Heterogeneity in Lymphocyte Spectrin Distribution: 
Ultrastructural Identification of a New Spectrin-Rich Cytoplasmonic Structure

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Abstract. Spectrin-like proteins are found in a wide variety of non-erythrocytoid cells where they generally occur in the cell cortex near the plasma membrane. To determine the intracellular distribution of α-spectrin (α-fodrin) in lymphocytes, we have developed an immunoperoxidase method to localize this protein at the ultrastructural level. Of considerable interest, particularly with regard to our efforts to determine the function of spectrin in this cell type, was the finding that its subcellular localization and its relationship with the plasma membrane can vary dramatically. Based on its position in the cell, α-spectrin can occur in two forms in lymphocytes: one that associates closely with the plasma membrane and another that occurs at some distance from the cell periphery, either as a single large aggregate or as several smaller ones. The single large aggregate of spectrin is a stable feature in a number of lymphocyte cell lines and hybrids which were used to examine its ultrastructural characteristics. A previously undescribed cellular structure, consisting of a meshwork of spectrin filaments and membranous vesicles, was identified in these cells. This structure could be induced to dissipate in response to membrane perturbants (e.g., hyperthermia and phorbol esters, known effectors of lymphocyte function and differentiation) and the patterns resulting from the redistribution of spectrin were a reflection of those observed routinely in lymphocytes in situ. The correlation between naturally occurring spectrin localization patterns and those seen after membrane perturbation suggested the possibility that spectrin distribution is indicative of particular maturation stages or functional states in lymphocytes. The implications of these findings with regard to the role of spectrin in lymphocyte function are discussed.

The plasma membrane of immunocompetent cells performs specialized functions that result in recognition of self and nonself and in subsequent effector activities. Structural features of the plasma membrane that enable them to perform these activities are not well understood. Recently, several well-characterized erythrocyte membrane-associated skeletal proteins including spectrin, ankyrin, and band 4.1, have been observed in non-erythrocytoid cells (for reviews see 3, 5, 7, 10, 19). However, the organization of these proteins in the lymphocyte and the role that they play in membrane-related phenomena of the immune response are not clear. With regard to spectrin (a major component of the erythrocyte membrane cytoskeleton [8, 19]), recent studies have addressed its role in crosslinking and "capping" of lymphocyte cell surface macromolecules (6, 17, 22). These studies have supported the assumption that, in this cell type, this protein has a submembranous localization and is tethered to membrane components in a manner analogous to that in the red blood cell. Accordingly, many tissue lymphocytes show a halo of fluorescence at the cell periphery when immunostained for spectrin (α-subunit), presumably reflecting a subplasma membrane localization (17, 22, 28).

However, we have observed a surprising heterogeneity in the subcellular distribution of spectrin in unperturbed lymphocytes (i.e., in the absence of exogenous ligand) (15, 24, 28): a large subpopulation contains a discrete aggregate of the protein at one pole of the cell and a smaller percentage shows several patches of varying size and distribution. These cells appear morphologically similar to lymphocytes exposed to crosslinking agents which induce cell surface "caps" and "patches" with coincident cytoskeletal protein clusters. Two fundamental issues that arise from these observations include (a) whether the spectrin found in aggregated form is actually associated with the plasma membrane and (b) whether there is a discernable structural basis for the polar spectrin immunofluorescence observed at the light microscope level. Answers to these questions would contribute toward understanding the role of spectrin in lymphocyte plasma membrane function. To characterize the nature of the various patterns of spectrin distribution and to establish the relationship of the protein with the plasma membrane in each case, we used an immunocytochemical approach to localize spectrin at the ultrastructural level in various lymphoid tissues and lymphocyte cell lines.
By means of a preembedding ultrastructural immunoperoxidase technique, we demonstrate in this study that lymphocytes differ considerably with respect to the amount and organization of spectrin at the plasma membrane, both in their natural state in situ and in vitro. Moreover, we show that many lymphocytes contain a previously undescribed spectrin-rich cytoplasmic structure of significant size (1–2 μm) across which can occur at some distance from the plasma membrane. Finally, we demonstrate that lymphocytes may rapidly convert from expressing one pattern of spectrin distribution to another and present evidence that the “patchy” configuration may represent an intermediate stage in this conversion. The possible physiological significance of the heterogeneity in spectrin expression in lymphocytes as well as putative functional roles of the observed spectrin-rich cytoplasmic structure are discussed.

Materials and Methods

Tissue Culture

DO.11-10 and BDK 38.2 T-cell hybrids (kindly provided by Drs. Marrack and Kappler, National Jewish Hospital, Denver, CO) were grown as described (33). The human leukemia T-cell line MOLT-4, was grown in 25-cm² polystyrene flasks and fed every other day with RPMI 1640 medium containing heat-inactivated (56°C, 30 min) FCS (10%; KC Biological, Inc., Lenesca, KS), antibiotics, and L-glutamine.

Antisera

The rabbit anti–chicken erythrocyte α-spectrin antisera used in this study has been described and characterized previously (28, 29). It was prepared by isolation of α-spectrin from two-dimensional gels (29) and has been shown to react with the 240-kD subunit of lymphocyte spectrin (22, 28), as well as the same subunit in numerous non-erythroid tissues of avian and mammalian origin. In a previous study (29), identical immunoreactivity in lymphocytes was found using antisera directed against α-fodrin (17). In the present study, similar results were obtained using an anti-chicken erythrocyte α-spectrin antisera prepared in goat (Note that the antigen is hereafter referred to as spectrin). Fluorescein- and peroxidase-conjugated goat anti–rabbit IgG were purchased from Miles Scientific Division (Naperville, IL).

Indirect Immunofluorescence

Immunolocalization of spectrin at the light microscope level was carried out as described previously (24, 28). Lymphocytes isolated from various lymphoid organs or cultured in vitro were washed in PBS, resuspended at high density, and allowed to settle on glass coverslips pretreated with Alcian blue to promote adhesion (31). Adherent cells were fixed in 2% formaldehyde for 15 min at room temperature, washed in PBS and permeabilized in 0.5% Triton X-100/PBS (PBS/Triton) for 5 min. Incubation with primary antisemur (diluted 1:30 in PBS/Triton) was carried out for 30 min at room temperature. After washes in PBS, fluorescein-conjugated goat anti–rabbit IgG was applied (diluted 1:150 in PBS/Triton) for 30 min. Coverslips were mounted in Elvanol (30), viewed with a Zeiss photomicroscope II equipped with an automatic camera and photographed with Kodak Tri-X film.

Immunocytochemical Localization of Spectrin in Tissue Lymphocytes

Small pieces of mouse thymus, spleen, or lymph node were fixed in periodate-lysine-paraformaldehyde fixative (PLP) (20) for 7 h at room temperature, cryoprotected by incubating overnight in 0.1 M phosphate buffer, pH 7.4, containing 30% sucrose, given a rapid freeze-thaw to permeabilize the cells and sectioned (30 μm) with a Vibratome tissue slicer (Oxford Instruments Polysciences, Warrington, PA). The free-floating sections were then incubated overnight (at 22°C) in rabbit anti–chicken erythrocyte α-spectrin (1:20, diluted in PBS/0.1% BSA containing 0.01% NaN₃) with gentle agitation. After extensive washing (90 min, six changes) in PBS/BSA, they were incubated in peroxidase-conjugated goat anti–rabbit IgG (1:50, diluted in PBS/BSA) for 1 h at 22°C. After washing (90 min, six changes) in PBS/BSA, they were fixed in 1.5-2% glutaraldehyde/0.1 M Na cacodylate buffer, pH 7.4 containing 5% sucrose for 1 h and washed (90 min; three changes in cacodylate buffer/7.5% sucrose, three changes in 50 mM Tris-HCl, pH 7.6/7.5% sucrose) before being incubated in 0.1-0.2% diaminobenzidine in Tris-HCl/7.5% sucrose for 15–30 min. H₂O₂ was then added to a final concentration of 0.01% and incubation was continued for 15–30 min to visualize the site of antibody binding. The tissue sections were then washed (45 min), post-fixed in 1% OsO₄ for 90 min at 4°C and processed for EM. Sections were counterstained with uranyl acetate and lead citrate and viewed in a Siemens 101 electron microscope. Control samples treated with preimmune serum showed no specific immunoperoxidase staining.

Results

Subcellular Localization of α-Spectrin in Tissue Lymphocytes

Tissue (spleen, thymus, and lymph node) lymphocytes, unperturbed by exogenous cross-linking agents, show considerable heterogeneity in the cellular location of spectrin as observed by indirect immunofluorescence (Fig. 1) (28). Lymphocytes may express (a) a ring-type or diffuse staining pattern; (b) several “patches” of staining of variable distribution, or (c) a large polar aggregate with little or no staining in the rest of the cell. The percentage of cells showing each of these patterns varies among the different lymphoid organs (28). The heterogeneity in lymphocyte spectrin subcellular distribution was examined at the ultrastructural level by immunostaining samples of murine lymph node, thymus, and spleen (data not shown for spleen). Tissues were dissected and fixed in PLP (20), cryoprotected with 30% sucrose, and given a rapid freeze–thaw before preparing 20–30-μm sections with a Vibratome tissue slicer. The free-floating sections were then stained for spectrin and processed for EM.

Uninterrupted staining of spectrin at the plasma membrane such as that seen in mammalian and avian erythrocytes...
Spectrin immunofluorescence in isolated tissue lymphocytes. Cells isolated from thymus (a) and spleen (b) show either a ring of fluorescence (arrow), a single large aggregate at one pole of the cell (arrowhead) or several patches of variable size and distribution (open arrow). Bars, 10 μm.

(23, 29) was rarely observed in tissue lymphocytes, although small 'dots' or short stretches (Fig. 2, a and b) of immunoprecipitate were observed along the periphery of many cells. Adjacent dendritic cells showed distinct, even staining at the plasma membrane (Fig. 2, c) indicating that the non-uniformity of membrane staining observed in lymphocytes was not the result of technical artifact. Points of contact between two cells were often decorated with dense precipitate. However, in a large subpopulation of lymphocytes, negative immunoreactivity was observed at the cell periphery suggesting that spectrin was entirely absent from this region (Fig. 2 d). In many of the latter cells (roughly 35% in spleen, 25% in thymus, and 50% in lymph node), the protein was confined to a single discrete polar aggregate in close association with the nucleus, Golgi apparatus or centrioles (Fig. 2, e, f, and g). Other lymphocytes showed a more diffuse pattern of staining at one pole of the cell (Fig. 2 h) or several smaller aggregates scattered throughout a particular area of the cytoplasm (Fig. 2 i). In many cases, more than one distinct large aggregate of spectrin occurred in different regions of the cell (Fig. 2 c). In some cells, a single large aggregate of the protein was detected close to the plasma membrane, often in association with surface microvilli (Fig. 2 j). This localization may account for the "capped" appearance observed by immunofluorescence in cells unperturbed by exogenously added ligand (28). In several cases, an arc of membrane staining was observed on either side of the aggregate (Fig. 2 i), and in some lymphocytes, small patches and a single larger aggregate were seen in the same cell.

Lymphocytes isolated from tissue and immunostained in suspension showed patterns of spectrin distribution that were similar to those observed in situ (data not shown).

**Immunocytochemical Localization of Spectrin in Cultured Lymphocytes: Identification of a Previously Undescribed Spectrin-rich Cytoplasmic Body**

Indirect immunofluorescence studies showed that long-term cultures of lymphocyte cell lines and hybrids also differ with respect to subcellular distribution of spectrin (24). While tissue lymphocytes show extensive heterogeneity in this regard (see above), cell lines homogeneously express one of three basic patterns. In the majority of lines examined the protein occurs only at the cell periphery (24), as previously reported for erythrocyte spectrin (8, 19, 23). In other lymphocyte lines, the cells express a discrete polar aggregate containing most or all of the cell's spectrin. In still other lines, the protein occurs both at the plasma membrane and within a focal aggregate in the cytoplasm (Fig. 3 a). We took advantage of the uniformity of these cell lines for a more detailed characterization of the unexpected spectrin-rich region observed in tissue lymphocytes. To this end, preembedding immunoperoxidase labeling of this protein in the T-cell hybrids DO.11.10 and BDK 38.2, homogeneous with regard to ex-
Figure 2. Immunoperoxidase localization of spectrin in lymphocytes in situ. (A) Sections of (a) thymus and (b) lymph node showing non-uniformity in membrane-associated staining of spectrin. Arrows indicate 'dots' and short stretches of immunoprecipitate. Some cells show little or no staining (arrowhead). (c) Micrograph showing the presence of a dendritic cell (D) in thymus. Note the even deposition of immunoprecipitate at the cell periphery (arrows) in contrast to the pattern observed in surrounding lymphocytes. Arrowheads denote cells with more than one distinct aggregate. (d) Section of thymus showing many cells lacking membrane-associated spectrin staining. (B) Many lymphocytes contain a large aggregate of spectrin (arrows) in close association with the nucleus, N (e and e') Golgi apparatus, G (f) or centriole, C (g). In some lymphocytes, spectrin is diffusely distributed at one pole of the cell near the plasma membrane (h). An "arc" of membrane-associated staining can sometimes be seen on either side of a spectrin aggregate (arrows in i). Note the small patches of stain found in some cells. Spectrin is also often accumulated in close association with surface microvilli (MV; j). Bars, 1 μm.

pression of this structure, was carried out and the immunochromically stained cells examined at the ultrastructural level.

Reaction product (marking the location of spectrin) was observed in a discrete region at one pole of the cells (Fig. 3, b–e), several microns from the cell surface and, usually, in close association with the centriolar complex and trans region of the Golgi apparatus. The stained area was generally oval in shape (although in some cases it was horseshoe-shaped; Fig. 3 c), 1–2-μm across, and frequently surrounded by many small membranous vesicles and cisternae.
Figure 3. (A) Immunolocalization of spectrin in cultured T-cell hybrids. (a) Immunofluorescence staining of spectrin in BDK 38.2 cells. Note the homogeneity of these cultured cells with respect to the presence of a focal accumulation of protein (arrows). (b) Ultrastructural immunoperoxidase localization of spectrin in DO.11.10 cells. Arrows indicate a region of dense immunoprecipitate several microns from the cell surface and in close association with the Golgi apparatus (G) (nucleus, N). Note the complete absence of staining at the plasma membrane (M). (c) DO.11.10 cell immunostained for spectrin showing that the aggregate is variable in shape. (B) (d and e) Higher magnification of the spectrin aggregate in DO.11.10 cells. Note its proximity to the Golgi region (G) and centriolar complex (C) and the numerous small vesicles and cisternae which frequently surround this spectrin-rich structure (arrowheads). Bars (a) 10 μm; (b–e) 1 μm.
Figure 4. Occurrence of the spectrin aggregate (arrow) in close association with regions of surface microvilli (MV) (a) and within a uropod (U) (b) in BDK 38.2 cells. Bars, 1 μm.

In some cells, similar staining occurred in close association with the plasma membrane, especially when the latter was invested with microvilli or where a uropod had spontaneously formed (Fig. 4) (as also seen in situ [refer to Fig. 2]).

The immunoprecipitate itself had a reticular appearance (Fig. 3, d and e) and was found to coincide with a previously unreported cytoplasmic structure composed primarily of a fine meshwork of densely packed filaments, as observed by conventional thin-section microscopy (Fig. 5). These filaments were 3–5 nm in diameter and ~100-nm long. The aggregate of filaments, which appeared more electron-dense than the surrounding cytoplasm, was not membrane-bound but excluded most other cellular organelles. In addition to membranous vesicles, it contained many electron-dense ribosome-like granules. Numerous intermediate filaments often surrounded and coursed through the structure (Fig. 5 c and d), as expected based on previous immunofluorescence data (15, also see reference 18).

The spectrin aggregate was absent in cell lines where immunofluorescent staining showed spectrin distributed at the cell cortex (e.g., MOLT-4; 24). Ultrastructural immunoperoxidase staining of the protein in these cells resulted in a uniform deposition of reaction product only in the region subadjacent to the plasma membrane (Fig. 6). Compared with tissue lymphocytes, cells grown in culture appear to have more spectrin distributed in a uniform manner at the cell periphery.

Evidence for Spectrin Redistribution in Cultured Lymphocytes

Previous immunofluorescence studies in this laboratory have shown that when lymphocytes containing aggregates of spectrin are exposed to membrane perturbants (e.g., phorbol esters, cis-unsaturated free fatty acids, or mild hyperthermia (12, 24)), the polar staining rapidly disappears and spectrin takes on a diffuse distribution. This phenomenon was studied at the ultrastructural level in cell lines containing cytoplasmic spectrin aggregates. Cells were briefly exposed to mild hyperthermia (40.5°C) or phorbol esters to monitor the morphological changes occurring in the spectrin aggregate as it dissipates. Under these conditions, a dramatic redistribution of the protein was observed (Fig. 7, a–d): in some cells, the aggregate took on a ring-like configuration; in others, small fragments appeared scattered throughout the cytoplasm; still others showed patches in association with the plasma membrane. The majority of cells under these conditions had lost their spectrin in the aggregated form and expressed the protein only at the cell periphery (Fig. 7 d). What was striking about the various patterns of staining observed was that they were a reflection of those seen routinely in normal lymphoid tissue (Fig. 8; also see Fig. 2, c and i). Since these patterns were the result of perturbations known to effect lymphocyte differentiation and cell function (2, 13, 14), it is possible that the natural variation in spectrin distribution observed in lymphoid tissue relates to different stages of differentiation or functional state in this cell type.

Discussion

The data presented here demonstrate that, in normal animal lymphoid tissues, there is considerable variability in the subcellular distribution of α-spectrin. Of particular significance is the observation that spectrin, which is evenly distributed near the plasma membrane in erythrocytes and many non-erythroid nucleated cells, is often entirely absent from the lymphocyte cell periphery and can occur as a single, large aggregate several microns from the plasma membrane. This aggregate appears to be a stable feature in certain lymphocyte subsets, as indicated by its constitutive expression in several lymphocyte cell lines (24). Antigen-specific, functional T-cell hybrids were found to be homogeneous sources of this cytoplasmic spectrin aggregate and were used to investigate its ultrastructural characteristics. As described in this paper, there exists in many lymphocytes a previously unreported discrete cytoplasmic region, of significant size relative to the amount of cytoplasm in these cells, consisting of a meshwork of filaments and membranous vesicles. The size of the filaments...
Figure 5. Identification of a previously undescribed spectrin-rich cytoplasmic structure in cultured lymphocytes. These micrographs show the presence of a nonmembrane-bound region (delineated by arrows), composed of a meshwork of fine filaments, that excludes most cellular organelles and is located in close association with the Golgi apparatus (G) and centriolar complex (C). Ribosome-like particles (short arrow) and membranous vesicles (V) are also found within the meshwork. Vesicles (v) are also frequently seen at the periphery of the structure (a, b, and d). Microtubules (probably emanating from the centriolar complex) and intermediate filaments (IF) often surround and course through the aggregate. (d) An enlargement of the boxed area in (c) showing numerous intermediate filaments (IF) at the periphery of the spectrin meshwork. (e) In some cases this spectrin-rich region is found near the plasma membrane especially when the latter is invested with microvilli (MV) (also see Figs. 2 and 4). Bars, 1 μm.
ments is consistent with reported values for spectrin molecules (19) and their identity was confirmed by our immunolocalization data. The position of this spectrin-rich structure can vary; in lymphocyte cell lines, we find it close to the *trans*-Golgi region whereas, in tissue cells, it is also frequently seen in association with the nucleus or the plasma membrane. Patterns of spectrin distribution in lymphocytes range from its presence exclusively in the region subadjacent to the plasma membrane to its focal accumulation in the cytoplasmic structure described above. Between these extremes, the protein is found in several smaller aggregates, either in the cytoplasm alone or in the cytoplasm and at the plasma membrane.

While the cytoplasmic aggregate of spectrin appears to be a stable structure in lymphocytes, we show that it can be induced to dissipate in response to certain external stimuli (e.g., hyperthermia and phorbol esters [12, 24]). EM shows that treatment of cells containing aggregates with these membrane perturbants results in a dramatic redistribution of the protein. Although the precise sequence of events that occur after exposure of the cells to hyperthermia or phorbol esters is unknown, it is possible to envision the following based upon the ultrastructural analysis of immunocytochemically stained lymphocyte cell lines: (a) disintegration of the spectrin aggregate and formation of several cytoplasmic fragments, (b) dispersal of these fragments and (c) migration of the protein to the cell periphery leading to an increase in its

*Figure 6.* Immunoperoxidase localization of spectrin at the plasma membrane. Note the uniform distribution of reaction product (arrows) at the cell periphery in human MOLT-4 cells. Bar, 1 μm.
Figure 8. Expression in tissue lymphocytes of patterns of spectrin distribution similar to those seen in perturbed cultured cells. a and b Show cells found in normal thymus that bear a resemblance in spectrin distribution to cells shown in Fig. 7 (cf. Fig. 7, a and c with Fig. 8). Bars, 1 μm.

Figure 7. Effect of hyperthermia on the subcellular location of spectrin in cultured cells. After treatment at 40.5°C, a variety of patterns of spectrin distribution are observed in BDK 38.2 cells including open rings (a) and dispersed fragments in the cytoplasm and/or at the plasma membrane (b and c). The majority of cells treated under these conditions have lost their aggregated spectrin and show distinct staining at the cell periphery (d). Bars, 1 μm.
to its structural function, new roles in this cell type which are yet to be characterized.

If the role of lymphocyte spectrin is viewed as analogous to that of the erythrocyte form, the cytoplasmic spectrin aggregate could be recognized as a pool of available protein which can be readily recruited according to the changing requirements of the membrane (such as changes in fluidity) in response to physiological stimuli. This region may be a source of preassembled spectrin-membrane packets (membranous vesicles are often found within the spectrin aggregate); rapid interchange between this cytoplasmic pool and the cell periphery may be facilitated by the absence of a bounding membrane confining spectrin within the aggregate. The dynamic character of lymphocyte spectrin differs from that of the red blood cell protein, where a constant amount is always found at the plasma membrane. This may reflect the more static nature of the erythrocyte membrane and its continuous need for the appropriate mechanical properties that spectrin provides. Our data may also explain results from biochemical analyses of the composition of purified lymphocyte plasma membranes showing considerable variability in the amount of spectrin associated with the membrane (cf. references 1 and 6). Lymphocytes which contain a large cytoplasmic aggregate of spectrin often, but not always, have undetectable amounts of the protein at the plasma membrane, whereas those with no aggregates often show distinct immunostaining at the cell periphery. A comparison of these cells would lead to different conclusions regarding the presence of spectrin as a component of the lymphocyte plasma membrane skeleton. It must be emphasized that our data do not exclude the possibility that cells showing little or no spectrin at the cell periphery, express the α, form (22).

With respect to additional roles for spectrin, it is likely that the aggregate in lymphocytes has functions in its own right. The presence of membranous vesicles within the structure and its frequent close association with the Golgi apparatus suggest the possibility that it may play a role in membrane trafficking (secretion and internalization) in this cell type. Support for involvement of spectrin in these processes comes from recent studies on the role of spectrin in the intestinal brush border (11, 25) and from the work of Perrin et al. (27) who demonstrate its participation in chromaffin granule secretion (also see reference 31).

We are currently attempting to relate differences in spectrin subcellular distribution to various functional states in lymphocytes. This, in conjunction with ongoing biochemical studies, should help to determine the role of this protein in immune function.

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