Relation between the Organization of Spectrin and of Membrane Lipids in Lymphocytes

Brian J. Del Buono, Patrick L. Williamson,* and Robert A. Schlegel

Department of Molecular and Cell Biology, The Pennsylvania State University, University Park, Pennsylvania 16802; and
* Department of Biology, Amherst College, Amherst, Massachusetts 01002

Abstract. In lymphocytes, the cytoskeletal protein spectrin exhibits two organizational states. Because the plasma membrane lipids of lymphocytes also display two organizational states, it was asked whether there is a relation between the organization of spectrin and of membrane lipids.

When mouse thymocytes were stained with merocyanine 540 (MC540), a fluorescent lipophilic probe that binds preferentially to loosely packed, disorganized lipid bilayers, some cells fluoresced brightly and some only dimly or not at all. When the same population was stained for spectrin by indirect immunofluorescence, the spectrin in some cells was uniformly distributed, while in others it was concentrated in a unipolar aggregate. Techniques enriching for mature thymocytes selected for cells displaying low MC540 fluorescence and aggregated spectrin, the same characteristics found in peripheral blood lymphocytes. Flow cytometric sorting of thymocytes based on MC540 phenotype simultaneously sorted them by spectrin phenotype. Finally, treatment with agents that alter the distribution of spectrin caused mature lymphocytes to display high MC540 fluorescence and uniform spectrin. Thus, a relation exists between the organizational states of spectrin and of membrane lipids in lymphocytes: aggregated spectrin is found in cells with tightly organized membrane lipids, uniform spectrin in those with loosely organized lipids. Spectrin may thus be involved in modulating membrane lipid organization in lymphocytes as it is in erythrocytes. Since loosely organized lipids may promote adhesion of blood cells to reticuloendothelial cells, spectrin may thereby be involved in transducing an internally generated adhesion signal to the lymphocyte surface.

Spectrin has been identified not only in erythroid cells, but also in a variety of nonerythroid cells including skeletal and cardiac muscle, gastrointestinal and respiratory epithelium (28), neural tissue (1, 7), platelets (10), and lymphocytes (23, 29). In erythrocytes, it comprises ~75% of the total mass of the cytoskeleton, existing as a heterodimer composed of a 240-kD subunit (band 1 or α-spectrin) and a 220-kD subunit (band 2 or β-spectrin) (6, 15). Nonerythroid α-spectrin is structurally and antigenically similar to α-spectrin from avian erythrocytes, while β-spectrin in most nonerythroid cells, including lymphocytes, is a 235-kD chain that does not cross react with antibodies to avian erythrocyte β-spectrin (7, 13).

In addition to its role in determining the shape and mechanical properties of the erythrocyte (39, 40), the spectrin network has been implicated in the maintenance of the normal asymmetric distribution of phospholipids across the two leaflets of the erythrocyte plasma membrane (24, 30, 43). This transbilayer asymmetry is probably responsible for the inner leaflet being more fluid and less tightly packed than the external one (22, 41, 44) because of the higher proportion of unsaturated fatty acids found in the phospholipids concentrated on the inside (42). If the interaction of spectrin with the bilayer is disrupted by treatment with mild oxidizing agents (16) or by disease (44), a looser lipid packing of the outer leaflet results from the introduction of inner leaflet lipids. This change in the organization of the outer leaflet can be detected with the fluorescent lipophilic probe merocyanine 540 (MC540),1 which inserts preferentially into bilayers in which the lipids are loosely packed (45). Owing to its permanent negative charge, MC540 is impermeant thereby allowing selective monitoring of the organizational state of the lipids of the outer leaflet (33). Thus the dye identifies erythrocytes and erythrocyte vesicles with abnormalities in their spectrin–cytomembrane network (8, 9, 44) which lead to looser packing of the outer leaflet.

Several analogies to this behavior in erythrocytes have been uncovered in lymphocytes. Binding of MC540 to lymphocytes indicates that the external lipids of mature quiescent cells are tightly packed, as in normal erythrocytes, while those of immature and activated cells are loosely packed, like those of immature and perturbed erythrocytes (11). Considering the relation between lipid organization and the state of the spectrin network in erythrocytes, the discovery of two different states of spectrin in lymphocytes is of considerable interest. When spectrin is visualized in lym-

1. Abbreviations used in this paper: CRT, cortisone-resistant thymocytes; HBS, Heps-buffered saline; MC540, merocyanine 540; PBL, peripheral blood lymphocytes; PNA, peanut agglutinin.
phocytes by immunological staining, it is found to be either uniformly distributed or concentrated in a unipolar aggregate (29). This situation raises the question of the relation between lipid and spectrin organizational states in lymphocytes.

We show here that in lymphocytes the organization of plasma membrane lipids and the distribution of spectrin are correlated, and describe conditions where these phenotypes can be simultaneously altered in vitro. These results suggest that spectrin may play a role in organizing the plasma membrane of lymphocytes, much as it does in erythrocytes.

**Materials and Methods**

**Reagents and Media**

Merocyanine 540 (MC540; Eastman Kodak Co., Rochester, NY) was prepared as a 1 mg/ml stock solution in 50% ethanol and stored at 4°C in the dark. Polyclonal antiserum to chicken erythrocyte α-spectrin was a generous gift of Dr. Elizabeth A. Repasky, Roswell Park Memorial Institute (Buffalo, NY). Potassium tetrathionate was purchased from BDH Chemicals (London, U.K.). Fluoresceinated secondary antibodies, heparin, neuraminidase, bovine serum albumin fraction V (BSA), hydrocortisone 21-acetate, PNA, mezeiren, and alcian blue 8GX were purchased from Sigma Chemical Co., St. Louis, MO). Heps-buffered saline (HBS) contained 137 mM NaCl, 3.7 mM KCl, and 10 mM Hepes, pH 7.3. Culture medium was MEM (Gibco, Grand Island, NY), supplemented with 5% (vol/vol) FBS.

**Cells**

Human venous blood was collected from normal, healthy volunteers into heparin (1 U/ml of whole blood). Murine peripheral blood was obtained via cardiac puncture into syringes containing 2 U of heparin (1 U/ml of whole blood). Murine peripheral blood was obtained via cardiac puncture into syringes containing 2 U of heparin (1 U/ml of whole blood). Lymphocytes were isolated from human and mouse peripheral blood by density gradient centrifugation, using Histopaque (Sigma Chemical Co.) or Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NY) as described (11).

Thymuses were obtained from untreated mice, or from mice injected intraperitoneally 48 h before thymectomy with 2.5 nag of hydrocortisone 21-or Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) as de-

**MC540 Staining and Analysis**

10^6 cells were suspended in 0.1 ml of HBS + 0.1% (wt/vol) BSA, and 0.01 ml of a 1:10 dilution of stock MC540 in HBS was added. Cells were incubated with dye for 3 min at room temperature in dimmed lighting, 0.9 ml of HBS was added, and cells were immediately analyzed on a flow cytometer (EPICS V; Coulter Electronics Inc., Hialeah, FL) equipped with an argon excitation laser tuned to 514 nm. Fluorescence was monitored through 515 nm barrier and 560-nm dichroic filters. Cells were also cyto-

**Immunofluorescent Staining for Spectrin**

Cells were stained for spectrin by indirect immunofluorescence, using a minor modification of the method of Repasky et al. (29). 10^6 cells in 1 ml HBS were allowed to settle for 10 min onto glass coverslips that had previ-

**Results**

**Unfractionated Thymocytes**

To test for a possible correlation between lipid and spectrin organizational states in lymphocytes, cells with differing affinity for MC540 are required. The thymus provides such a source, containing subpopulations of lymphocytes which differ in their ability to bind MC540 (II). When freshly iso-

**Phorbol Ester Treatment**

Cells were treated with phorbol esters as described by Pauly et al. (25). Briefly, thymocytes or PBL were incubated at 37°C, 5% CO2/air, in culture medium with or without 10 ng/ml PMA or mezerein. At various times, cells were removed from culture, washed twice with HBS, and examined.

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the thymic medulla, the location of mainly mature cells, while the less mature cells of the thymic cortex display mainly uniform spectrin. It might therefore be expected that high MC540 fluorescence corresponds with uniformly distributed spectrin. Conversely, low MC540 fluorescence might correspond to aggregated spectrin. This expectation was tested by examining thymocytes from mice of different ages with populations shifted toward high or low MC540 fluorescence.

As a mouse ages, the population of thymocytes shifts from predominately immature cells at 5 wk, to mainly mature cells at 15 wk (35). As indicated in Fig. 2, cells displaying aggregated spectrin and cells binding low amounts of MC540 both increased in frequency with increasing donor age. As a second means of biasing the population of thymocytes toward more mature cells, mice were injected with hydrocortisone, a treatment that selectively eliminates immature cells (2). As shown in Fig. 3, >90% of the cortisone-resistant thymocytes (CRT) displayed low MC540 binding and aggregated spectrin. Together these results indicate that tightly packed lipids and aggregated spectrin are characteristic of mature thymocytes, while loose packing and uniformly distributed spectrin are found in less mature cells.

Fractionated Thymocytes

It is also possible to physically enrich for mature versus immature cells by fractionation procedures. Two separate approaches were used. In the first method, thymocytes were incubated with the lectin PNA, which selectively agglutinates less mature thymocytes (27). Agglutinated and nonagglutinated populations were collected and their phenotypes recorded in Fig. 3. Greater than 90% of the nonagglutinated thymocytes (PNA-NA) displayed both low binding of MC540 and aggregated spectrin, while nearly 95% of the agglutinated cells (PNA-A) displayed instead high MC540 binding and uniform spectrin. To eliminate the possibility that agglutination induced the phenotypes observed in PNA-A cells, samples of PNA-NA cells were incubated with neuraminidase to cleave terminal sialic acid residues from surface glycoproteins, exposing penultimate galactose residues and allowing the cells to be agglutinated by PNA (27). Despite being agglutinated, these more mature thymocytes (Fig. 3, PNA-NA') still exhibited low MC540 binding and aggregated spectrin.

Figure 2. Age dependence of MC540 and spectrin phenotypes in murine thymocytes. Thymocytes were prepared from mice aged 5, 10, or 15 wk, stained with MC540, and examined by flow cytometry, or stained for spectrin, and classified according to the aggregated or uniform phenotype. Low and high MC540 subpopulations are defined by the gate in Fig. 1 a. (Open bars) Aggregated spectrin; (shaded bars) low MC540. Bar heights represent means and error bars the standard deviations of seven experiments.

Figure 3. Effects of enrichment for mature thymocytes upon MC540 and spectrin phenotypes. Cortisone-resistant thymocytes (CRT) were prepared from mice injected with hydrocortisone. Thymocytes from uninjected mice were fractionated with PNA into nonagglutinated (PNA-NA) or agglutinated (PNA-A) subpopulations. Aliquots from the PNA-NA subpopulation were treated with neuraminidase, then agglutinated with PNA (PNA-NA'). All cells were stained and classified with respect to MC540 and spectrin phenotypes, and compared to unfractionated populations (U). (Open bars) Aggregated spectrin; (shaded bars) low MC540. Bar heights represent the means and error bars the standard deviations of five experiments.
None of the above methods actually separated thymocytes on the basis of either of the phenotypes being examined. Cells can, however, be fractionated directly on the basis of their MC540 staining using fluorescence-activated cell sorting (McEvoy, L., R. A. Schlegel, P. Williamson, and B. J. Del Buono, manuscript submitted for publication). Thymocytes sorted into populations based on the intensity of their MC540 fluorescence (defined as in Fig. 1 a) were stained for spectrin. 94% of those cells in the sorted population with low MC540 binding demonstrated aggregated spectrin (Fig. 4 a). Nearly all (>99%) of those sorted cells with high MC540 binding displayed uniformly distributed spectrin (Fig. 4 b). Therefore, separation of thymocytes on the basis of their MC540 phenotype simultaneously separates according to spectrin phenotype.

Peripheral Blood Lymphocytes

When mature thymocytes enter the circulation and assume their role as T lymphocytes, they retain their low MC540 binding phenotype (11). As seen in Fig. 5 a, >95 % of PBL from mice bound low amounts of MC540 and displayed aggregated spectrin. Because B lymphocytes were also contained in these samples, the correlation of low MC540 binding and aggregated spectrin must also hold true for B cells in the peripheral circulation. Similar results were obtained with human PBL (data not shown).

Phenotype Conversion

Together, the preceding results provide strong evidence for a relation between plasma membrane lipid organization and spectrin distribution in lymphocytes. In erythrocytes, agents that alter the organization of spectrin result in alteration in the organization of plasma membrane lipids as detected by a change in MC540 binding (44). Therefore, the possibility of a similar simultaneous conversion in lymphocytes was examined.

Pauly et al. (25) have reported that the phorbol esters PMA and mezerein induce lymphoid cell lines exhibiting aggregated spectrin to display a uniform distribution. Accordingly, CRT with aggregated spectrin were incubated with each of these agents and then examined for MC540 staining and spectrin distribution. As seen in Fig. 6, both PMA and mezerein induced large decreases, to <5%, in both the percentages of cells with aggregated spectrin and with low MC540 binding. Results similar to these recorded at 24 h were observed as early as 30 min after addition of phorbol ester. In contrast, when PBL were similarly treated, either phorbol ester caused only small decreases in the percentages of cells with aggregated spectrin and low MC540 fluorescence (Fig. 7). Similar results were obtained with human PBL (data not shown).

Although PBL were largely refractory to these agents, they proved sensitive to treatment with potassium tetrathionate. This mild, permeant oxidizing agent cross-links spectrin in peripheral blood erythrocytes through disulfide bond formation (16), and concomitantly increases binding of MC540 (44). As shown in Fig. 7, incubation of PBL with tetrathionate caused large decreases, to <3%, in the percentages of cells exhibiting aggregated spectrin and binding low amounts of MC540, compared with untreated cells. In control experiments, PBL were incubated with tetrathionate and dextrose, which allows internal glutathione levels to be maintained and thus prevents internal oxidation by tetrathionate. Under these conditions >85% of the PBL displayed aggregated spectrin and bound low amounts of MC540, indicating that increased binding of MC540 was not attributable to oxidation of external membrane components.

Given the reciprocal responses of CRT and PBL to phorbol esters, the effects of tetrathionate on CRT were examined. As shown in Fig. 6, this treatment had no effect on either the
splecticn or MC540 phenotypes of CRT; nearly 90% of untreated and tetrathionate-treated cells displayed aggregated spectrin and bound low amounts of MC540.

**Discussion**

The results presented here indicate that the packing of plasma membrane lipids and the distribution of spectrin in lymphocytes are correlated. This conclusion is derived from three sets of findings. First, the two phenotypes vary coincidentally with maturation. Second, experimental interconversion of one phenotype simultaneously interconverts the

![Figure 6. Effects of agents known to alter spectrin organization on MC540 and spectrin phenotypes in CRT. CRT were treated with the phorbol esters PMA or mezerein, or with potassium tetrathionate (Tet), and MC540 and spectrin phenotypes determined. (Open bars) Aggregated spectrin; (shaded bars) low MC540. Bar heights represent means and error bars the standard deviations of five experiments.](image)

![Figure 7. Effects of agents known to alter spectrin organization on MC540 and spectrin phenotypes in murine peripheral blood lymphocytes. PBL were treated with PMA or mezerein, or with potassium tetrathionate in the absence (Tet) or presence (Tet + Dex) of dextrose, and MC540 and spectrin phenotypes determined. (Open bars) Aggregated spectrin; (shaded bars) low MC540. Bar heights represent means and error bars the standard deviations of five experiments.](image)

other. And third, the phenotypes copurify: separation of cells on the basis of lipid organization also separates them according to spectrin distribution.

When thymocytes are enriched according to maturity by either in vivo or in vitro manipulation, immature cells exhibit uniform spectrin and loosely packed lipids, while mature cells display aggregated spectrin and tightly packed lipids. Thymocytes emigrating to the peripheral circulation retain both the mature spectrin and MC540 phenotypes, but can be transformed back to the spectrin phenotype characteristic of immature cells by mitogenic stimulation (18). From this result it would be expected that stimulated cells would bind high amounts of MC540; such is in fact the case (11).

An analogy can be drawn between these maturation changes and those that occur during erythrocyte maturation. The staining phenotype of immature erythrocytes resembles that of immature thymocytes in that both cell types bind high amounts of MC540 (11, 17, 32, 33). Conversely, mature erythrocytes and thymocytes both bind little dye (26, 32, 33). In erythrocytes loss of transbilayer phospholipid asymmetry is almost certainly responsible for conversion from the nonstaining to the staining phenotype, as disordering inner leaflet lipids are introduced into the outer leaflet. Whether a similar mechanism is responsible for conversion of lymphocytes is currently not known.

Similarly, the exact mechanism by which lipid and spectrin organization might be linked is not known. In erythrocytes it has been postulated that phospholipid asymmetry, and thereby lipid packing, is maintained by a direct interaction between phospholipids and the cytoskeleton, particularly spectrin (16, 44). Indeed, in vitro studies have shown that spectrin binds directly to purified phospholipids (21), particularly phosphatidylycerine (19) which is restricted to the inner leaflet of the erythrocyte bilayer (24); other cytoskeletal proteins to which spectrin is linked also bind phospholipids (6, 38). Direct interaction between these cytoskeletal proteins and adjacent membrane lipids may thus provide a mechanism by which spectrin restricts the phospholipid molecules with which these proteins interact to the inner leaflet. Such a model explains simply why disruption or elimination of the spectrin network leads to loss of lipid asymmetry. Considering that similar spectrin-binding proteins exist in lymphocytes (3-5, 12, 14), a mechanism similar to that in erythrocytes may be operative in lymphocytes. It is not intuitively clear, however, how the spectrin distributions observed in lymphocytes might be simply fitted into a direct interaction model: a mechanism exactly analogous to the one in erythrocytes might have predicted low MC540 binding by cells with uniformly distributed spectrin, exactly the opposite of the correlation observed. Further work is required to clarify this issue.

An intriguing finding of this study is that mature lymphocytes from differing sources are demonstrably different in their responsiveness to reagents affecting spectrin and MC540 phenotypes. Thymocytes displaying a mature phenotype are much more susceptible to phorbol ester-mediated lipid and spectrin rearrangements than are peripheral blood T lymphocytes displaying the same phenotype, while the latter are more responsive to treatment with tetrathionate. These results suggest that "mature" thymocytes are not simply exported lymphocytes of the peripheral type, but that additional maturation events may distinguish them; a possibility.
widely debated (36). The system described here delineates structural differences between these maturational states, and may provide a means of elucidating the biochemical events that interconnect them.

The question remains as to the possible functional significance of different organizational states of membrane lipids and spectrin in lymphocytes. Data are accumulating that indicate that in erythrocytes, maintenance of a tightly packed exterior leaflet is requisite for their continued circulation in the blood. When transblayer phospholipid asymmetry is lost, lipid packing in the external leaflet is loosened, leading to increased interaction of erythrocytes with endothelial cells and macrophages (20, 31, 34). A similar situation may pertain to lymphocytes. Immature cells (with loosely packed lipids) bind to macrophages and other stromal cells in bone marrow and thymus. Their mature, circulating counterparts (with tightly packed lipids) by definition do not interact with cells that line or accompany them through the peripheral blood; yet when stimulated, they revert to the immature lipid phenotype, leave the circulation, and home to the peripheral lymphatic tissues (II). A loosening of the packing of membrane lipids may therefore provide a signal for the adherence of lymphocytes to reticuloendothelial cells. It should be remembered throughout that it is the status of externally exposed lipids that is being assessed. The spectrin-based cytomembrane network, however, is internal. Thus, a spectrin–lipid linkage may be responsible for transducing an internally generated homing signal from the inside to the outside of the cell.

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