The binding of an appropriate ligand to its specific receptor on the membrane of T cells triggers a cascade of events involved in T cell activation. An important yet unanswered question is how the mitogenic signals are transmitted through the cytoplasm and into the nucleus. The present study was carried out to determine changes in the microtubule (MT) system following T cell activation. Fluorescence microscopy was employed to examine the organization of the microtubule network in human peripheral blood T cells in response to four different mitogens (phytohemagglutinin, concanavalin A, anti-CD3, and phorbol 12-myristate 13-acetate). The microtubules increase in length, number, and complexity of distribution 20 h after mitogenic stimulation. Using an in situ direct analysis protocol consisting of selective extraction of cells with detergent and Ca**+, 11 protein species, which fulfill the operational definition of microtubule-associated proteins (MAPs), were identified in resting human T cells. Alterations in the expression of these protein species were studied following mitogenic stimulation. These alterations in MAPs expression were also found in purified blast cell fractions indicating that they were specific changes occurring in activated T cell populations. These observations suggest a role for MT and MAPs in the cascade of human T cell activation.

The role of the cytoskeleton, especially MT and MAPs, in the transduction of proliferative signal(s) in lymphocytes remains poorly understood (19–22), mainly due to technical difficulties in examining MT microscopically and analyzing MAPs biochemically. We have overcome these technical problems by adapting an in situ direct analysis protocol consisting of selective extractions of cells followed by two-dimensional gel electrophoresis (18, 23). With this analytical methodology, we have identified for the first time several protein species operationally defined as MAPs (23) in human T lymphocytes. The identification of MAPs by this selective extraction procedure is based on two major criteria: (a) that the presence of putative MAPs is absolutely dependent on the simultaneous presence of intact MT in situ, and (b) conditions which depolymerize MT would release these proteins from cytoskeletal preparations (34, 35). The major advantage of this selective extraction procedure lies in its ability to identify MAPs which are associated with MT in situ (23). In contrast, most of the previous work on the identification of MAPs has relied on the reconstitution of MT through repeated cycles of temperature-dependent assembly and disassembly in situ (reviewed in Ref. 14). One major drawback of this in situ reconstitution procedure is that it cannot be used to identify MAPs from cells and tissues which have a low concentration of tubulin, such as lymphocytes, which does not allow the

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1 The abbreviations used are: MT, microtubules; MAPs, microtubule-associated proteins; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PMA, phorbol 12-myristate 13-acetate; PHA, phytohemagglutinin; ConA, concanavalin A; FBS, fetal bovine serum; EGTA, ethylenbis(oxethylenlenesulfonato)tetraacetic acid; PIPES, 1,4-piperazinediethanesulfonic acid.
vitro assembly of MT. In addition, using a recently modified procedure for indirect immunofluorescence, we have observed marked alterations in the organization of the MT network in human T cells following mitogenic stimulation.

MATERIALS AND METHODS

Cells—Buffy coats were obtained from apparently healthy blood donors. Resting peripheral blood T lymphocytes were purified by centrifugation through an Isolymph gradient (Gallard and Schlessinger), followed by a nylon wool column separation (24). The preparations yielded 95–94% T cells as determined by staining with an anti-human CD3 monoclonal antibody (Oncogen) followed by flow cytometric analysis.

Mitogenic Stimulation of Human T Cells—Mitogenic lectins phytohemagglutinin (PHA-M) (Gibco Laboratories) and concanavalin A (ConA) (Sigma), phorbol 12-myristate 13-acetate (PMA) (Sigma), and an anti-CD3 antibody (64.1) were used individually to stimulate T cells. T cells were always maintained at a density of 2 × 10⁶ cells/ml in RPMI medium with 10% heat-inactivated fetal bovine serum (FBS). Mitogens were added at various doses, and the cells were plated out onto 96-well plates (Costar). DNA synthesis was measured by determining the amount of [3H]thymidine incorporated into tri-chloroacetic acid-insoluble material by scintillation counting.

Immunofluorescent Staining of MT in T Cells—To visualize MT, T lymphocytes were pelleted onto glass slides in a Cytospin 2 (Shandon) centrifuge. Cells were fixed with 3.7% formaldehyde in a microtubule stabilizing buffer (PM2G) containing 0.1 M PIPES, 1 mM MgSO₄, 2 mM glycerol, 2 mM EGTA, pH 6.5, followed by gentle extraction with 0.5% Nonidet P-40 in phosphate-buffered saline. MT were stained using an indirect immunofluorescence protocol with a polyclonal rabbit anti-tubulin primary antibody (Polyscience) and a rhodamine-conjugated sheep anti-rabbit IgG antibody (Cappel Laboratories) as the secondary antibody.

Metabolic Labeling—Purified resting human T cells were suspended in RPMI 1640 medium supplemented with penicillin, glutamine, and 10% heat-inactivated FBS. The cells were incubated overnight at 37 °C in 7% CO₂, washed twice with Hanks’ balanced salt solution and then labeled with [35S]methionine (Du Pont-New England Nuclear) centrifuged. Cells were fixed with 3.7% formaldehyde in a microtubule stabilizing buffer (PM2G) and extracted using a modification of the selective extraction procedure (26) as 4 h after treatment (Fig. 3E).

Electrophoresis and Fluorography—Ca²⁺ extracts were analyzed on 7.5% SDS-PAGE (25) as well as on two-dimensional gels with isoelectric focusing (pH 4.3–7.2) in the first dimension and SDS-PAGE in the second (26). Fluorography was performed using ENHANCE (Du Pont-New England Nuclear). Dried gels were exposed to photographic Kodak X-Omat AR film at −80 °C. Integrated intensities of each protein spots on autoradiograms were quantitated using a Visage 60 densitometer (BioImage). All data were obtained from at least three separate donors for each experiment.

Fractionation of Lymphoblast Population of Activated Human Peripheral Blood T Cells—Discontinuous Percoll gradients were used to separate lymphoblasts from 20-h PHA-stimulated human T cell populations on the basis of their density difference (27). Gradients between 50% and 30% with a 10% differential between layers usually ensured a good separation of blast cells. The fraction with a density < 1.056 g/ml contained about 74% blast cells with a viability of 98% as determined by the Trypan Blue exclusion assay.

RESULTS

Mitogenic Stimulation of Purified Human T Cells—The optimum doses for mitogenic stimulation of human T cells with PHA, ConA, anti-CD3, and PMA were determined using [3H]thymidine incorporation and scintillation counting. At 2 × 10⁶ T cells/ml, the optimum doses for PHA, ConA, anti-CD3, and PMA were 5 μl/ml, 5 μg/ml, 2.5 μl/ml, and 2 ng/ml, respectively (Fig. 1). Time course studies showed that DNA synthesis in PHA-stimulated T cells peaked between 48 and 72 h following mitogenic stimulation, and that no DNA synthesis was observed within the first 24 h (Fig. 2).

Organization of MT Network in Resting and Stimulated Human T Cells—We examined the effect of PHA stimulation on the MT network organization in human T cells. Resting T cells contain a clearly defined microtubule organizing center from which all MT (although relatively few) appear to emanate toward the periphery (Fig. 3A). On stimulation with PHA (1.25 μl/ml), an increase in the number and length of MT was evident at 20 h (Fig. 3B), and the changes became more pronounced at 48 h (Fig. 3C) and 72 h (Fig. 3D). Furthermore, MT are distributed in a more complex network in PHA-stimulated cells (compare Fig. 3A with 3B, 3C, and 3D) showing an increased abundance in distribution along the cell periphery. Similar alterations were observed in T cells stimulated with ConA, anti-CD3, and PMA (data not shown). These results indicate that marked alterations in the MT network organization are associated with T cell proliferation. It should be noted that incubation with nocodazole (33 μM), a MT depolymerizing agent, resulted in the depolymerization of MT as evidenced by the absence of a MT network as early as 4 h after treatment (Fig. 3E).

Identification of MAPs in Resting Human T Cells—Although extraction of metabolically labeled T cells with 0.5% Nonidet P-40 removed 85–90% of the soluble cell protein, it did not affect the cytoplasmic MT which were preserved in their original distribution resembling those shown in Fig. 3, A–D, as assessed by immunofluorescence. However, subsequent extraction of the Nonidet P-40-resistant fraction with buffers containing 5 mM CaCl₂ released MT depolymerization products containing tubulin, MAPs, and a set of background polypeptides. Autoradiograms obtained following SDS-PAGE and two-dimensional electrophoresis of these Ca²⁺ extracts (+MT, Fig. 4A) were compared to those obtained from parallel extracts derived from cells which had been pretreated with nocodazole and therefore contained no intact MT (−MT, Fig. 4B) as also confirmed by immunofluorescence (Fig. 3E). That treatment with nocodazole effectively removes most of the tubulin during Nonidet P-40 extraction without affecting action was further verified by SDS-PAGE (−MT, Fig. 5) and two-dimensional electrophoresis (Fig. 4B).

Thus, only those proteins which are (a) present when MT are intact and (b) solubilized in the presence of excess Ca²⁺ have been defined as MAPs. By this operational definition, proteins in the −MT extract (Fig. 4B) whose integrated intensity was reduced by at least 50% compared to the integrated intensity of identical proteins in the +MT extract (Fig. 4A) were considered MAPs. Comparison of the protein profiles of these extracts from different donors revealed that at least 11 proteins qualified as MAPs by the above criteria are present in resting human T cells. The nomenclature of the MAPs identified here has been arbitrarily set so that the first number indicates the apparent molecular weight (M, × 10⁻⁶) and the second number refers to the isoelectric point (pI) of the protein identified as a MAP. Among the species indicated by arrowheads in Fig. 4, three MAPs are of the same M, as α-tubulin (54/5.1, 54/5.5, and 54/6.0) and three MAPs are of the same M, as β-tubulin (50/5.1, 50/5.4, and 50/6.0) but with different pI values. Fig. 6 schematically summarizes the MAPs profile in resting human T cells.
MT, MAPs, and Human T Cell Activation

**FIG. 1.** Dose-response curves for PHA, ConA, PMA, and anti-CD3 mitogenic stimulation in human peripheral blood T cells. T cells were purified and cultured at 2 x 10^6 cells/ml in 96-well plates in RPMI containing 10% heat-inactivated FBS. Mitogens at varying doses were added to the wells at the time of plating. After incubation for 45 h, the cells were pulse-labeled with [³H]thymidine (1 μCi/well) for 3 h and then harvested onto glass fiber filter discs. The incorporation of [³H]thymidine was measured to determine the optimum dose for stimulation of DNA synthesis.

**FIG. 2.** Kinetics of PHA stimulation of human peripheral blood T cells. Purified T cells were cultured as described above. PHA (5 μl/ml) was added at the time of plating, and the cells were harvested at the indicated time points. Three h prior to harvesting, the cells were pulsed with [³H]thymidine, and the incorporation of [³H]thymidine was measured.

**FIG. 3.** Organization of MT network in resting and PHA-stimulated human T cells. Resting and stimulated human T cells were fixed and stained for MT visualization. Presented here are micrographs showing MT in resting (A) and PHA-stimulated T cells for 20 h (B), 48 h (C), and 72 h (D). Note the absence of a MT network in T cells incubated with nocodazole, a MT depolymerizing agent, for 4 h prior to detergent extraction in resting T cells (E).

Alterations in the Expression of MAPs Following Stimulation with PHA—Stimulation of resting human T cells with PHA (5 μl/ml) induced a dramatic alteration in the expression of MAPs. As early as 2 h following stimulation with PHA, there was a modest increase in the level of α- and β-tubulin along with an increase in the expression of MAPs 50/5.1, 50/5.4 and 50/6.0, 52/5.2, 54/5.1, 54/5.5, and 54/6.0. These increases were more apparent at 20 and 48 h (Fig. 7). There was a considerable decrease in the level of MAP 107/4.8 and 30/4.6 at 20 h and 48 h following stimulation, whereas the amount of MAPs 87/4.8 and 121/5.8 remained essentially unchanged.

Alterations and Quantitation of Altered Expression of MAPs in T Lymphocytes Stimulated with Different Mitogens—Based on the assumption that the expression of proteins which play an important role in cell cycle progression should be uniform in any mitogenic response, regardless of the stimulatory agent, we examined the MAPs profile in human T cells following stimulation with a variety of mitogens. Twenty h after stimulation with mitogens, when the cells are in the middle to late G1 phase of the cell cycle, alterations were observed in the expression of MAPs. For example, marked increases were observed in the integrated intensity of MAP 52/5.2, reaching 294-3500% of untreated control cells in PHA-treated cells, 200-805% in ConA-treated cells, 347-933% in anti-CD3-treated cells, and 279-1026% in PMA-treated cells. MAPs 50/5.1, 50/5.4, 50/6.0, 54/5.1, 54/5.5, and 54/6.0 showed increases ranging from 200%-1971% with all mitogens tested. In contrast, the integrated intensity of MAPs 107/4.8 and 30/4.6 showed a decrease of 25%-91% over untreated control cells with all four mitogens. Fig. 8 is a representative profile of MAPs in T cells isolated from a single donor and stimulated with four mitogens. Thus, all four mitogens (PHA, ConA, anti-CD3, and PMA) produce similar patterns of changes in
MT, MAPs, and Human T Cell Activation

Identification of MAPs in resting human T cells using a selective extraction method. Autoradiograms were obtained following two-dimensional gel electrophoresis of Ca²⁺ extracts derived from 10⁶ cells with an intact MT network, +MT (A), and those derived from cells preincubated with nocodazole and thus containing no intact MT network, −MT (B).

Confirmation of the effective removal of tubulin during Nonidet P-40 extraction of nocodazole-treated cells. Fluorograph of Ca²⁺ extracts from resting human T cells (+MT) and nocodazole-pretreated T cells (−MT) reveals the absence of tubulin from the latter, whereas the level of actin remains unchanged.

The expression of MAPs 20 h following stimulation (Table I). It should be noted that the direction of these alterations (increase or decrease) is consistent among donors, although the extent of responses varies from donor to donor.

MAPs Profile in Fractionated Lymphoblasts—The results from studies with mitogen-activated normal human T cells raised an important question, that is, whether the alterations in the levels of tubulin and MAPs occurred as a result of contribution by blast cells. To assess this possibility directly, human T cells activated with PHA for 20 h were fractionated using a discontinuous Percoll density gradient. The blast cells by virtue of their lower density floated to the top of the gradient and were collected. They were labeled with [³⁵S]-methionine and then processed for the identification of MAPs. As presented in Fig. 9B, the profile of MAPs of such PHA-treated and Percoll gradient-fractionated lymphoblasts showed similar changes as seen in unfractionated T cell populations stimulated with mitogens for 20 h (compare with Fig. 7). These changes include a marked increase in the integrated intensity of MAP 52/5.2, moderate increases in MAPs 50/5.1, 50/5.4, 50/6.0, 54/5.1, 54/5.5, and 54/6.0, and a decrease in MAPs 107/4.8 and 30/4.6.

DISCUSSION

Much of the current understanding of the structure and function of the cytoskeleton has come from the identification...
FIG. 8. Alterations in the profiles of MAPs in human T cells stimulated with mitogens for 20 h. Composite autoradiograms showing MAPs profile in T cells stimulated with PHA, ConA, anti-CD3, and PMA for 20 h. Arrowheads point to MAPs showing altered expression with $M_r$ ranging between 50,000 and 54,000 (A), 87,000 and 121,000 (B), and 25,000 and 33,000 (C).

TABLE I

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<th>anti-CD3</th>
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of major protein components of cytoskeletal elements. Microtubules, one of the three principal filamentous elements of the cytoskeleton, are formed through self-association of their major constituent protein, tubulin, which is a heterodimer composed of $\alpha$ and $\beta$ subunits (13). Modulations of these highly conserved proteins may be necessary for the different structural and functional requirements of various cell types. These modulations may result either directly from covalent modifications of tubulin or indirectly via molecular alterations in MAPs. We have demonstrated (Fig. 3) a well defined MT network in resting human T cells. Stimulation of resting T cells with mitogens such as PHA, ConA, anti-CD3, and PMA leads to alterations in the MT network, with an increase in the number and length of MT emanating from the MT organizing center. In addition, most MT appear to be distributed in a highly organized fashion along the periphery of the cell. This may represent either a redistribution of MT in the peripheral area of the cell and/or an increase in the synthesis of tubulin which then assembles into MT polymers. These findings suggest that mitogenesis may alter the MT dynamics of the cell. Indeed, it has been well established that the unpolymerized tubulin pool regulates the synthesis of new...
Regulation of α-tubulin genes also occurs by post-transcriptional stabilization of either mature message or intranuclear precursors (31, 32). It has also been reported that an increase in the transcription of tubulin gene occurs early during T cell activation (33). This could be explained by a decrease in the tubulin pool, due to its increased utilization in the formation of MT polymers in activated T cells, which promotes transcription of the tubulin gene.

Since MAPs are important in regulating MT dynamics, we undertook the task to identify MAPs in resting T cells in order to determine if the expression of these proteins changed following mitogenic stimulation. Using a modification of the selective extraction procedure described by Solomon (23), we have identified a set of operationally defined MAPs in resting human T cells.

Using this procedure, we have previously identified MAPs in Swiss 3T3 cells and observed a rapid phosphorylation of these proteins through distinct mitogenic pathways (18). In resting human T cells, 11 proteins have been identified which fit the operational definition of MAPs (Figs. 4 and 6). This novel profile of MAPs is very different from those described in brain and other cultured cells. This in itself is not surprising since there is ample evidence for compartmentalization of MAPs among specific cell types (14, 38, 39, 40). Among the 11 species, MAPs having the same α- or β-tubulin might be unique MAPs. However, the possibility of their being isoforms of tubulin or post-translationally modified tubulin cannot be ruled out. The 11 species of MAPs identified above may still represent only a minimum profile of MAPs as those proteins which are bound to MT as well as to some other cytoskeletal element may not be completely removed together with tubulin during nocodazole depolymerization or may not be solubilized by Ca2+ and hence will not fit into the criteria for MAPs (23). For the most part, the blast cell fraction and unfractionated mixed cell populations show similar alterations in the profile of MAPs, with a few exceptions (Fig. 9).

It should be noted that one of the four donors tested shows a decrease in the expression of MAP 50/5.1 while another donor shows a decrease in the expression of MAPs 50/5.4 and 54/6.0 (data not shown). It is not clear if these variations are due to a unique response to mitogens of these individual donors. Nevertheless, the pattern of an increase or decrease in MAPs was always consistent in all donors tested with all four mitogens, although the extent of change varied among donors. This is not unexpected since the degree of response to various mitogens varies among individual donors. Since the donors were randomly selected for this study, it is possible that factors such as age, sex, and the general health state might have contributed to the wide range of responses to mitogens.

The strikingly similar pattern of expression of MAPs in response to the four mitogens used in this study may occur at several levels in the signalling cascade. One possibility is that by virtue of the association between surface receptors and cytoskeletal elements (8–12), the ligand receptor binding may elicit a direct effect on MT and/or MAPs resulting in subsequent transcription of genes encoding for the same proteins. Another possibility is an induction of identical second messengers by the various mitogens which then induces the transcription of MAP genes. A third possibility is the induction of diverse second messengers with a final converging common pathway which in turn exerts the transcriptional or post-transcriptional regulation of genes encoding MAPs. In addition, it is tempting to speculate a similar feedback regulation of MAP genes by either tubulin dimers or MAPs themselves, perhaps resembling the regulation of tubulin expression.

T cell activation is a multistep process of fundamental importance to the body’s successful mounting of an immune response. Most circulating cells in the peripheral blood are resting (G0) T cells, which can be activated to enter the cell cycle by a variety of ligands such as antigen in the context of major histocompatibility complex gene products, antigens against certain cell surface determinants, and mitogenic lectins such as ConA and PHA (1). MAPs are substrates for several protein kinases including a Ca2+/calmodulin-dependent kinase (44), a cAMP-dependent kinase which is bound to the brain MAP 2 (45, 46), and protein kinase C (18). Covalent modification of these MAPs in T cells may lead to an alteration in the integrity of cytoplasmic MT and might play a role in the transduction of a mitogenic signal(s) across the cytoplasm from the membrane to the nucleus. In lymphocytes, mitogens induce a departure from G0 and a slow transition through a lag phase, G1, which lasts for 26–30 h prior to DNA synthesis (47). It is during this lag phase that sequential gene expression is induced by extracellular signals such as mitogens, growth factors, and interleukins (48–51), which finally culminates in DNA synthesis and cell division. Thus, the G1 interval accommodates both the stage-specific events necessary to initiate the S phase and the ongoing continuous activities related to cell mass accumulation (52) which have been speculated to be two mutually exclusive events (53). The preferentially enhanced expression of MAPs following mitogenic stimulation of T cells might represent products of cellular genes that respond to early steps of mitogenesis and whose function in cell growth is required at later steps. The coordinated synthesis and interaction of these proteins produced in the G1 phase of the cell cycle has been speculated to play an important role in the regulation of entry of cells into the S phase of the cell cycle and the onset of mitosis (54–56). We have recently observed marked differences in the expression of these proteins in human leukemic T cells in comparison with normal T cells. Thus, the altered expression of MAPs following T cell activation might be important in cell cycle progression.

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