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Appearance of cytoskeletal components on the surface of leukemia cells and of lymphocytes transformed by mitogens and Epstein–Barr virus

(actin/tubulin/lactoperoxidase iodination/immunofluorescence)

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ABSTRACT **Lactoperoxidase iodination and two-dimensional electrophoresis of the labeled proteins have demonstrated well-characterized cytoskeletal proteins (actin and tubulins) on the surface of human lymphocytes undergoing blastogenic transformation and of certain malignant human cells. Such proteins could not be detected on the surface of normal resting human lymphocytes. The most prominent cytoskeletal protein identified on the surface membrane of mitogen-transformed T and B lymphocytes was actin. In Epstein–Barr virus genome-positive Burkitt's lymphoma and lymphoblastoid cell lines and in two leukemia cells, the major iodinated membrane protein components were actin and α_1-, α_2-, and β-tubulins. These proteins were firmly connected to the cytoplasmic skeleton and could not be removed by Triton X-100. Concurrent immunofluorescence studies with specific antibodies and F(ab')$_2$ fragments confirmed the appearance of cytoskeletal components on the surface of live and fixed lymphocytes, in parallel with the biochemical data, and indicated that such cytoskeletal proteins formed distinctive patterns on the cell surface, ranging from small patches to large projections. Five-hour labeling with [^{35}S]methionine indicates that all such cells released large quantities of labeled actin and tubulins into the culture medium. These materials were not readsorbed to the membrane surfaces of the cells.**

The cytoskeletal framework, composed principally of microfilaments and microtubules, is normally confined to the interior of the cell and to the inner surface of the cell membrane (1–4). Earlier studies have documented marked increases in relative rates of synthesis of major skeletal proteins (actin and tubulin) in polyclonally activated normal human T and B lymphocytes, as well as in Epstein–Barr virus (EBV) genome-positive lines and in three leukemia cell lines (5). Such cells also release large quantities of labeled actin and tubulin into the culture medium, with no evidence of a loss of cell viability (5). In view of recent reports of extensive shedding of membrane materials and submembrane anchorage components by mouse mastocytoma cells (6), an attempt has been made to ascertain whether a similar mechanism was operative in the transformed human lymphocytes studied in this laboratory. The results indicate that blastogenic stimulation of normal lymphocytes or malignant transformation of such cells (or both) is associated with the appearance of large concentrations of cytoskeletal proteins on the cell membrane surface. This observation is in marked contrast to the absence of such components on the surface of normal human lymphocytes.

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MATERIALS AND METHODS

Enriched populations of T and B lymphocytes from healthy volunteers were prepared from defibrinated blood (7) and were stimulated *in vitro* with concanavalin A (Con A) (T cells) or pokeweed mitogen (PWM) (B cells) as described (8). EBV genome-positive cells used in the present study were: (*i*) Burkitt's lymphoma lines Daudi, Maku, and Solubo; (*ii*) AW-Ramos and EHRA-Ramos, lines that had been superinfected *in vitro* with EBV (type P3HR-1); and (*iii*) lymphoblastoid lines SJVB and GUK. The cells used also included leukemia cell lines 1301 and K562, HeLa cells (S-3), and primary fibroblast human lines grown and maintained as described (9). For pulse labeling, iodination, or immunofluorescence microscopy, the cells were washed three times with balanced salt solution and, as judged by the vital dye exclusion test, only cell populations exceeding 95% viability were used. Cells were pulse labeled and samples were prepared for two-dimensional electrophoresis as described (5). Cell surface proteins were iodinated by the lactoperoxidase method as described by Marchalonis (10). The cells were washed with phosphate-buffered saline and, if the viability of the cells was below 95%, the dead cells were removed by centrifugation over Ficoll (11). The viable cells were then pelleted and lysed in lysis buffer (12). Surface iodinated Ig was specifically immunoprecipitated as described (8). For comparison purposes, actins were isolated from rhesus monkey striated muscles and from AW-Ramos, Daudi, and 1301 cell lines by the procedure of Spudich and Watt (13). Purified chicken gizzard actin containing 90% γ-actin and approximately 10% β-actin was generously provided by Sergio Abreu (Cornell University Medical College, New York). Microtubules from pooled AW-Ramos and 1301 cell lines and from acetone-extracted powder of rhesus monkey and dog brain tissue and liver were prepared by a modification (14) of the procedure of Shelanski *et al.* (15), with two cycles of polymerization. All preparations were analyzed by the two-dimensional electrophoretic technique of O'Farrel (12), except that the second-dimension electrophoresis was on 10% polyacrylamide/NaDodSO$_4$ gels. The pH gradient of the first-dimension gels was measured with a Bio-Rad proPhiler. Crosslinked hemoglobins were used as the standards for molecular weight determinations (16). The second-dimension gels of [^{35}S]methionine-labeled proteins were processed for fluorography as described by Bonner and Laskey (17). The gels of unlabeled and of iodinated proteins were stained with Coomassie blue and were dried in a cellophane sandwich. For visualization of ^{125}I-labeled pro-

Abbreviations: EBV, Epstein–Barr virus; Con A, concanavalin A; PWM, pokeweed mitogen.

teins, the gels were exposed to x-ray films between two Cronex 4-DuPont intensifying screens at room temperature.

The IgG fraction (18) of antisera to tubulin, actin, smooth muscle myosin, tropomyosin, and α-actinin was prepared in rabbits as described (19–21). Each immunoglobulin fraction was further purified by affinity chromatography with homologous antigen (22). Immunofluorescent staining was performed as described (20). Live cells were treated with 0.4 mg of sodium azide per ml in balanced salt solution prior to staining. Dried smears were fixed by exposure to formalin fumes for 10 min or immersion in 10% buffered formalin (pH 7.4) for 2 min. Internal structures were not stained in live or fixed cells, as shown by the failure to stain mitotic figures with antibodies against tubulin or mitochondria with antibodies against human mitochondria. For visualization of internal structures, the formalin-fixed smears were dipped sequentially in 0.2% Triton X-100 (for 10 min), acetone/water, 1:1 (vol/vol) (for 4 min), acetone (for 4 min), and phosphate-buffered saline (for 10 min) prior to immunofluorescent staining.

RESULTS

The two-dimensional electrophoretic patterns of iodinated cell surface membrane proteins were complex but distinct for each cell type used in this study. The pattern of iodinated proteins from the Daudi cell line shown in Fig. 1*A* is representative of the patterns encountered. A group of proteins of 68,000 daltons and intermediate pI heterogeneity was identified as μ Ig chains and proteins of 22,000 daltons were identified as light Ig chains by using anti-human IgM antibodies and carrier IgM for specific immunoprecipitation. In normal or PWM-transformed lymphocytes, the pI heterogeneity for μ, γ, and light chains was much greater, with frequent appearance of a continuous radioactive line across the entire pH gradient. In contrast, only single well-defined radioactive spots for μ and light Ig chains were observed in the AW-Ramos cell line. In Con A-stimulated T cells, no iodination of Ig chains could be detected.

Four major cell surface iodinated proteins (numbered arrows in Fig. 1*A*) occurred as the common surface proteins in all transformed lymphocytes and in two leukemia cells. As seen in Fig. 1*B*, these four proteins were also abundant in the cell lysate. In contrast, Ig chains were scarce in the lysate, despite their heavy iodination. To assist in the identification of these major proteins, we extracted pooled Daudi cells by the procedure used for preparation of cytoplasmic actin. The resulting preparation (Fig. 1*C*) contained two major and two minor proteins comigrating upon two-dimensional electrophoresis with the four iodinated proteins of Fig. 1*A*. These proteins migrated to the same position as the well-characterized β-actin and β-, α_1-, and α_2-tubulin samples from various known sources, which were run on parallel gels. Fig. 2 compares the two-dimensional electrophoretic positions of α-actin from striated muscle of rhesus monkeys, chicken gizzard γ-actin containing small amounts of β-actin, and actin prepared from pooled AW-Ramos and 1301 cells. In addition, cell surface actin from Daudi cells (spot 1 in Fig. 1*A*) also migrated to the same position as the actin from AW-Ramos cells, actin prepared from monkey liver or HeLa cells, or actin copolymerizing with microtubules obtained from monkey brain (data not shown). The other major proteins in Fig. 1 (spots 2–4) were identified as tubulins by comparing their positions with the positions of proteins extracted from purified brain microtubules (Fig. 2 *D* and *E*) and proteins prepared from cytoplasmic microtubules isolated from pooled unlabeled AW-Ramos cells (Fig. 2*F*). In EBV genome-positive cells, α_1- and α_2-tubulins were present in almost half the quantity of β-tubulin. The pI values and molecular weights of tubulins extracted from microtubules of human lymphoid cells and of monkey and dog brains agreed closely with the two-dimensional electrophoretic data of Berkowitz *et al.* (23) with calf brain tubulins and of Kelly and Cotman (24) with rat brain tubulins.

The remarkable accessibility of actin and tubulin to lacto-

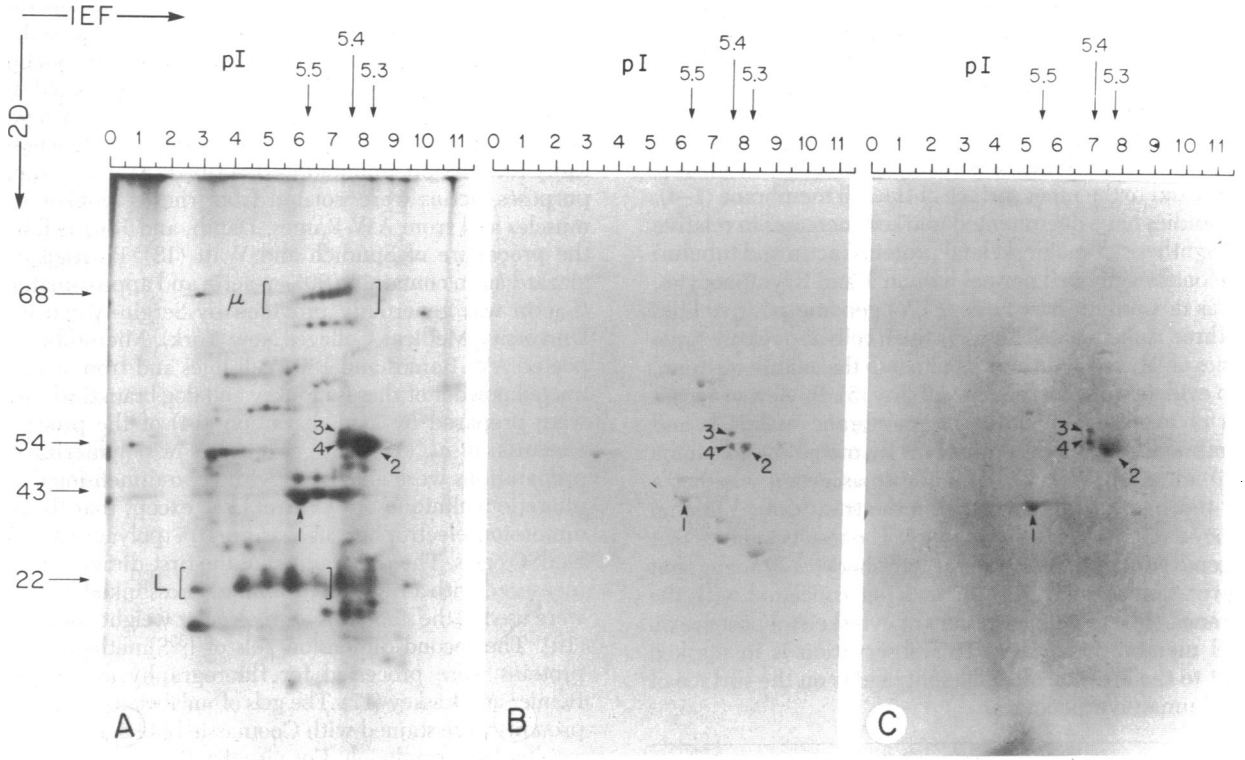

FIG. 1. (*A*) Two-dimensional electrophoretic fluorogram of iodinated proteins of the Daudi cell line. IEF, isoelectric focusing; 2D, second dimension. The activity of the sample applied on first-dimension electrophoresis was 6×10^5 dpm; the gel was exposed on x-ray film for 4 days. Sizes in kilodaltons are indicated at the left. L and μ, light and heavy Ig chains. (*B*) Coomassie blue-stained gel used for the fluorogram. (*C*) Coomassie blue-stained proteins extracted from Daudi cells by the procedure for preparation of actin.

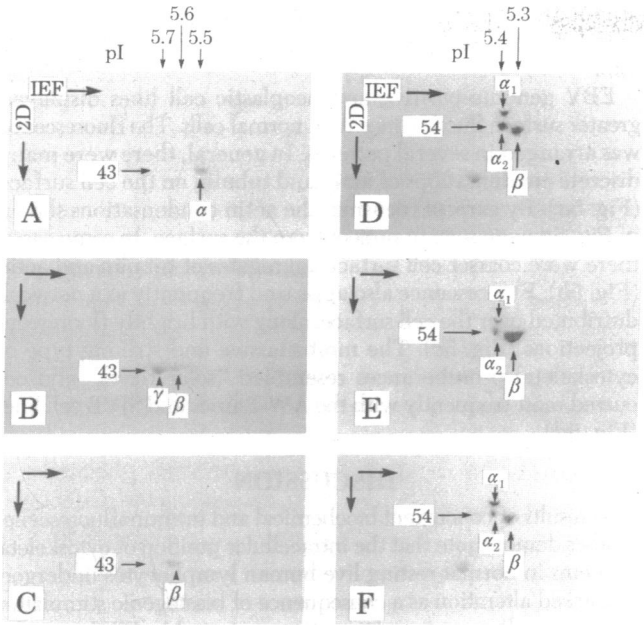

FIG. 2. Two-dimensional electrophoresis of actins and microtubule proteins. (*A*) α-Actin from striated muscle of rhesus monkeys; (*B*) chicken gizzard γ-actin; (*C*) actin prepared from pooled AW-Ramos and 1301 cell lines. (*D–F*) Microtubule proteins from monkey brain (*D*), dog brain (*E*), and AW-Ramos cells (*F*). The gels were stained with Coomassie blue; 43-kilodalton and 54-kilodalton proteins are indicated. IEF, isoelectric focusing; 2D, second dimension.

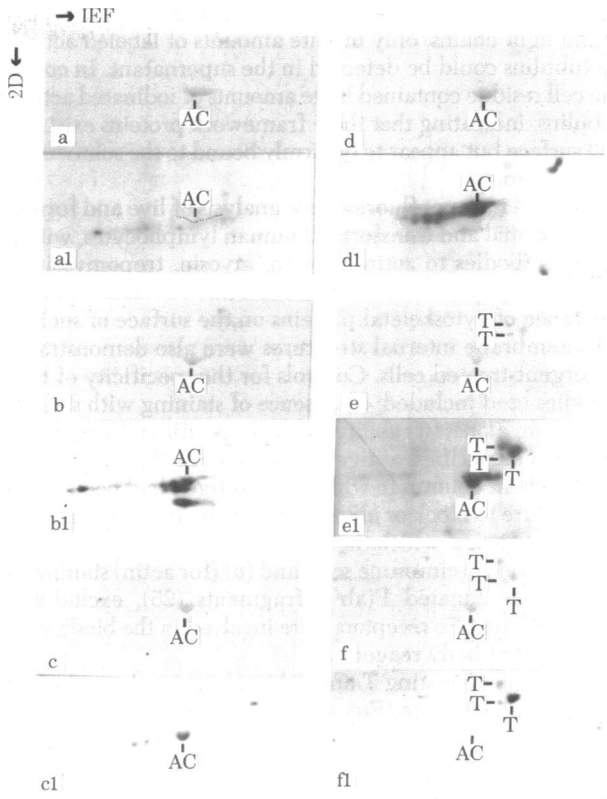

FIG 3. Two-dimensional electrophoretic fluorograms of lactoperoxidase radioiodinated surface proteins from unstimulated normal lymphocytes (*a1*), PWM-stimulated lymphocytes at day 4 (*b1*), Con A-stimulated cells at day 4 (*c1* and *d1*), AW-Ramos cell line (*e1*), and cell line 1301 (*f1*). The corresponding Coomassie blue-stained gels (*a–f*) are shown above the fluorograms. The dotted area of *a1* shows the position of the Coomassie blue-stained actin in *a*. IEF, isoelectric focusing; 2D, second dimension; AC, actin; T, tubulin.

peroxidase iodination observed in Daudi cells prompted a study of the cell surface proteins present in other lymphocytes. As noted in Fig. 3*a*, actin was the major cytoplasmic protein stained by Coomassie blue in normal resting lymphocytes; such actin was, however, inaccessible for lactoperoxidase iodination (Fig. 3*a1*). When enriched B lymphocytes from the same subject were stimulated *in vitro* with PWM and lactoperoxidase iodination was performed on the fourth day of culture, however, actin now appeared as a major labeled cell surface protein (Fig. 3*b1*). As shown in Fig. 3 *c1* and *d1*, similar results were obtained in normal human T lymphocytes stimulated with Con A. Iodination of tubulins was weak in all of these cells and could be demonstrated only after prolonged exposure of the gels. These results were repeated with several individual cell donors, yielding the same data.

Fig. 3*e* illustrates the two-dimensional electrophoretic pattern of iodinated proteins obtained from cultures of AW-Ramos cells. As in Daudi cells, actin and tubulins occurred as major iodinated proteins on the cell surface. Similar results were obtained with all other EBV genome-positive cells studied. As noted in Fig. 3*f1*, the tubulins (and to a somewhat lesser extent, actin) were also accessible to iodination in cultures of cell lines 1301 and K-516. There was no evidence of such framework proteins on the surface of HeLa cells or of monolayers of normal human fibroblasts.

A significant array of evidence points to the role of lactoperoxidase iodination as a specific label for cell surface protein components. The pattern of proteins iodinated by this technique differs radically from that of stained total cell protein. As noted in Fig. 3*b*, actin and other proteins in the range 43,000–35,000 daltons were almost all equally labeled with ^{125}I, but nonactin proteins were not detectable in total cell lysates. In Fig. 1*A*, Ig chains were heavily iodinated but were not seen in Coomassie blue-stained gels, further militating against the possibility that the iodination of actin was due to the iodination of intracellular proteins in a few dead cells. When the permeability of Daudi cells was increased by treatment with 0.1% Triton X-100 in isotonic solution and the cells were subsequently iodinated with lactoperoxidase, the resulting Coomassie blue-stained proteins closely overlapped the ^{12}I-labeled spots. In contrast to the untreated cells, there was very little, if any, iodination of light and μ Ig chains. In another type of control, several cell lines were iodinated with and without lactoperoxidase; the amount of acid-precipitable radioactivity for the same number of cells iodinated in the absence of lactoperoxidase was between 0.7% and 1.2% of the amount incorporated in the presence of lactoperoxidase, showing that endogenous peroxidases participated only minimally in the reaction.

One possible explanation for the accessibility of usually intracellular actin and tubulins to iodination in transformed cells might be that these components became readsorbed to the cell surface after membrane material was shed. To assess this possibility, we dialyzed the supernatant from [^{35}S]methionine-labeled AW-Ramos cells against balanced salt solution containing 0.1% unlabeled methionine in order to eliminate free [^{35}S]methionine. Lymphocytes stimulated with Con A for 4 days were then incubated in this supernatant for 1 hr at 37°C. The cells were washed once in balanced salt solution, were lysed, and were subjected to two-dimensional gel electrophoresis. Despite the presence of large amounts of labeled actin and tubulins in that particular supernatant, the Con A-stimulated cells failed to pick up any labeled actin or tubulin.

In a search for a better understanding of physical relationships between cell surface framework proteins and the membrane, iodinated viable Daudi and EHRA-Ramos cells were exposed to 0.5% Triton X-100 in phosphate-buffered saline for

2 min at 0°C. Although this procedure released all labeled Ig μ and light chains, only minute amounts of labeled actin and no tubulins could be detected in the supernatant. In contrast, the cell residue contained large amounts of iodinated actin and tubulins, indicating that these framework proteins exist on the cell surface but appear to be firmly bound to the submembrane cytoskeleton.

Parallel immunofluorescence analysis of live and formalin-fixed normal and transformed human lymphocytes, with specific antibodies to actin, tubulin, myosin, tropomyosin, and α-actinin, provided further corroborative evidence of the appearance of cytoskeletal proteins on the surface of such cells. Submembrane internal structures were also demonstrated in detergent-treated cells. Controls for the specificity of the antibodies used included: (*i*) absence of staining with secondary antiserum alone; (*ii*) ability to block specific staining by direct addition of purified antigen or by passage through an immunoabsorbent column to which that antigen had been coupled; (*iii*) failure to block or absorb antisera by using antigens other than that against which the antiserum was directed; (*iv*) failure to stain with preimmune sera; and (*v*) (for actin) staining with directly conjugated $F(ab')_2$ fragments (25), excluding the possibility that Fc receptors were involved in the binding of the primary antibody reagents.

Normal live resting T and B lymphocytes showed minimal surface fluorescence (Fig. 4*a*); after detergent treatment, fluorescence of cytoskeletal proteins in the cytoplasm was evident, with few if any surface projections. Polyclonally activated T and B lymphocytes displayed an ordered surface fluorescence array of fine stippling or of coarser cell surface condensations with antibodies against actin (Fig. 4*b*) and against tubulin and, to a lesser extent, with antibodies against myosin and against α-actinin. Many cells (usually T more than B) possessed fine surface villous projections, which stained for actin and tubulin (Fig. 4*b*, right lower quadrant). Massive condensations of actin, tubulin, and, rarely, myosin (very rarely, α-actinin and tropomyosin) could be seen at the surface of some cells (Fig. 4*d*). These projections did not detach. Internal structures, including mitotic figures, were observed only in Triton-treated cells by use of antibodies against tubulin (Fig. 4*c*) and against myosin.

EBV genome-positive and neoplastic cell lines displayed greater surface fluorescence than normal cells. The fluorescence was arranged in several patterns. In general, there were many discrete condensations of actin and tubulin on the cell surface (Fig. 5*a*). By careful focusing, the actin condensations shown in Fig. 5*a* were seen to project from the surface. In some cases, there were coarser cell surface aggregates of tubulin and actin (Fig. 5*b*). Fluorescence also appeared frequently as a network distributed over the cell surface, along with brightly fluorescent projections (Fig. 5*c*). The most massive and striking type of cytoskeletal protuberances resembled "solar flares" and occurred most frequently with the AW-Ramos and SJVB cell lines (Fig. 5*d*).

DISCUSSION

The results of concurrent biochemical and immunofluorescence studies demonstrate that the intracellular position of cytoskeletal proteins in normal resting live human lymphocytes undergoes a marked alteration as a consequence of blastogenic stimulation or after malignant transformation, as tested in EBV genome-positive and leukemia cell lines. Actin and tubulins are readily detectable on the cell membrane surface of such cells. As a result of the high sensitivity of the immunofluorescence tests used, other mechanochemical and cytoskeletal proteins, such as smooth muscle myosin, tropomyosin, and α-actinin, have also been detected on the cell surface in certain cases. The absence of such framework proteins on the cell surface of normal live lymphocytes and their appearance after mitogenic stimulation, viral (EBV) infection, or malignant transformation (or all three) suggest that this change may be related to a common transitional differentiation pathway shared by human lymphocytes in response to these different agents. The absence of tubulin and actin on the surface of HeLa cells and human diploid fibroblast monolayers is in keeping with a possible specificity of the phenomenon for a particular cell type. The data also indicate

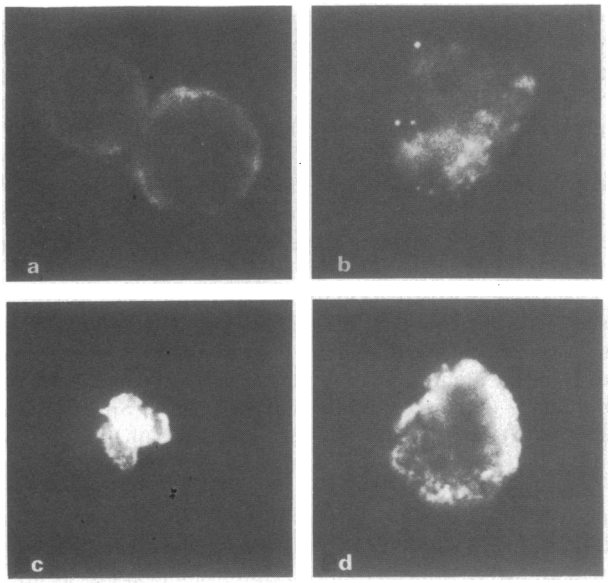

FIG. 4. Immunofluorescent oil immersion microphotographs of normal lymphocytes. (*a*) Maximal staining of normal, unstimulated, human B cell with antibody against actin. (*b*) Human B cell stained with antibody against actin 4 days after exposure to PWM. (*c*) Detergent-treated human T cell stained with antibody against tubulin 4 days after exposure to Con A; the mitotic figure is clearly visible. (*d*) T cell stained with antibody against actin 4 days after stimulation with Con A. (×400.)

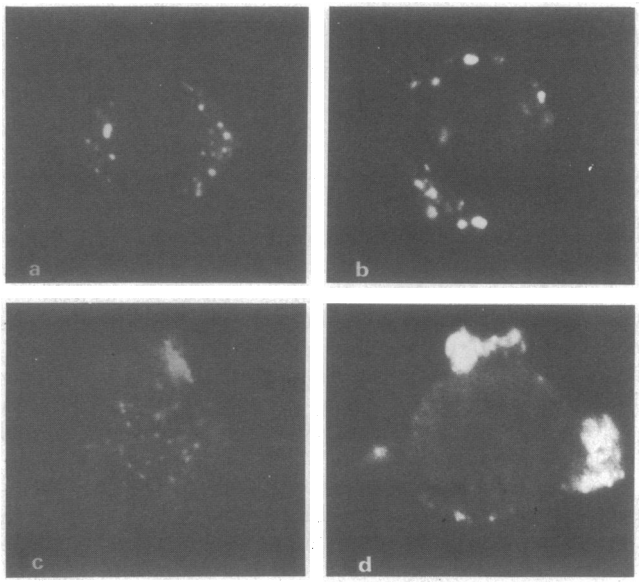

FIG. 5. Immunofluorescent oil immersion microphotographs of lymphoid cell lines. (*a*) AW-Ramos cell visualized with antibody against actin. Projection of some patches from the surface can just be made out. (*b*) SJVB lymphoblastoid cell stained with antibody against tubulin. (*c*) 1301 leukemia cell showing a pattern developed with antibody against tropomyosin. (*d*) AW-Ramos cell showing protrusions, one of which appears to be detached, stained with antibody against tubulin. (×400.)

that the release of large quantities of labeled actin and tubulins by such activated cells (5) is not a consequence of an actual shedding of submembrane anchorage elements into the supernatant, a process that had earlier been implicated in the interpretation of this result (6).

The distribution of cytoskeletal proteins shown by immunofluorescence techniques ranged from fine projections to an accumulation of large masses of protein on the cell surface. The similar patterns observed in any given cell line when antibodies against actin, tubulin, myosin, and α-actinin were used suggest that these proteins are associated with a common type of subcellular structure at defined areas of the cell membrane. Such proteins may be continuous with the cytoplasmic framework and may protrude through the plasma membrane in certain areas of the surface because they remain bound to the cells after treatment with nonionic detergents. In these particular regions, the continuity of the membrane lipid bilayer may therefore be interrupted by closely associated masses of cytoskeletal proteins. A protrusion of microtubules through the cell membrane has been reported in fibroblasts (26), although this observation is disputed by most investigators (2, 3). Another possibility may be that the framework proteins are integral components of the cell membrane, as has been documented for tubulin at the synaptosomal cell junction in higher organisms (27) as well as in scallop gill cilia (28). A physicochemical basis for such associations is also provided by the recent finding that tubulin binds strongly with, and can be embedded within, simple phospholipid bilayers (29).

The presence of cytoskeletal proteins has been reported on the cell surface in several other studies. Olden *et al.* (30) found that some fibroblast myosin may be accessible to iodination on the cell surface and is distributed uniformly over the surface as revealed by specific antibodies, but other workers have failed to confirm this finding (31). A sparse distribution of myosin has been reported on the outer membrane surface of platelets (32, 33). By using lactoperoxidase iodination, Owen *et al.* (34) have reported that actin is present on the surface of normal resting B lymphocytes, although they failed to demonstrate such actin by immunofluorescence staining with antibodies against actin. The present study has not confirmed this report when normal human B lymphocytes obtained from defibrinated blood were used.

Microtubules and microfilaments participate in various cell functions, making any interpretation of the biological significance of their appearance on the surface of transformed lymphocytes particularly difficult. It might be concluded, however, on the basis of the data presented that this event may be related to a common step in cell differentiation, occurring whenever human lymphocytes are triggered away from their normal resting stage. One possible explanation might be that the appearance of actin and tubulins on the surface of such cells reflects a switch to the production of different subisotypes of actin or tubulins. From an immunological standpoint, the appearance of cytoskeletal proteins on the surface of transformed lymphocytes and leukemic cells might constitute a new immunogenic challenge to the host and might be related, for example, to the appearance of smooth muscle antibodies in patients with lymphoma (35, 36). The presence of actin on the surface of such cells might also hold important implications for the regulation of immunoglobulin-mediated signals between lymphocyte subsets in view of the capacity of actin to form soluble complexes or crosslinked matrices with immunoglobulins (37). An additional area worthy of particular consideration is related to the possible direct role of cytoskeletal membrane components upon lymphocyte interaction and cooperation.

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