Spectrin Immunofluorescence Distinguishes a Population of Naturally Capped Lymphocytes in Situ

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ABSTRACT
Immunofluorescence analysis of mammalian lymphocytes using antiserum directed against chicken erythrocyte α-spectrin revealed a lymphocyte population in which spectrin antigen was arranged in the form of a discrete cap (hereafter referred to as capped lymphocytes). This subset could be easily distinguished from other lymphocytes in which the spectrin antigen was diffusely distributed near the plasma membrane (noncapped lymphocytes). The subset of capped lymphocytes could be visualized in situ and in isolated cells in the absence of added ligand. Using frozen sections of lymphoid organs that were fixed in formaldehyde prior to the immunofluorescence procedure, capped lymphocytes were found in characteristic locations depending on the tissue examined. In the thymus, the major population of medullary lymphocytes were capped whereas cortical lymphocytes were mostly noncapped. In Peyer’s patches, capped lymphocytes were interspersed with noncapped lymphocytes throughout the tissue. In the spleen, capped lymphocytes were concentrated in the periarterial lymphoid sheath of the white pulp and in lymph nodes they were found predominantly in the paracortical and cortical regions. Capped lymphocytes were not visible in the thymus until just before birth and did not appear in the spleen until 3 d after birth. When lymphocytes were isolated from lymphoid organs, fixed in formaldehyde and prepared for immunofluorescence, capped and noncapped lymphocytes were still identifiable and present in the same relative proportions as seen in situ. Results identical to those described above are obtained using antiserum directed against guinea pig fodrin. Natural capping of proteins previously shown to co-migrate with a variety of cell surface macromolecules after cross-linking may be a new means of identifying various stages of lymphocyte activation or differentiation.

MATERIALS AND METHODS
All mice used in these experiments were kept in the animal colonies at Roswell Park Memorial Institute. Data depicted in this study was obtained from Balb/c female mice although similar results were obtained using Balb/c male mice, C57Bl/6 and DBA/2 mice, and various other mammals (hamster, gerbil, and cat).

For single cell suspensions, lymphoid organs were minced into small fragments with scissors and the fragments placed into phosphate buffered-saline (137 mM NaCl, 3 mM KCl, 2 mM KH2PO4, and 8 mM Na2HPO4, pH 7.4). After 1 min of gentle resuspension of the fragments with a Pasteur pipette, the large clumps of cells and connective tissue were allowed to settle out by gravity and the suspension of single cells was washed two times. Cells in PBS were then allowed to settle for 5 min onto coverslips that had been treated with Alcian blue, which promotes the adherence of cells (30). Coverslips with adherent cells were placed into 2% formaldehyde in PBS for 10 min and then washed two times before incubation with antisera or Triton-containing buffers.

For imprint preparations, spleens and thymuses were removed and placed on a clean glass surface. A new razor blade was used to cut each organ in half; the exposed surface of cells was affixed to a clean, nonacidic blue-treated coverslip by gently pressing the piece of tissue onto the coverslip, cut-side down, and then quickly pulling it away. Approximately five imprints per cut were...
made on a given coverslip. The coverslip was then placed into formaldehyde fixative for 10 min and prepared for immunofluorescence. The time from the death of the animal to the insertion of the coverslip into fixative never exceeded 1 min while the duration of time between cutting the organs in half and affixing them onto the coverslip was only a few seconds.

For preparation of frozen sections, various lymphoid organs were rapidly removed from mice that had been killed by cervical dislocation (or decapitation in the case of embryos or mice <10 d of age). Organs were placed into O.C.T. compound (Tissue-Tek; Miles Laboratories, Naperville, IL) and frozen at \(-80^\circ\)C. Sections 6-8 μm thick were made using an IEC cryostat set at \(-20^\circ\)C. Each section was melted onto a glass coverslip which was then immediately placed into formaldehyde fixative. After 10 min, coverslips were rinsed in several changes of PBS before incubation with antisera or Triton-containing buffers.

Immunofluorescence procedure using anti-chicken erythrocyte α-spectrin antisemur was performed as described previously (29). Antifodrin antisemur (24) was the generous gift of Dr. Mark Willard and was used at a dilution of 1 to 50. The same immunofluorescence procedure as used for spectrin was used for fodrin. Cells were photographed using Kodak Tri-X film on a Zeiss Photomicroscope II equipped with an automatic camera. Zeiss fluorescein filter set #17 was used for observation and photography. Conditions for photography and printing were optimized for portrayal of the cells with the caps of spectrin antigen. However, in some cases, (particularly the higher magnification photographs) the staining intensity of the caps was so bright that printing conditions led to the overdevelopment of other regions, resulting in an artificially dim representation of the noncapped cells (e.g., Fig. 2/7). SDS PAGE was based on the discontinuous Tris-glycine system of Laemmli (22) as modified and described previously (21). Isolated splenic cells were placed into 0.17 M ammonium chloride to lyse red blood cells, washed two times, and the final pellet of approximately 3 x 10^6 cells was resuspended in 1% SDS-sample buffer. The sample was boiled for 1 min before loading it onto a gel. Preparation of mouse erythrocyte ghosts and chicken erythrocyte membranes was as described previously (20, 29). Immunooautoradiography was performed as described previously (19, 29), except that only 1 μCi of radiiodinated protein A (Amersham Corp., Arlington Heights, IL) was used per gel. Exposure time for Fig. 4 was 2 d with a Dupont Cronex intensifying screen. Coomassie blue-stained gels were photographed prior to drying and exposure to X-ray film.

Several control experiments were run to test the specificity of the immunofluorescence staining of spectrin. Using preimmune rabbit serum followed by fluorescein-conjugated goat anti-rabbit antiserum, or fluorescein-conjugated goat anti-rabbit antiserum alone, negligible fluorescence was obtained. As previously described (29), if the antiserum is adsorbed with α-spectrin antigen, specific fluorescence is eliminated. Finally, if intact lymphocytes were not extracted with Triton X-100 prior to the addition of antisera, only background levels of fluorescence are seen indicating that the antibody must enter the cell to bind antigen. These results indicate that the fluorescence depicted in this study is specific for α-spectrin antigen and is not due to nonspecific binding of antiserum to lymphocytes.

RESULTS

When freshly-isolated lymphocytes from 1 mo-old mice were allowed to adhere to coverslips, formaldehyde-fixed, and prepared for immunofluorescence using α-spectrin antiserum, we consistently observed that the antigen was in a capped configuration in a large number of cells. Fig. 1, a and b depict lymphocytes isolated from spleen and thymus, respectively. Among splenic lymphocytes, ~35% of the lymphocytes were capped whereas in the thymus ~45% were capped. Other lymphocytes in which the antigen was diffusely distributed capped where as in the thymus ~45% were capped. Other lymphocytes isolated from spleen and thymus, respectively.

Among lymphocytes from the small intestine of a 1 mo-old mouse stained with antisera used in this study was further analyzed by immunooautoradiography (Fig. 4). As expected from previous reports (25, 28), both antisera reacted with the same 240-kd lympho-
cytolyte protein that co-migrates with chicken and mouse erythrocyte α-spectrin and both reacted with chicken erythrocyte α-spectrin, although the cross-reactivity of antifodrin with the chicken protein was considerably less than that of the anti-α-spectrin. However, at the exposure duration used here, neither antiserum showed cross-reactivity with mouse erythrocyte α-spectrin indicating that the reaction obtained in the lymphocyte lane and by immunofluorescence was not due to contaminating erythrocytes. Although some biochemical differences have been reported between the antigens recognized by antifodrin and anti-α-spectrin (3, 10, 16, 17), the immunnoautoradiographic results indicate that in lymphocytes, the two antisera were cross-reacting with the same protein complex. In addition, double immunofluorescence experiments using both antisera on the same capped and noncapped lymphocytes show that the two antisera have identical distributions (not shown).

DISCUSSION

The phenomenon of capping (31) is a segregation of specific plasma membrane-associated macromolecules to one discrete region of the membrane. Usually associated with this energy-requiring activity are (a) cross-linking by ligands, which appears to initiate capping and (b) coordinated movement of both surface macromolecules and several submembraneous cytoskeletal proteins (see reviews in references 6, 13, 14). This latter observation has led to the general assumption that the forces required for capping are generated by these membrane-associated cytoskeletal proteins; yet, the mechanism of linkage between the cytoskeleton and cell surface groups in the lymphocyte has never been established. Alternative hypotheses regarding cap formation have also been proposed (4, 8). Although capping has provided a useful model system for determining interrelationships between various membrane proteins, the physiological significance of redistribution of these groups is unknown.

In this report, we have presented our morphological investigations concerning lymphocytes that have a naturally-occurring capped configuration of α-spectrin, a protein previously reported to co-cap with various cell surface macromolecules on T and B cells only after the addition of cross-linking ligands (28). However, all of the experiments described in that study were done on various lymphocyte cell lines that apparently do not exhibit natural capping of spectrin. Therefore, a physiologic factor may be required to maintain lymphocyte spectrin in a capped configuration, or capped lymphocytes may only be seen in a specific phase of differentiation. Our results also differ from previous reports of “spontaneous” capping of various cytoskeletal proteins, such as myosin, since spontaneous capping is dependent upon (and subsequent to)
FIGURE 2 Anti-α-spectrin immunofluorescence of frozen sections of mouse spleen and thymus. (a) Low magnification view of a section of 2 wk-old mouse spleen with a colony of capped lymphocytes (colony indicated by arrowheads). Capped lymphocytes can be better visualized in b, which is a higher magnification view of a colony of capped cells (arrows) in the white pulp of the spleen of a 1 mo-old mouse. The border between capped lymphocytes and other noncapped lymphocytes is approximately delineated by the curved arrows. The artery of the white pulp is shown in longitudinal section in the upper right portion of the figure. A similar colony (indicated by arrowheads) cut in transverse section is shown in c; artery is indicated by short arrow. (d) Large expanse of capped lymphocytes (arrow) seen in the thymus of a 2 wk-old mouse. In the thymus, capped lymphocytes are found primarily in the medulla as shown in a low magnification view in e. The border area between cortex and medulla delineated by the rectangle in e is shown in higher magnification in f. Capped cell is indicated by arrow. Bars, 20 μm.

the onset of lymphocyte motility in vitro (7) and is not seen in rounded (i.e., nonmotile) freshly isolated cells as are shown in Fig. 1 of this paper. Using another antiserum that reacts with the same 240-kd protein as does anti-α-spectrin in lymphocytes, i.e., anti-guinea pig fodrin, we have also observed the occurrence of capped cells in situ. This protein has also been previously reported to co-cap with either antibody cross-linked surface immunoglobulin or histocompatibility antigens.
of isolated murine splenic lymphocytes (25). It is apparent therefore, that there may be two populations of lymphocytes with respect to the distribution of spectrin or fodrin: one in which the spectrin/fodrin antigen is uniformly distributed (and therefore available for induced capping with ligand as previously described) and a second subset in which the spectrin/fodrin is already capped prior to the addition of ligand. Both of these lymphocyte subsets (capped and non-capped) can be visualized in Fig. 1 using isolated cells and in various locations in situ as shown in Figs. 2 and 3 of this paper. Our observation of naturally capped cells does not preclude the possibility that these lymphocytes are responding to cross-linking ligands in their physiological environment, which stimulates capping of membrane-associated proteins. In this case, perhaps certain surface macromolecules will be found in a capped configuration, which is coincident to that of spectrin.

The differentiation state of lymphocytes may be important with regard to whether or not the cells are capped since in the thymus, (see Fig. 2, e and f), capped lymphocytes were restricted to the medulla where lymphocytes differ developmentally in several respects from cortical lymphocytes (11). During erythropoiesis, it has been shown that membrane regions of increased spectrin concentration are associated with decreased deformability and endocytic activity (32, 33). Perhaps similar modifications of membrane deformability due to spectrin capping will be observed during lymphocyte maturation. A possible pre-existing polarity with respect to membrane deformability could fulfill the constraints of the model of capping proposed by Berlin and Oliver (4) in which interaction of a particular receptor or ligand-receptor complex with a region of membrane that is altered in its association with underlying cytoskeletal elements could either arrest passage of the complex (resulting in formation of a cap) or facilitate its passage to another region of the membrane. The close association of spectrin with integral membrane proteins, and its function in modulation of membrane integrity and deformability as demonstrated in the erythrocyte, makes this protein a good candidate for local reconstruction of membrane properties in the lymphocyte. A pre-existing polarity of membrane-associated cytoskeletal proteins (which could significantly alter the deformability of certain regions of the membrane) could also provide experimental support for the "membrane-flow" hypothesis of cap formation proposed by Bretcher (8).
Analysis of spectrin capping (or other as yet undescribed naturally capped proteins) may help us to recognize the physiological significance of in situ capping with regard to lymphocyte activation and/or differentiation. This work is currently being extended to determine whether or not any exogenously or endogenously derived macromolecules on the cell surface exist in a capped configuration that is coincident to that of spectrin and to characterize the surface phenotype of the subset of capped lymphocytes in various lymphoid tissues.

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