hard to evaluate. The fluorescence decay of diphenylhexatriene is nonsingle-exponential, a reflection of the heterogeneity of diphenylhexatriene location. Since the plasma membrane is less than 20 percent of the total membranes in lymphocytes [21], the changes in the diphenylhexatriene order parameter are dramatic indeed and could be indicative of generalized membrane alterations. The functional consequence of mitogen stimulation might arise from changes in membrane phospholipid metabolism. While the relative amounts of the different types of phospholipids in membranes appears to be unaffected by mitogen,

the incorporation of a wide variety of radioisotopes into the lipids is affected. Mitogen treatment causes increased rates of incorporation of glucose, glycerol, and choline [22], the methyl group of methionine [23], inositol [24], oleate [25] and of glucosamine into membrane glycolipids [26]. In some cases this enhanced uptake is evident within the first hour. Another early event in mitogen stimulation is apparent in the cytoskeleton [27–29]. Membrane proteins appear to be attached to the actin-like fibers of the cytoskeleton. Thus, modification of cell surface proteins is certain to be critically involved in the mitogen-induced cytoskeletal alterations [30]. The plasma membrane is composed of about 50% protein by weight. If, then, the proteins undergo structural rearrangement upon stimulation, the membrane lipid bilayer structure may be secondarily affected: such a secondary effect would be manifest in gross changes in membrane structure which occur upon stimulation.

In addition to the early changes in the order parameter upon succinyl-concanavalin A stimulation, additional changes were observed during the cell cycle. The most marked differences in order parameter occurred within the first 48 h after stimulation, during which time there was little or no DNA synthesis. At day 3 of culture DNA synthesis was maximal and the order parameter reached its lowest value; at this time there was no difference in the order parameter between control and stimulated cells. Collard et al. [31] have observed that two days after stimulation of human lymphocytes with concanavalin A, there is a decrease in membrane fluidity indicated by diphenylhexatriene fluorescence anisotropy. Their results are, then, in general agreement with ours. On the other hand, in leukemic 1210 cells, membrane fluidity appeared to be constant during the cell cycle [32]; however, whether these observations apply to non-neoplastic lymphocytes is not known. Two reports are particularly relevant to our studies: (1) an electron paramagnetic resonance study of spin-labelled human lymphocytes stimulated with phytohemagglutinin-P [33] and (2) a diphenylhexatriene-fluorescence polarization study of human lymphocytes stimulated with *Wistaria floribunda* mitogen [34]. The first work showed a transient increase in the order parameter within the initial 30 min of mitogen exposure, followed by return to the control value within the next 30 min. These studies were not carried out beyond the first hour of stimulation. Since both the probe and the mitogen used in the spin-label studies were different from those used in our studies it is not clear whether this transient change in the value of the order parameter can be compared with our studies. In particular, phytohemagglutinin-P and succinyl-concanavalin A are markedly different in terms of the induced changes in cell surface morphology and agglutination. The study by Toyoshima and Osawa [34] reports a transient change in the calculated microviscosity within the first 30 min of exposure to the mitogen, *W. floribunda* mitogen, and a rapid return to control levels within the next 30 min. However, this work also indicated that there was a decrease in the calculated microviscosity of the lipid bilayer of lymphocytes after 3 days of mitogen incubation. Our results appear to be consistent with the long-term effects of mitogen observed by Toyoshima and Osawa [34]. We should point out that their analysis of fluorescence anisotropy is different from ours in that we have separated the biphasic components of the anisotropy; our order parameter is calculated from a measured r_∞ value, while their values were obtained by solution of the Perrin equation. Furthermore, the mitogens used in their studies are different from succinyl-concanavalin A. It may be that the early changes in anisotropy (within the first 2 hours) that we observe are due to the fact that succinyl-concanavalin A is divalent and produces little or no cross-linking, capping and agglutination. However, the long-term effect of succinyl-concanavalin A is similar to that of phytohemagglutinin-P and *W. floribunda* mitogen, i.e., it is mitogenic.

Finally, we may point out that in all these studies, including ours, variations in fluidity may not be uniquely assigned to the plasma membrane because diphenylhexatriene distributes into all the cell membranes. These results can only be interpreted in greater detail in conjunction with studies that distinguish between different membrane components.

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