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Relationship between membrane lipid mobility and spectrin distribution in lymphocytes

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We have previously established that T and B lymphocytes in situ are remarkably heterogeneous with respect to the cytoskeletal protein spectrin. Since in erythrocytes spectrin is known to play an important role in the regulation of membrane fluidity, lipid organization and lateral mobility of membrane proteins, we have sought to determine if the heterogeneous patterns of spectrin distribution that we have observed are related to possible differences in membrane lipid organization in these various subsets. To this end, we have utilized a fluorescent pyrene-labelled phospholipid as a probe of the lipid lateral mobility and have examined two related T cell systems maintained in vitro, DO.11.10 cells and a spontaneously arising variant, DO.11.10V. In these (and other cloned in vitro systems) we have previously observed that the cells homogeneously express one of the kinds of spectrin distribution patterns observed in situ. Thus the uniformity of staining of these systems permits us to address whether the various patterns of spectrin distribution may be predictive of differences in membrane lipid properties. Here we show that in cells in which there is little or no spectrin at the plasma membrane (DO.11.10) that the lipids in the plasma membrane are considerably less mobile than in its related variant in which spectrin is diffusely distributed within the cell and at the plasma membrane. From this and previous results, we conclude that differences in the distribution of the cytoskeletal protein spectrin among lymphocytes may be a useful parameter in helping to predict the status of membrane lipid organization.

Spectrin; Membrane fluidity; Lipid; Cytoskeleton; Lymphocyte

1. INTRODUCTION

The structural organization and physical properties of the lymphocyte plasma membrane is believed to be fundamentally important to the subset-specific immune functions of these cells [1]; yet, there are few recognizable clues to help in recognizing differences in plasma membrane structure among lymphocytes. We have been seeking morphological criteria which may signal differences among lymphocyte subsets. This kind of information can also provide new means for determining how alterations in plasma membrane organization affects immune function.

Previously, we have observed a significant natural heterogeneity in the cytoplasmic and plasma membrane patterns of the cytoskeletal protein spectrin among lymphocytes in primary and secondary lymphoid organs in situ, and among various long-term cell lines and hybridomas [2–4]. We have also observed that the onset of heterogeneity of spectrin distribution is developmentally regulated and is affected by plasma membrane events such as activation [4,5]. One parameter in which the organization of spectrin differs among lymphocytes is in the extent of association of this protein with the inner surface of the plasma membrane. While many cells do have a ring-like pattern of staining, reflective of a distribution at or near the inner surface of the plasma membrane, other cells have distinct patches or aggregates of spectrin at the plasma membrane or within the cytoplasm; still others appear to have no detectable spectrin at the membrane at all. In many of this latter set of cells, there is instead a large cytoplasmic aggregate of spectrin that often occurs near the trans-Golgi region and sometimes near the nucleus.

In erythrocytes, where spectrin has been far more extensively characterized, several lines of investigation have shown that the positioning of spectrin and its lateral associations with other membrane-associated molecules is linked to the maintenance of plasma membrane organization and phospholipid asymmetry and fluidity [6,7]. It has also been suggested that direct interactions of the skeleton, particularly spectrin, with phospholipids may provide the mechanism by which phospholipid asymmetry is maintained [8,9]. If spectrin plays a similar role in lymphocytes, then the extensive natural heterogeneity in spectrin distribution patterns that we have observed would suggest that lymphocyte plasma membrane organization among various lymphocytes should differ. In a previous study that first supported this hypothesis, Del Buono et al. [10] concluded that murine lymphocytes with little or no membrane spectrin staining (i.e. cells with cytoplasmic aggregates) had 'tightly packed' lipids based upon the observation that these cells were enriched in populations of cells sorted for weak or negative staining with the dye Merocyanine 540 (MC540) whereas cells sorted for bright staining with MC540 were enriched for cells that had spectrin distrib-

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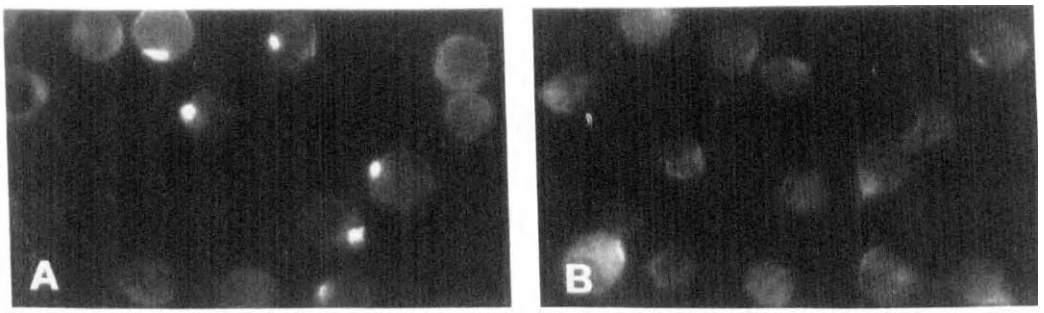

Fig. 1. Spectrin immunofluorescence of DO 11.10 (A) and variant (B) cells. In (A) cells possess a distinct polar aggregate of spectrin with little staining in other regions of the cell, whereas cells in (B) possess a more diffuse staining pattern with occasional very small patches of stain near or at the plasma membrane.

uted diffusely at the plasma membrane. MC540 is a fluorescent lipophilic probe which is thought to insert preferentially into bilayers where the lipids are loosely packed [11].

One problem associated with studies using isolated murine tissue lymphocytes is that purified populations of cells expressing only one spectrin pattern are difficult to obtain for direct study of membrane lipid properties. Moreover, isolation procedures themselves may cause alterations in membrane lipid organization or spectrin distribution. In the present study, we aimed to further characterize and more directly determine the possible relationship between plasma membrane state and spectrin distribution by using two related T lymphocyte hybridroma lines that homogeneously express distinct spectrin distribution patterns. In addition, these cells differ considerably in their activation potential [5]. For this study, we have chosen phospholipid-pyrene derivatives which can function as probes for lipid mobility as well as lipid packing measurements.

Pyrene derivatives have been used to monitor membrane 'fluidity' in a variety of systems: liposomes [12–14], lipid–peptide interactions [15,16], and plasma membranes [17–19]. The ability of the probe to form excimers, reflected by changes in spectroscopic properties [20], has been utilized to measure the lateral mobility of membrane lipids [16,21]. In this study, we found significant differences in terms of eximer formation of phospholipid–pyrene derivatives between the two related T lymphocyte systems that differ in terms of spectrin distribution. This work extends observations regarding membrane lipid organization and how it relates to spectrin distribution and will contribute to a better understanding of events related to T cell activation.

2. MATERIALS AND METHODS

2.1. *Chemicals*

N-(1-pyrenesulfonyl)Dipalmitoyl-L-α-phosphatidylethanolamine (pyrene-PE), and 3-palmitoyl-2-(1-pyrenedecanoyl)-L-α-phosphatidylcholine (pyrene-PC) were purchased from Molecular Probes, Inc. Eugene, OR. Rhodamine-6G was purchased from Sigma Chemical Co., St. Louis, MO, Dulbecco's phosphate-buffered saline (PBS) was from Gibco Lab., Grand Island, NY.

2.2. *Cell lines*

The T-cell hybridoma DO 11.10 was originally supplied by Dr. P. Marrack and was grown on supplemented Dulbecco's Modified Eagle Medium as previously described [5,22]. Variants arose spontaneously after subculturing in this laboratory and were maintained under conditions similar to DO 11.10 cells. Their activation-related properties have been previously characterized [5]. Variants are not responsive to antigen-dependent activation. The variant population is very uniform (practically 100% of cells have dispersed spectrin), while approximately 85% of cells in the DO11.10 population express a single large cytoplasmic spectrin aggregate [5].

2.3. *Pyrene-PE and pyrene-PC labelling and analysis*

Cells were washed and resuspended in PBS. Pyrene-labelled phospholipids were added to the cell suspension from the tetrahydrofuran stock solution (1 mg/ml) to a given final concentration. After incubation for 15 min at room temperature cells were washed twice and resuspended in PBS again. The fluorescence spectra were taken when the sample was excited at 347 nm. The ratio of excimer intensity to monomer intensity was measured as a function of concentration of pyrene in cell membrane. We used the excimer–monomer ratio as a parameter to compare the apparent lateral diffusion of probe in the membrane [16,20,23,24]. The amount of pyrene incorporated into cell membranes was determined by comparing the monomer maximum after treatment with Triton X-100 to the standard curve. The probe concentration in a single cell was estimated by dividing the total pyrene concentration by the number of cells in the sample.

2.4. *Energy transfer from pyrene labelled phospholipids to rhodamine-6G*

The energy transfer efficiency depends, among other factors, on the extend of overlap of the emission spectrum of the donor (pyrene) with the absorption spectrum of the acceptor (rhodamine), and the distance between these molecules [25–27]. We estimated the energy transfer efficiency using the extent of quenching of donor (pyrene moiety) by various concentrations of acceptor (rhodamine 6G) [26]. We determined the quenching efficiency of donor located in the plasma membrane by the slope of change in the fluorescence intensity ($F_0/F-1$) vs. rhodamine-6G concentration, according to the Stern–Volmer formula [26]. The fluorescence intensity ratios were linear with rhodamine-6G concentration (the correlation coefficient was higher than 0.99 for at least five concentrations of the quencher estimated by linear regression analysis). The energy transfer from pyrene to water-soluble rhodamine-6G was accomplished by adding the acceptor to a labelled cell suspension from a PBS stock solution (1 mg/ml) to a given final concentration.

To estimate the pyrene-PC accessibility to rhodamine-6G we applied the modified Stern–Volmer equation [28]. When the fluorophore is in two positions from which one is not quenched, the equation can be written as:

$F_o(F_o-F) - 1/([Q]\ Kf)+1/f$

where F_o and F are fluorescence intensities in the absence and pressure at Rhodamine 6G respectively, [Q] is rhodamine 6G concentration in the equous phase, K is the Stern-Volmar constant and f is the fraction of fluorophore accessible to quencher. If fraction f equals 1, then all fluorophores are accessible to the quencher.

All data were corrected for inner filter effect and light scattering using methods described elsewhere [26,29]. We were careful to prepare samples with the same concentrations of cells and quenchers to further eliminate possible errors due to those effects. All fluorescence experiments were carried out at 22°C with an SLM 8000 C spectrofluorometer equipped with thermoregulated sample chamber (SML AMINCO, Urbana, IL).

Live cells labeled with pyrene analogs were observed with a fluorescence microscope (Olympus CH-2, Olympus Optical Co. Ltd., Japan) equipped with a mercury lamp. The confocal microscopic fluorescence images were obtained with a fluorescence confocal microscope (Bio-Rad MRC 600; GB) equipped with an argon laser.

3. RESULTS

Whereas a large percentage of cells (80–90%) in the DO.11.10 culture express a single, large cytoplasmic aggregation of spectrin, with little or no staining at the plasma membrane, the cells of the variant culture express a diffuse pattern of spectrin in the cytoplasm and at the plasma membrane. Representative samples of both cells types are shown in Fig. 1 (see also ref. 5). To establish the influence of the probes on lymphocytes, we measured their viability, activity and spectrin distribution after labelling. We did not observe any changes with respect to control samples. Probe distribution among cells was studied with fluorescence microscopy. Visible excimer emission was observed in all or nearly all cells of each sample. No subpopulation of cells appeared to dominate the fluorescence emission. The comparison of fluorescence and phase contrast images did not reveal a noticeable number of unlabeled cells. Fig. 2 shows a series of representative confocal micrographs of crossections of a typical DO11.10 lymphocyte labeled with pyrene-PE. Similar results are obtained with the variant cells (results not shown). The figure shows that the probe is located mainly on the plasma membrane. A large number of cells have been examined and no significant amounts of fluorescent vesicles within the cell were observed. Similar results were obtained with pyrene-PC labeled lymphocytes. Consequently we assumed a uniform distribution of labeled phospholipids between cells, and a label location primarily in the plasma membrane.

We utilized the ability of pyrene moieties to form excimers as a result of diffusional collisions to examine the lateral mobility of labelled phospholipids within the plasma membrane. The fluorescence emission spectra of phospholipids labelled with pyrene exhibited a typical broad excimer peak around 490 nm and two sharp mon-

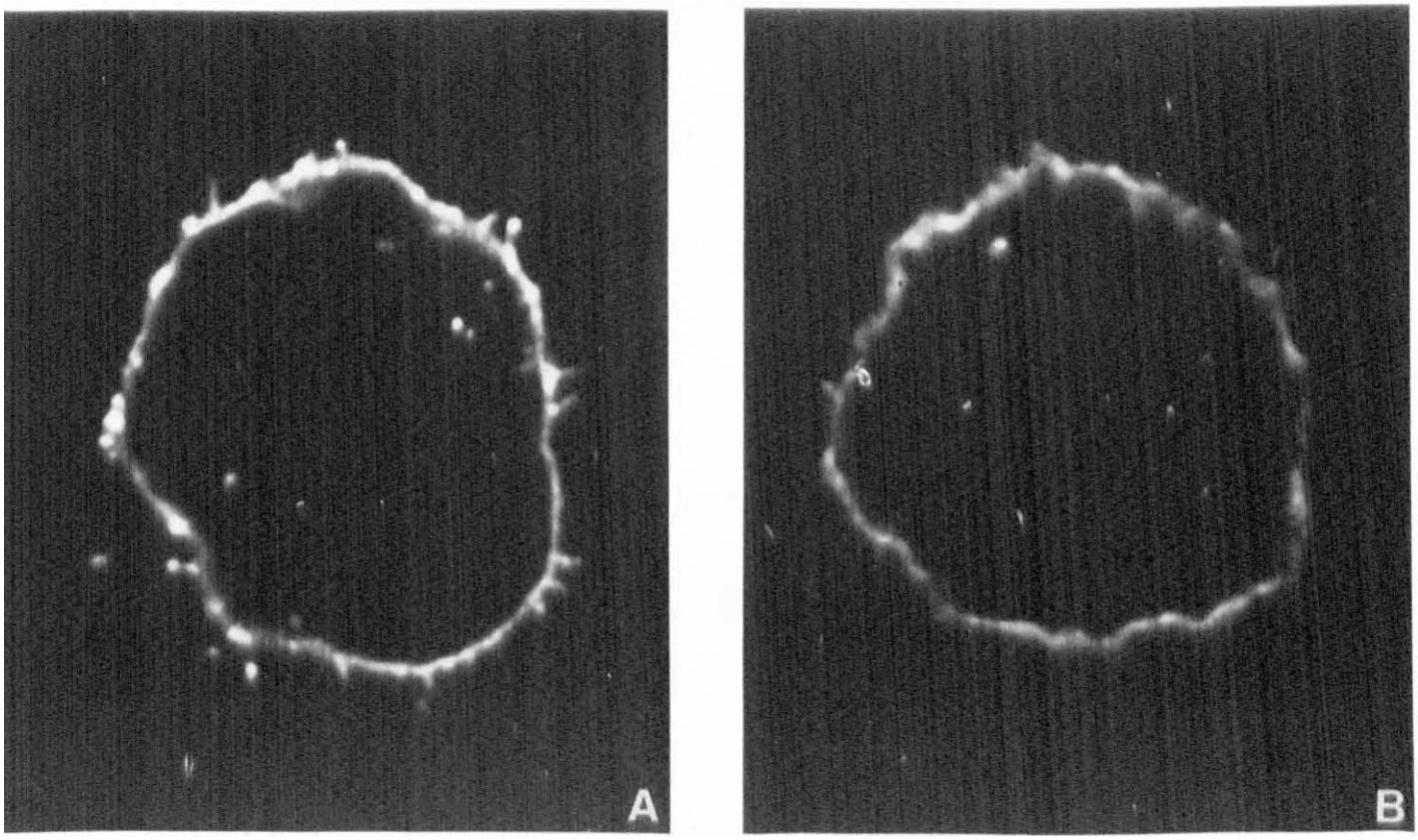

Fig. 2. Central crossections of typical (A) and variant (B) DO11.10 cells labeled with pyrene-PE. The images were obtained using a confocal microscope. Identical results were obtained with pyrene-PC.

omer peaks around 382 nm and 400 nm. A similar spectra profile of pyrene labelled phospholipids has been demonstrated previously by Somerharju et al. [30], Lakowicz [31], Kaneda [19], and Galla [25].

Due to a slow flip-flop ratio [32,33] the pyrene labelled phospholipids are mostly located in the outer leaflet of the plasma membrane. We measured the excimer/monomer ratio of pyrene labeled phospholipids after incorporation into the cell's plasma membrane as a function of time. The ratio did not change more than 10% within 30–40 min (results not shown) of this magnitude of change of fluorescence intensities represents a result of probe flip-flop, its effect on the observed E/M ratio differences between cell line is not significant. Stable excimer/monomer ratio allows us to assume that the probe redistribution within plasma membrane is completed within the incubation period. The pyrene-PE is considered to be useful for the investigation of the lateral mobility of the head groups of phospholipids, since the fluorophore of this probe is covalently bound to the amino group of phosphatidylethanolamine and is believed to be located at the lipid–water interface of the membrane [19]. The pyrene on PC is attached to 10th carbon on the hydrocarbon chain; therefore since the pyrene is located deep in the hydrophobic region of the membrane, it is sensitive to the molecular mobility of that region of the bilayer.

Fig. 3 represents the excimer–monomer ratio of DO 11.10 and DO 11.10V (variant) lymphocyte lines measured with pyrene-PE. The ratio is plotted as a function of the pyrene concentration per cell. The measured excimer–monomer ratio is significantly higher for the variant cells than for DO 11.10 cells. The consistently higher excimer–monomer ratio indicates that the labelled lipids in the variant cells' plasma membrane are more mobile than those in DO11.10 cells. This behavior was observed for a variety of cell and probe concentrations as indicated in Fig. 3.

The experimental results on energy transfer are shown in Figs. 4 and 5. The pyrene-PC incorporated into plasma membrane of the variants with dispersed spectrin (Fig. 5) quenched more efficiently than in DO 11.10 cells (Fig. 4). At the same time the quenching efficiency of pyrene-PE, in which the pyrene moiety is in the aqueous phase, does not depend on the type of cell (Figs. 4 and 5). This indicates that the concentration of rhodamine-6G at the membrane surface is similar for both cell types and that fluorescence lifetime and location of pyrene moiety is similar in both types of cells. Consequently it might be translated to a shorter average distance between fluorophores in variant cells since the energy transfer depends, among other factors, on the distance between interacting fluorophores. Its changes may be correlated to the ability of pyrene to move perpendicularly to the membrane surface or the ability of the rhodamine-6G to penetrate the membrane. The low level of pyrene-PC quenching in DO11.10 cells might

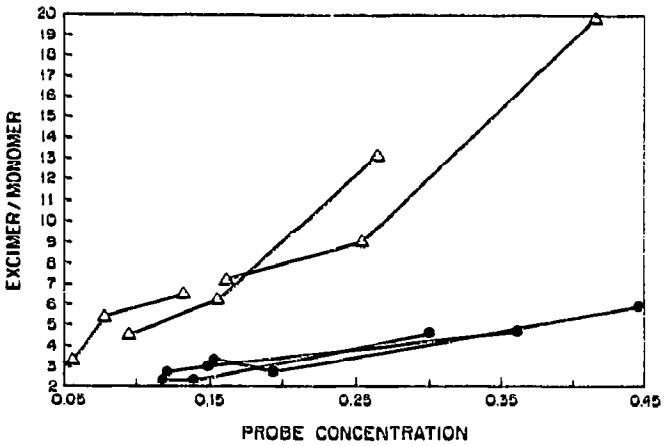

Fig. 3. The excimer–monomer ratio for the pyrene-PE probe incorporated into the lymphocyte plasma membrane. The data are presented for three cells concentrations (1, 2 and 4×10^6 cells/ml) of DO 11.10 (●) and variants (△). The probe concentration in the plasma membrane is measured as fluorescence intensity per cell at 400 nm after Triton X-100 treatment.

also be explained with the assumption that the probe is transferred to the intracellular side of plasma membrane which, in consequence will limit the ability of rhodamine 6G to quench the pyrene moiety. However, quenching data analyzed with the method introduced by Lehrer [28] revealed that all fluorophores are accessible to the rhodamine 6G since the values of Fig. 5 close to unity given by the intercept of the x-axis (Fig. 6). This result allows us to assume that most of labelled phospholipids stay on the outer leaflet of the plasma membrane, within the time range of our experiments. It has been shown previously that pyrene-PC's transversal movement is very slow in lipid vesicles and resealed erythrocyte ghosts [17,32,33]. This further supports our conclusion concerning the data obtained with pyrene-PC.

4. DISCUSSION

In this study, we used derivatives to compare the state of plasma membrane lipids in DO 11.10 cells in which spectrin is densely packed within the cytoplasm, or in a spontaneously arising variant, in which the spectrin is more diffusely distributed within the cell and at the plasma membrane. This system is being studied to help determine the physiological significance of the naturally occurring heterogeneity in spectrin distribution found among tissue lymphocytes in situ, in which cells expressing the patterns of spectrin described above occur naturally in a significant proportion of T and B cells. One parameter in which these lymphocytes in situ may differ is the membrane lipid mobility based upon (1) spectrin's known role in regulating membrane properties in the erythrocyte and (2) previous studies by others using lymphocytes in which lipid packing (as determined by incorporation of merocyanine 540) was shown to be

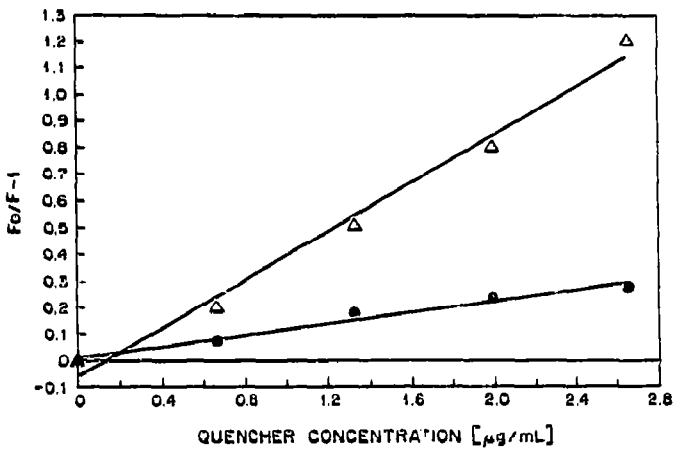

Fig. 4. The energy transfer from pyrene-PE (△) and pyrene-PC (●) to rhodamine-6G in the DO11.10 T lymphocytes. The energy transfer efficiency is expressed as the donor's (pyrene) decrease of fluorescence intensity as a function of acceptor concentration. Cells were resuspended in the PBS buffer to the concentration 4×10^6 cells/ml.

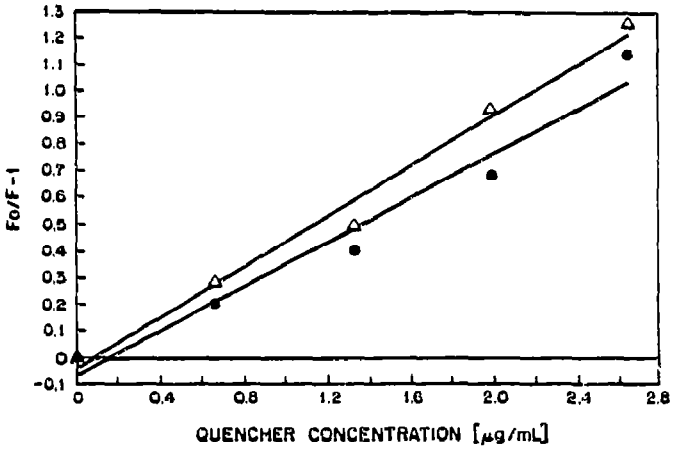

Fig. 5. The energy transfer from pyrene-PE (△) and pyrene-PC (●) to rhodamine-6G in the variant cells. All conditions are the same as in Fig. 3.

different in subsets of tissue lymphocytes that vary in spectrin distribution [9] and our own studies showing that spectrin's distribution within lymphocytes is sensitive to free fatty acid perturbation [34]. We have shown here that membrane lipid mobility, as judged by pyrene excimer formation in two T-cell hybridoma subsets in which spectrin is either diffusely distribution or densely packed, differs considerably. Notably the differences in lipid mobility, i.e. cells with a densely packed aggregate having a decreased mobility associated with pyrene probes, is consistent with a previous observation related to lipid packing and organization. In this previous report, tissue lymphocytes that stained brightly with merocyanin 540 contained no aggregates of spectrin whereas cells with aggregates stained only dimly.

Pyrene phospholipids derivatives have been used to determine the mobility of lipid fraction in artificial and reconstituted membrane systems [17,33]. The realization of using phosphatidylethanolamine labelled with pyrene as an indicator of the plasma membrane lipid mobility, is based on the assumption that excimer formation is limited by lipid diffusion oly [21] and that the probe's lateral distribution is uniform within the plasma membrane. It further requires that the pyrene moiety's concentration is known and its lifetime is constant. In our experiments, the total pyrene concentration in the lymphocyte plasma membrane was estimated with Triton X-100 treatment (described in section 2). As seen from Fig. 2 the average probe concentration per cell is similar for both cell types. As far as excimer lifetime is concerned, it has been shown in a number of systems [17,35,36], that the pyrene attached to the lipid headgroup of phosphatidylethanolamine is located at the lipid-water interface and is not free to change its position. Consequently, its lifetime, which depends on the environment and the quencher concentration, will not change significantly [25].

The energy transfer efficiency between pyrene-PE and rhodamine-6G is practically the same for both cells as shown in Figs. 3 and 4, which indicate that the pyrene moiety of labeled molecules in both cell systems are at the same location and have similar properties. The acceptor concentration and properties are the same in all samples since rhodamine-6G does not partition into the plasma membrane. In the energy transfer experiments we show that the estimated energy transfer efficiency between pyrene-PC and rhodamine-6G depends on cell type and is higher for the variants. The efficiency of the energy transfer depends on (1) the distance between fluorophores, (2) donor's lifetime, and (3) diffusion of fluorophores [27,35]. When one fluorophore (rhodamine-6G) is able to diffuse freely, in the aqueous medium, the efficiency of energy transfer depends mainly on the average transversal distance between fluorophores [19,35,37]. The translocation of the pyrene moiety attached to the phosphatidylcholine hydrocarbon toward the membrane surface would cause a decrease of pyrene lifetime due to the polarity difference between membrane interior and surface. But the energy transfer efficiency depends more strongly on distance (sixth power) than on lifetime (linear relation). We conclude, therefore, that the observed differences in energy transfer efficiency are due mainly to changes in the average transverse distances between donor and acceptor. In other words, the pyrene moiety is closer, on average, to the membrane surface in the variant cells, because the molecular packing in the membrane of these cells allows the pyrene moiety attached to the hydrocarbon chain of phospholipid to move more freely normal to the plane of the membrane surface. This explanation is consistent with data obtained for pyrene-PE excimer–monomer ratio and upon the theory that the increase transversal

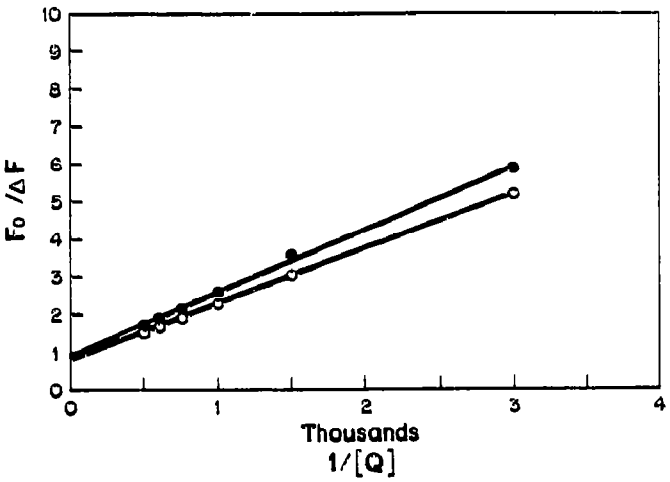

Fig. 6. Modified Stern–Volmar plot of the quenching of pyrene-PC in DO11.10 lymphocytes by rhodamine 6-G. Plots were obtained for two probe concentrations in incubation buffer: (●), 3.4 μg/ml and (○), 1.17 μg/ml.

mobility causes increase energy transfer efficiency [25,27–39].

The differences in the excimer/monomer ratio measured with pyrene-PE might be alternatively explained by differences in the lateral distribution of probe on the surface of plasma membrane. Our observations can not provide satisfactory arguments to exclude this possibility. However, the hypothesis that the lipid fluidity is changed is consistent with the energy transfer results, whereas the domain model could not easily be related to the pyrene-PC experiments. It is not certain whether the difference in molecular packing is caused by difference in the lipid membrane organization or differences in the lipid composition. The labeled phospholipids are believed to locate mainly in the outer leaflet of the plasma membrane. Therefore all conclusions are pertinent only to the outer leaflet of the plasma membrane.

In summary, our data support and extend the observation that the heterogeneity of spectrin distribution observed in situ is reflective of differences in membrane lipid organization which may, in turn, affect the functional properties of various subsets of lymphocytes.

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