

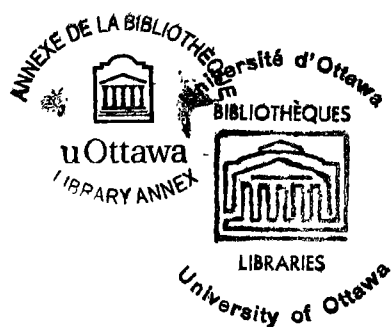
Changes in the Microtubule and Tubulin Contents  
of Lymphocytes During Activation.

by

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## ABSTRACT

Changes in the microtubule system of lymphocytes were examined during manipulation of two lymphocyte functions. Splenic lymphocytes from nude mouse, which were 95% B cells, were induced to cap surface Ig by incubation with anti-Ig antibody. Cells which patched or capped surface Ig showed an altered microtubule distribution by immunofluorescent staining. To determine if there was a change in microtubule assembly during capping, resting and capped cells were examined by electron microscopy. No significant difference was found in the number of microtubules in sections through the MTOCs of resting cells and of capped cells. This suggests that the change in immunofluorescent staining pattern results from a change in the spatial organization of the microtubules.

Immunofluorescent staining showed increased microtubule networks in stimulated mixed cell and nude mouse B cell populations. The tubulin contents in resting populations of mixed cells and of nude mouse B cells were 3.6% and 2.9% respectively. These increased to 5.1% and 3.2% after 24 h of stimulation. The difference in tubulin contents of 2.9% and 4.4% in live and unfractionated (live and dead) resting B cell populations, and the difference between mixed, B cell,

and T cell populations emphasizes the need to use selected populations for these determinations.

The microtubule networks of T cell populations stimulated for 0, 24 and 48 h were examined by immunofluorescent staining and electron microscopy. Increased microtubule networks were seen in cells which had increased in size during stimulation. The most extensive networks were seen in 48 h stimulated blast cells. The increase in microtubule number during stimulation was determined by electron microscopy of the MTOC regions of cells in resting and stimulated populations. The mean number of microtubules per section through the MTOC increased from  $15.7 \pm 7.2$  in resting cells to  $26.3 \pm 9.2$  and  $35.1 \pm 14.6$  in 24 h and 48 h stimulated populations respectively.

Direct measurements of tubulin and actin contents, as a percent of total cell protein, and of total cell protein (TCP) contents were made on resting and stimulated T cell populations from which contaminating dead cells had been removed. As a percent of TCP, tubulin increased from 0.8% in resting cells to 1.2% in a 24 h stimulated population, and to 1.3% in a 48 h stimulated population. The TCP contents were 25, 29 and 39  $\mu\text{g}/10^6$  cells in resting, 24 h and 48 h stimulated populations respectively. The total tubulin content increased 2.5 fold, from 0.20  $\mu\text{g}/10^6$  resting cells to 0.51  $\mu\text{g}/10^6$  cells in a 48 h stimulated population. As per-

cent of total cell protein, the actin content increased from 2.3% to 3.1% in the first 24 h of stimulation, but dropped to 2.5% in 48 h stimulated cells. The total actin content per cell, however, increased over 48 h of stimulation from 0.58 to 1.0  $\mu\text{g}/10^6$  cells.

I have used these numbers to estimate the total and polymerized tubulin contents. During stimulation the total tubulin content increases from 0.20 pg in a resting T cell to 1.0 pg in a blast cell. The percent of tubulin in polymer form increased from 65% in a resting cell to 93% in a blast cell, and the concentration of soluble tubulin dropped from 0.74 mg/mL in resting cells to 0.12 mg/mL in stimulated cells. The large increases in tubulin and microtubule contents during stimulation make lymphocytes a model system to study the regulation of tubulin synthesis and of its assembly into microtubules.

## RESUME

Lors de la manipulation de deux fonctions lymphocytes, on a pu remarquer des modifications au niveau de leur système microtubulaire. Des splénocytes, dont 95% sont des cellules B provenant d'une souris nue, ont été induits à la formation de capuchons Ig superficiels par incubation avec l'anticorps anti-Ig. Au moyen de l'immunofluorescence, on a observé que la distribution des microtubules a été modifiée chez les cellules où il y a eu formation de plaques ou de capuchons Ig superficiels. Afin de déterminer s'il y a eu une modification dans l'assemblage des microtubules lors de la formation des capuchons, on a observé des cellules capuchonnées et au repos, au microscope électronique. En ce qui concerne le nombre de microtubules, aucune différence importante n'a été notée entre les cellules au repos ou capuchonnées dans les sections comportant des centres d'organisation microtubulaire (MTOC). Ceci laisse croire que les modifications observées par l'immunofluorescence dans la distribution des microtubules proviennent d'un changement dans leur organisation spatiale. Par l'immunofluorescence on a observé une augmentation marquée des réseaux microtubulaires dans les populations stimulées des cellules mixtes et des cellules P de souris nues. Dans les cellules mixtes et dans les cel-

lules B de souris nue, au repos, la teneur en tubuline était respectivement 3,6% et 2,9%, pour ensuite augmenter à 5,1% et 3,2% après 24 h de stimulation. Ces différences de 2,9% et de 4,4% dans la teneur en tubuline de cellules B non-stimulées vivantes et les cellules P non-fractionnées (vivantes et non vivantes), et la différence entre les cellules mixtes, les cellules B et les cellules T démontrent qu'il est important de se servir des populations sélectionnées établir la teneur en tubuline des différentes cellules.

A l'aide de la technique de l'immunofluorescence et du microscope électronique, on a procédé à l'observation des réseaux microtubulaires des populations de cellules T qui ont été stimulées pendant 0, 24 et 48 h. On a noté une augmentation des réseaux de microtubules dans les cellules qui avaient augmenté de volume lors de la stimulation. Les réseaux les plus vastes ont été observés chez les cellules blastiques stimulées pendant 48 h. L'augmentation du nombre de microtubules lors de la stimulation a été déterminée par un examen au microscope électronique des régions MTOC de populations cellulaires au repos et stimulées. Le nombre moyen de microtubules par section dans la région MTOC a augmenté de  $15,7 \pm 7,2$  chez les cellules au repos à  $26,3 \pm 9,2$  et  $35,1 \pm 14,6$  chez les populations stimulées respectivement pendant 24 et 48 h.

On a effectué, chez des populations de cellules T au repos et stimulées dont on avait retiré les cellules mortes contaminants, des mesures directes de la teneur en tubuline et en actine, en tant que pourcentage du contenu protéique total de la cellule, et du contenu protéique de la cellule (TCP). La tubuline est passée de 0,8% du TCP chez les cellules au repos, à 1,2% chez les cellules stimulées pendant 24 h, et à 1,3% chez les cellules stimulées pendant 48 h. Chez les populations cellulaires qui ont été stimulées pendant 0, 24 et 48 h, la teneur en TCP était respectivement de 25, 29 et 39 ug/10<sup>6</sup> cellules. Le contenu total de tubuline s'est multiplié par 2,5, passant de 0,20 ug/10<sup>6</sup> cellules chez une population au repos, à 0,51 ug/10<sup>6</sup> cellules après une stimulation de 48 h.

L'actine a augmenté de 2,3% du TCP à 3,1% après les 24 premières heures de stimulation, mais a diminué à 2,5% après 48 h de stimulation. Toutefois, le contenu total d'actine par cellule a augmenté de 0,58 à 1,0 ug/10<sup>6</sup> cellules au cours d'une période de stimulation de 48 h.

On a utilisé ces résultats pour évaluer la quantité de tubuline total et de tubulin polymérisée. Lors de la stimulation, la teneur total de tubuline a augmenté de 0,20 pg chez une cellule T au repos à 1.0 pg chez une cellule en blastogénèse. La proportion de tubuline sous forme de polymère est passée de 65% chez une cellule au repos à 93%

chez une cellule en blastogénèse, et la concentration de tubuline soluble a diminué de 0,74 mg/mL dans les cellules au repos à 0.12 mg/mL dans les cellules stimulées. La forte augmentation de la teneur en tubuline et en microtubules, lors de la stimulation des lymphocytes, nous permet de nous servir des lymphocytes comme système modèle pour l'étude de la régulation de la tubuline et de son assemblage en microtubules.

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## section I

### INTRODUCTION

The components of the cytoskeleton interact with each other and with cellular membranes to participate in maintaining cell shape and in other cellular functions. In order to understand how the cytoskeletal components work together, it is important to understand how they are organized individually.

The microtubule network is thought to have a structural role, rather than a contractile role like the microfilaments. The organized yet dynamic nature of the microtubule network is illustrated by the changes it undergoes during mitosis. Disassembly of the interphase microtubule network, assembly and disassembly of the spindle apparatus, and then assembly of interphase microtubule networks in the daughter cells suggests a highly regulated system.

The introduction will be concerned with microtubule organization and mechanisms of regulating assembly of microtubules in vivo, particularly in several tissue culture cells that have been used to study the roles of the microtubule organizing center, tubulin and microtubule associated proteins in regulating microtubule assembly.

## 1.1 MICROTUBULES

Microtubules are found in all eukaryotic cells. The participation of microtubules in a wide variety of cellular functions has been realized for some time (Porter, 1966). Microtubules are a structural component of the locomotory organelles cilia and flagella (Tilney et al., 1973). The loss of cell shape was correlated with the disappearance of cytoplasmic microtubules induced by microtubule disrupting treatments (Tilney and Gibbons, 1969; Brown and Bouck, 1973). Cell shape was regenerated and the microtubules reappeared during subsequent recovery from the treatment. Microtubule integrity was shown to be necessary for metaphase chromosome movement (Brinkley and Cartwright, 1971), and protein transport in nerve axoplasm (Fernandez et al., 1971). Movement of pigment granules in melanophores (Byers and Porter, 1977) and of cytoplasmic granules in nerve cells (Stearns, 1980) appeared to be directed by microtubules, while the motive force could have been provided by the other cytoskeletal elements. Motility of cell surface proteins can be directed by (Fogers et al., 1981) or restricted by (Oliver et al., 1980) the microtubule system.

The major microtubule proteins, alpha and beta tubulins, have MWs of 50,000 and 48,000 respectively. The primary structure of the tubulins has recently been determined by protein sequence analysis of porcine brain alpha (Ponstingl

et al., 1981) and beta (Kraus et al., 1981) tubulins, and by nucleotide sequencing of cloned cDNA copies of embryonic chick brain mRNA for alpha and beta tubulins (Valenzuela et al., 1981).

Alpha and beta tubulin associate to form heterodimers, the basic subunit of microtubules. Microtubules are cylindrical structures 24 nm in diameter. Each tubule is composed of 13 protofilaments arranged in a parallel fashion to make the tubule wall. Protofilaments consist of heterodimers stacked end to end.

Microtubule elongation is a result of the net addition of tubulin subunits to the polymer, but the exact mechanism is not clear (for a review see Kirschner, 1978). Preferential growth of brain microtubule subunits onto the distal end of isolated flagellar axonemes demonstrated polarity of in vitro microtubule assembly (Allen and Borisy, 1974). There is net assembly at one (plus) end and net disassembly at the other (minus) end of steady state microtubules in vitro. This results in a flux of subunits through steady state microtubules which is referred to as treadmilling (Marqolis and Wilson 1978). The property of treadmilling in vitro implies that microtubules with the minus end anchored to a microtubule organizing center (MTOC) would be more stable, and assemble at lower tubulin concentrations than free microtubules (Kirschner, 1980).

### 1.1.1 Spatial and Temporal Organization

MTOCs are structures which can initiate the growth of microtubules (for one recent review see Raff, 1979). The distribution of microtubule initiation sites on the MTOC can determine to a large extent, the spatial organization of the microtubule network (reviewed in Tucker, 1979). One example where the MTOC determines the precise patterning of cytoplasmic microtubules is found in the quadriflagellate alga Polytomella agilis. Parallel arrays of cytoplasmic microtubules are initiated from the rootlets which act as organizing centers in vivo. Isolated basal-body rootlet complexes initiated the in vitro assembly of microtubules from exogenous tubulin in the in vivo pattern (Stearns and Brown, 1981).

The extent to which the precise pattern of cytoplasmic microtubules is determined by the MTOC in other cell types is less clear. Well spread tissue culture cells, such as fibroblast cell lines, have a radial pattern of microtubules which extend between the central MTOC and the cell periphery (Weber et al., 1975). The MTOC in these cells, also referred to as the centrosome, consists of a centriole pair and pericentriolar material (for a review see Peterson and Berns, 1980). During recovery from a microtubule disrupting colcemid treatment, microtubules were first seen around the centrosome. At later times of recovery, the microtubules had

extended to the cell periphery (Weber et al., 1975). The radial pattern of microtubules which extends from the cell center to the periphery, is determined at least in part by the MTOC.

Microtubules have been shown at the electron microscopic level to interact with other filaments. Numerous connections were seen between the microtubules and the microtrabecular lattice in the cell margins of fish erythrocytes (Byers and Porter, 1977). In cultured chick embryo fibroblasts, microtubules were seen to be inserted onto by both actin filaments and the microtrabecular lattice. Stereo images revealed that the microtubules frequently changed direction at sites where the actin filaments made contact, and that the microtubule pattern was greatly altered if the microfilament system was disrupted with cytochalasin B (Schliwa and van Blerkom, 1981). These observations suggest that other cytoskeletal elements influence the spatial organization of microtubules.

Temporal organization of microtubules is well illustrated by changes in the microtubule networks of dividing cells. In mitotic cells the cytoplasmic microtubule network disappears before the spindle apparatus begins to form (Weber, 1976). Microtubules can then be seen in the spindle apparatus but are not found throughout the rest of the cell (McIntosh, 1979). Following cytokinesis the cytoplasmic microtu-

bules are reassembled in the daughter cells (Osborn and Weber, 1977).

## 1.2 COMPONENTS OF MICROTUBULE ASSEMBLY

Among the possible components of regulation of microtubule assembly are the MTOC, the availability of assembly competent tubulin, microtubule associated proteins (MAPs), and calmodulin-Ca<sup>2+</sup> mediated microtubule disassembly.

1. MTOCs are organelles which, by definition, organize microtubules. The existence of MTOCs was proposed to account for the initiation and directional control of precise arrays of microtubules seen in cells (Pickett-Heaps, 1969). MTOCs have been shown to initiate microtubule assembly in vivo (Osborn and Weber, 1976) and in vitro (Brinkley et al., 1980). Kinetic (Bergen et al., 1980) and morphological (Heideman and McIntosh, 1980) analyses showed that the growing, or plus end of the microtubule was distal to the MTOC. Hence the minus, or disassembly end was attached to the MTOC. The MTOC could conceivably regulate microtubule assembly by determining the number of initiation sites and, keeping in mind the in vitro property of treadmilling, by controlling the disassembly end of the microtubule.
2. The assembly of microtubules is ultimately dependent on the availability of assembly competent tubulin.

Association and disassociation rate constants were determined for the addition of dimers to the plus and minus ends of microtubules during in vitro assembly (Bergen and Borisy, 1980). Microtubules will assemble in vitro when the tubulin concentration is higher than the critical concentration (0.3 mg/ml in Bergen and Borisy, 1980), such that the rate of assembly at the plus end is higher than the rate of disassembly at the minus end. The concentration of unpolymerized tubulin in 3T3 cells has been estimated to be 1.2 mg/mL (Hiller and Weber, 1978), which is considerably higher than the critical concentration for in vitro assembly. The soluble tubulin, however, is not all necessarily available for assembly into microtubules.

Both the synthesis and the post translational modification of tubulin may regulate the availability of tubulin for assembly. In 3T6 cells, when the intracellular level of unpolymerized tubulin is increased by disrupting the microtubule network with colchicine or nocodazole, there was a rapid decrease in the level of tubulin mRNA synthesis (Cleveland et al., 1981). Due to the short half life of tubulin mRNA in these cells, the rate of tubulin synthesis dropped soon after (Ben-Ze'ev et al., 1979; Cleveland et al., 1981). A decrease in the unpolymerized tubulin levels was induced by stabilizing microtubules with taxol or

by sequestering free tubulin into paracrystals with vinblastine. This resulted in a slight increase in tubulin mRNA production, and tubulin synthesis. These studies indicate that, in these cells, the rate of tubulin synthesis may be dependent on the level of unpolymerized tubulin.

Post translational modification of tubulin may potentially influence its assembly properties. Tyrosylation of the alpha tubulin carboxyl terminus, by tyrosyltubulin ligase, did not influence the *in vitro* assembly properties (Rabin and Flavin, 1977). However, the activity of tyrosyltubulin ligase in synchronized CHO cells has been shown to change through the cell cycle, with a peak activity detected during mid S (Forrest and Klevecz, 1978). This suggests that tyrosylation may play a role in *in vivo* assembly.

Another example of post translational modification of tubulin is phosphorylation. Tubulin isolated from the S and M stages of the HeLa cell cycle contained 2-3 times more covalently bound phosphate than tubulin isolated during G1 or G2 (Piras and Piras, 1975). The role of phosphorylation in regulating tubulin assembly has not been concretely established.

3. The role of microtubule associated proteins (MAPs) in microtubule assembly has been extensively studied with in vitro assembly systems, using microtubule proteins isolated from brain (for a review see Kirchner, 1978). Two classes of brain MAPs thought to be responsible for the observed stimulation of microtubule assembly are the high molecular weight (HMW) proteins of MW 286,000 and 271,000 on SDS gels (Murphy and Borisy, 1975) and the tau proteins MW 58,000 to 65,000 (Weingarten et al., 1975). The tau proteins are unrelated to HMW proteins as determined by peptide mapping (Cleveland et al., 1977).

MAPs were incorporated into microtubules in stoichiometric rather than catalytic amounts (Sloboda et al., 1976). The HMW proteins were seen distributed along the length of in vitro assembled microtubules by electron microscopy (Murphy and Borisy, 1975). The mechanism by which MAPs promote in vitro assembly is not clear, but it is suggested that they function by adding onto the tubules and stabilizing them against disassembly (Murphy et al., 1977). If the same mechanism works in vivo then microtubule disassembly could be controlled by controlling dissociation of the MAPs.

MAPs have been localized to microtubules in tissue culture cells using immunofluorescent staining techniques. Both interphase and spindle microtubules of several tissue culture cells stained with primary antibodies made against brain tau (Connolly et al., 1977) and brain HMW proteins (Sherline and Schiavone, 1977; Connolly et al., 1978). The staining patterns suggested that the in vivo distribution of MAPs was continuous along the lengths of the microtubules in these cells.

Immunofluorescent staining and biochemical techniques have shown that a particular MAP can be wide spread through a species (Bulinski and Borisy, 1980; Duerr et al., 1981) or can be cell type specific (Duerr et al., 1981; Olmstead and Lyon, 1981).

4. Calmodulin mediates  $Ca^{2+}$  regulation of several cell enzyme systems (reviewed in Means and Dedman, 1990). The in vitro assembly of microtubules depends on the ability of the assembly buffer to chelate  $Ca^{2+}$  (Weisenberg, 1972). Calmodulin or  $Ca^{2+}$  alone, in  $\mu M$  amounts, only slightly reduced in vitro assembly of microtubules. However,  $10 \mu M$   $Ca^{2+}$  and  $0.4 \mu M$  calmodulin together totally inhibited microtubule assembly (Marcum et al., 1978). The interphase microtubules of permeabilized BSC-1 monkey cells were sensitive to  $Ca^{2+}$  levels above  $4 \mu M$ . If drugs such as stelazine

which inhibit calmodulin were used then millimolar levels of  $Ca^{2+}$  were needed to disassemble the microtubules (Schliwa et al., 1981).

Immunofluorescent staining of calmodulin showed that it was evenly distributed throughout the cytoplasm of interphase cells and not specifically associated with the microtubules (Welsh et al., 1978). In mitotic cells, however, the tubulin and calmodulin staining patterns coincided. Calmodulin was concentrated at the poles of the spindle apparatus, and fluorescence extended towards the chromosomes (Welsh et al., 1979). Immunolocalization at the electron microscopic level showed calmodulin to be concentrated where the microtubules showed extensive lateral interaction, and not around single tubules or the MTOC (DeMey et al., 1980). This does not support the idea that calmodulin plays a role in microtubule disassembly since bundles of microtubules are thought to be more stable than single microtubules.

Three of the best studied systems which illustrate the potential of these four components in the regulation of microtubule assembly, are interphase and mitotic HeLa cells, normal and SV40 transformed 3T3 cells, and differentiating neuroblastoma cells.

### 1.2.1 HeLa cells

The MTOCs of synchronized HeLa cells were tested for their ability to initiate in vitro microtubule assembly. After cold disassembly of intrinsic microtubules, cells were lysed into a solution containing brain microtubule proteins. Following incubation at 37°C to promote microtubule assembly, centriolar complexes were isolated and examined by electron microscopy. Assembly of microtubules was only seen on the centriolar complexes isolated from mitotic cells (Telzer and Fosenbaum, 1979) suggesting a cell cycle dependent change in initiation capacity of the MTOC.

An increase in tubulin content, determined by <sup>3</sup>H-colchicine binding activity, during G2 was found in HeLa cells released from hydroxyurea block (Lawrence and Wheatley, 1975). They interpret this to mean that the increase in tubulin content could play a role in regulating spindle formation. Tubulin in the S and M stages of the cell cycle contained 2-3 times more covalently bound phosphate than tubulin from cells in G1 or G2. This seemed to result from an increased protein kinase activity associated with the microtubules (Piras and Piras, 1975). The role of phosphorylation in the regulation of microtubule assembly is not known.

A more recent analysis has found that the tubulin content, as a percent of total cell protein, was the same in both exponentially growing and mitotic enriched HeLa cul-

tures (Bulinski et al., 1980B). It was also found that the tubulin from each population was the same in its ability to assemble in vitro and to co-assemble with carrier brain microtubule proteins, and in the ability of alpha tubulin to act as a substrate for tyrosyltubulin liqase (Bulinski et al., 1980). In addition, the amount and type of MAPs were the same in extracts from both cell types. This suggests that microtubule assembly is being regulated at the MTOC level.

### 1.2.2 3T3 cells

Mouse 3T3 cells display a radial pattern of microtubules which grow from a central MTOC to the cell periphery. The cells display an altered microtubule network following transformation with SV40 (Brinkley et al., 1975; Weber et al., 1975). To determine if there is a change in the initiation capacity of the MTOC following transformation, Brinkley and coworkers tested the ability of MTOCs in lysed 3T3 and SV40 transformed (SV3T3) cells to initiate the growth of microtubules in vitro, using exogenous microtubule proteins. They reported a decrease in the in vitro initiation capacity of the MTOCs following transformation which was comparable to the decrease in microtubule number seen in vivo following transformation. In addition, the average lengths of in vitro assembled microtubules initiated by MTOCs from 3T3 cells were twice as long as the microtubules initiated by MTOCs

from SV3T3 cells (Brinkley et al., 1980), suggesting that MTOCs exert some control over microtubule length. This group has more recently reported no differences in microtubule numbers, as determined by electron microscopy, in the centriolar regions of 3T3 and SV3T3 cells but that there were fewer microtubules in the cell periphery of SV3T3 cells (Zimmer et al., 1981). They suggest that the microtubules in 3T3 cells are longer, extending to the cell periphery, and possibly doubling back. They do not explain the apparent contradiction between these and the earlier results.

Reports of changes in total and polymerized tubulin contents in 3T3 and SV3T3 cells have not been consistent. Determinations using  $^3\text{H}$ -colchicine binding indicated a 50% increase in total and polymerized tubulin contents following transformation by several viruses including SV40 (Tichorn and Peterkofsky, 1979). A 50% decrease in total and polymerized tubulin contents following SV40 transformation has also been reported (Fine and Taylor, 1976). They determined tubulin content by measuring the percent of radioactivity incorporated in vivo into tubulin subsequently isolated from 1D gels.

Others, using more sensitive methods, have shown no change in tubulin content following transformation. The total tubulin content was determined to be 3.3% of total cell protein in both 3T3 and SV3T3 cells by radioimmunoassay

(Hiller and Weber, 1978), and by the percent of label incorporated in vivo into tubulin subsequently isolated by immunoprecipitation (Chafouleas et al., 1981). These tubulin values are an order of magnitude higher than the values obtained by  $^3\text{H}$ -colchicine binding assay.

Tau like MAP proteins have been isolated from the microtubules in 3T3 cells and shown to participate in in vitro microtubule assembly (Duerr et al., 1981). Immunofluorescent staining using antibody prepared against brain HMW proteins stained microtubules in 3T3 cells continuously along their length (Sherline and Schiavone, 1977). No one has looked at the MAP content of SV3T3 cells.

Radioimmunoassay determinations showed a doubling in the calmodulin content of 3T3 cells following SV40 transformation. The increase was due to a two fold higher level of synthesis relative to the rate of degradation in transformed cells (Chafouleas et al., 1981). They interpret this to mean that an increase in the calmodulin- $\text{Ca}^{2+}$  mediated disassembly is, at least in part, responsible for the decreased microtubule content in transformed cells.

### 1.2.3 Neuroblastoma cells

Neuroblastoma cells, when induced to differentiate by serum deprivation, grow long neurites. Disruption of the microtubules with colchicine prevented neurite formation, and

resulted in neurite retraction (Daniels, 1972). Electron microscopy revealed that the number and density of microtubules in neuroblastoma increased during differentiation, due to bundles of microtubules found in the neurites (Morgan and Seeds, 1975). Multiple MTOCs in undifferentiated cells were the initiation sites for cytoplasmic microtubules. During differentiation the MTOCs aggregated at the site of neurite formation, before outgrowth of the neurite (Spiegelman et al., 1979A).

Radioimmunoassay determinations showed that the total tubulin content was 4 pg per cell in both undifferentiated and differentiated neuroblastoma cells. The percent tubulin in polymer form, however, increased from 14% to 55% during differentiation (Olmstead, 1981) corresponding to the increased microtubule content seen by electron microscopy.

A 215K MW MAP can be isolated from differentiated neuroblastoma, which is not detectable in undifferentiated cells (Olmstead and Lyon, 1981). This suggests that the appearance of the MAP regulates the microtubule assembly and could play a role in microtubule dependent neurite differentiation.

The results of these three well studied systems indicate that the regulation of microtubule assembly may be accomplished in a different manner in the different cell types.

In these three systems the MTOC exhibited a change in initiation capacity which was correlated with a change in microtubule organization. The tubulin content remained the same. There could be as yet undetected changes in tubulin, such as the phosphorylation seen in HeLa cells, which alter the assembly characteristics of the tubulin. MAPs play an important role in microtubule assembly during differentiation of neuroblastoma cells. Any role they play in the changes in microtubule assembly seen during the cell cycle of HeLa cells or following SV40 transformation of 3T3 cells is not evident. Calmodulin, whose involvement in microtubule (dis)assembly is not at all clear, is implicated by association in contributing to the decrease in microtubule assembly in 3T3 cells following transformation.

### 1.3 LYMPHOCYTE ACTIVATION

Lymphocytes function in immune responses in higher vertebrates. B lymphocytes provide humoral, or antibody mediated immunity, and T lymphocytes function in cellular immunity. Activation of resting lymphocytes is necessary for immune response. Lymphocyte activation is the process whereby lymphocytes in G0 are induced to enter and complete cell division.

Polyclonal mitogens such as the plant lectins phytohemagglutinin (PHA) and concanavalin A (con A) can be used to ac-

tivate a large portion of a resting lymphocyte population in vitro (Oppenheim and Rosenstreich, 1976). Con A is a T cell mitogen; that is, it only activates T lymphocytes although it binds to the surface of both T and B Lymphocytes (Greaves and Bauminger, 1972). B lymphocytes are subsequently activated by stimulated T lymphocytes (Potash, 1980). Secondary stimulation, along with different responses of individual lymphocytes, result in a very heterogenous response to in vitro activation (reviewed in Wedner and Parker, 1976).

Many morphological and biochemical changes occur during activation. Increases in  $Ca^{++}$  uptake (Allwood et al., 1971) and  $Na^+$  and  $K^+$  transport across the plasma membrane (Kaplan, 1978) are detectable early in activation. Increases in cAMP and cGMP levels early in activation, and at the onset of S phase have been reported, but the results are by no means consistent (for a review see Hume and Weideman, 1980). Increased membrane fluidity resulting from changes in phospholipid metabolism (Pesch et al., 1977), and increased uptake of metabolites such as sugars, amino acids and nucleosides during activation have also been reported (Wedner and Parker, 1976).

Protein synthesis increases early and continues to increase throughout activation (Hausen et al., 1969). rRNA synthesis increases after about 10 h. Appearance of new mRNA in the cytoplasm before this time is due to processing

and transport of preformed mRNA (Mitchell et al., 1978). Increased DNA synthesis, which can be detected by 30 h, peaks between 48 h and 60 h and is followed by cell division. Incorporation of  $^3\text{H}$ -thymidine into DNA at 48 h, by virtue of its simplicity, is used as a general measure of stimulation in the population (Wedner and Parker, 1976).

In activated lymphocytes, both the cytoplasmic and nuclear volumes have increased but the nucleus / cytoplasm ratio has gone down. The chromatin has become decondensed (see for example Biberfeld, 1971). In the cytoplasm of activated lymphocytes, ribosomes and mitochondria have increased in number, the Golgi apparatus is highly developed, and the microtubule system is more extensive (for one example see Biberfeld, 1971).

### 1.3.1 The microtubule network in lymphocytes

Anti-Ig induced Ig capping is inhibited by high concentrations of con A. Colchicine released the inhibition of Ig capping, presumably by depolymerizing the microtubules (Edelman et al., 1973). These workers suggest that the freezing of receptors by con A is due to a direct association of the con A receptors with the microtubules, and that the mobility and distribution of cell surface receptors is modulated by the microtubules.

The inhibitory effect of anti-microtubule drugs on several aspects of lymphocyte mitogenesis such as increases seen in amino acid transport (Greene et al., 1976), RNA synthesis (Hausser et al., 1976), and DNA synthesis (Gunter et al., 1976; and many others), led to the suggestion that the microtubule system plays a key role in the regulation of lymphocyte mitogenesis. Other studies however, have found that colchicine levels which inhibit DNA synthesis had little effect on other aspects of stimulation such as lymphokine production (Sherline and Mundy, 1977) or phospholipid metabolism (Resch et al., 1977).

Edelman and coworkers have determined the time course of inhibition of activation produced when colchicine was added at varying times of culture (Wang et al., 1975; Gunther et al., 1976). From their results they proposed a model in which microtubules are necessary for the initiation of DNA synthesis brought on by the binding of mitogen to the cell surface. More recent studies have found that colchicine will inhibit DNA synthesis if added after the initiation and commitment period of activation (Pudd et al., 1979; Resch et al., 1981). These authors suggest that inhibition of DNA synthesis is part of the general disruption of cellular metabolism resulting from colchicine mediated microtubule disassembly.

Despite the potential importance of the microtubule system in lymphocyte responses, very few studies have specifically examined the microtubule system of lymphocytes or the changes in microtubule assembly which accompany these responses.

The microtubule network has been suggested to play a role in the redistribution of lymphocyte cell surface receptors. Using electron microscopy to directly look at the microtubules, it was shown that the mobility of con A receptors was restricted in cells with intact microtubule networks (Oliver et al., 1980). The restriction of mobility is released, and the con A receptors cap, when the microtubules are disassembled. Intact microtubule networks have been shown, by immunofluorescent staining and electron microscopy, in lymphocytes which have capped surface Ig (Pogers et al., 1981). These authors also suggested that there was a change in the microtubule networks, seen by immunofluorescent staining, in capped cells.

Increased microtubule networks have been detected by immunofluorescent staining and electron microscopy in con A stimulated mouse splenic lymphocytes (Rudd et al., 1979) and by electron microscopy of PHA stimulated human peripheral lymphocytes (Biberfeld, 1971).

The MTOCs of stimulated mouse splenic T lymphocytes were shown to initiate the in vitro assembly of more microtubules

from exogenous tubulin in a lysed cell system than the MTOCs from resting T cells (Schweitzer and Brown, 1981), demonstrating an increase in the initiation capacity of the MTOC during activation. No one has looked at the amount or type of MAPs in resting or stimulated cells.

Tubulin determinations made using a  $^3\text{H}$ -colchicine binding assay showed an increase in the total and polymerized tubulin contents following PHA stimulation of mixed populations of human peripheral blood lymphocytes (Pipeleers et al., 1977; Sherline and Mundy, 1977). Determinations made after 36 h of PHA stimulation showed not only an increase in tubulin content per cell but also that tubulin increased from 0.43% to 0.97% of the soluble cell protein (Sherline and Mundy, 1977). Tubulin determinations made by directly measuring tubulin specific peptides, showed that tubulin represents 4.1% of total cell protein in normal human peripheral blood lymphocytes (Anderson, personal communication). The tubulin content varied widely between donors, possibly due to the state of their immune system. Comparison of tubulins from human normal and chronic lymphocytic leukemia lymphocytes found no differences between the tubulins as a percent of soluble cell protein (1.2% by  $^3\text{H}$ -colchicine binding assay), in ability to polymerize in vitro or in cross reactivity with anti-tubulin antibody following transformation (Liebes et al., 1980).

#### 1.4 PURPOSE OF THESIS

Microtubules have been implicated in lymphocyte immune function. However, very little is known about microtubule organization in these cells. The activating lymphocyte is a potential model system to study the regulation of tubulin synthesis, and of its assembly into microtubules.

Problems with past studies of the tubulin and microtubule contents in lymphocytes are due in part to the use of poorly defined systems. Mixed populations were used, and the extent of response during stimulation was not monitored. Difficulties arise in comparing the reported values for tubulin content in mixed lymphocyte populations, determined by  $^3\text{H}$ -colchicine binding assay, to values reported for other studies on lymphocytes, or studies on other tissue culture cell types.

As an initial step in a long term study I have defined a system which can be used to examine the regulation of tubulin synthesis, and of microtubule assembly in lymphocytes. In this thesis I have used selected cell populations to minimize heterogeneity of the populations, and I have used two manipulations which induce changes in microtubule organization.

Capping of cell surface Ig is induced by incubating cells with anti-Ig antibody. Immunofluorescent staining showed an

altered microtubule network in cells which had patched or capped surface Ig. No difference in microtubule number was detected by electron microscopy of resting and capped cells. The rapid change in microtubule organization seen during capping does not seem to be useful for looking at changes in microtubule assembly, but may be useful for looking at rapid changes in spatial organization.

T lymphocytes were induced to activate by con A. Direct tubulin determinations showed a large increase in tubulin content during activation. A corresponding increase in microtubule number was seen by electron microscopy of resting and stimulated T cell populations. The coordinated increase in tubulin and microtubule contents suggests a role for tubulin synthesis in the regulation of microtubule assembly during activation of lymphocytes.

## section II

### MATERIALS AND METHODS

#### 2.1

##### PREPARATION OF CELLS

##### 2.1.1 Mixed lymphocyte populations

To isolate mixed cell populations the spleens from Balb/c mice were removed and disrupted on a wire screen into 4 mL of medium (RPMI 1640 and 10% fetal bovine serum). Large clumps were settled through a calf serum gradient. Cells were spun down and resuspended in 0.17 M NH<sub>4</sub>Cl for 7 min to lyse the red cells. Red cell membranes were removed by spinning the cells through calf serum. All operations were carried out on ice. Lymphocytes were resuspended and cultured in medium at 37°C.

##### 2.1.2 B lymphocyte populations

B cell populations were isolated from the spleens of RNC nu/nu mice, as described above for Balb/c mice. The percent of B lymphocytes in the population was determined by staining cell surface Ig. 50 ul of cells at 10<sup>7</sup>/mL in RPMI 1640 were incubated at 37°C with 200 ug/mL rhodamine conjugated goat-anti-mouse Ig (Rh-GaMIg) for 7 min. During this time

the cells were settled onto poly-l-lysine coated coverslips. Following incubation with antibody the cells, on coverslips, were washed for 15 sec in phosphate buffered saline (PBS: 7.65 g NaCl, 0.725 g NaH<sub>2</sub>PO<sub>4</sub>, 0.212 g KH<sub>2</sub>PO<sub>4</sub> per liter, pH 7.0), fixed for 30 sec in 3% paraformaldehyde in PBS at pH 7.0. Cells were then washed twice for 1 min in PBS and mounted on a slide in 50% glycerol (V/V) in PBS at pH 7.8. Samples were examined with epifluorescent and phase contrast optics and 400 cells were examined to determine the percent of Ig bearing cells in the population.

Poly-l-lysine coated coverslips were prepared immediately before use. Clean coverslips were flooded with 0.1% poly-l-lysine in water for 1 min. Coverslips were rinsed twice with deionized water, then air dried.

Cells on poly-l-lysine coated coverslips were processed by carefully transferring the coverslips to weighboats containing the wash and fix solutions.

### 2.1.3 T enriched populations

Balb/c mice were killed by cervical dislocation. The spleens were removed and disrupted on a wire screen into 4 mL of medium. T enriched populations were isolated by the method of Bourguignon et al. (1979). Total splenocytes were suspended at  $3-4 \times 10^8$ /mL in 100 ug/mL wheat germ agglutinin and layered on top of a cushion of 50% calf serum. The B

lymphocytes and red cells clumped and settled through the gradient. After 30 min the top layer of cells, consisting of T lymphocytes and contaminating red cells, was removed and washed in 0.2 M N-Acetyl D-glucosamine to bind free wheat germ agglutinin. Any contaminating red cells were lysed by incubation in 0.17 M NH<sub>4</sub>Cl for 7 min. The lymphocytes were spun through a calf serum gradient to isolate them from red cell membranes, then suspended in medium for culturing. All operations were carried out on ice.

The percent of contaminating B cells in the T lymphocyte population was determined by staining cell surface Ig, as described above. At least 400 cells were examined to determine the percent of Ig positive (B) cells. Only populations containing less than 5% Ig bearing cells were used in the subsequent experiments.

#### **2.1.4 Live cell populations**

Live cell populations were prepared (method of vonBoehmer and Shortman, 1973) for the protein determinations, and the cell size profiles measured on a Coulter counter channelizer model Zf. To remove dead cells, cultured cell populations were collected by gentle centrifugation. The pellets were resuspended into a low ionic strength buffer (LISB) to a density of  $5 \times 10^7$ /mL. The LISB was NaCl, KCl, MgSO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub> each at 4.2 mM; 2.8 mM CaCl<sub>2</sub>; 25.2 mM Hepes; 38.5 mM glucose and 246 mM sorbitol at pH 7.0. In LISB, the lead

cells clumped together, and were separated out when the cell suspension was passed over a nylon wool filter in a pasteur pipet. All operations were done on ice. The filtrate contained the live cell population. Cell viability was determined by exclusion of the vital dye trypan blue.

## 2.2 LYMPHOCYTE ACTIVATION

### 2.2.1 Cell culture

Lymphocytes were cultured at a density of  $1.8 \times 10^6$ /mL in media supplemented with 2 mM L-glutamine, penicillin-streptomycin (100 units/mL-100 ug/mL) (Difco) buffered to pH 6.9 with HEPES. Cells were cultured in sealed tissue culture flasks at 37°C. Cultures were supplemented every 24 h with 10% volume of complete medium to improve cell viability over long culture times. Cell numbers were counted on a haemocytometer, and cell viability was determined by exclusion of the vital dye trypan blue.

### 2.2.2 Lymphocyte activation

Mixed and T lymphocyte populations were stimulated by the addition of Concanavalin A (Con A, Calbiochem) to the optimal dose of 3 ug/mL. The activation of Con A stimulated populations was assayed by an increase in cell size during stimulation, and by  $^3\text{H}$ -thymidine incorporation at 48 h of stimulation.

To stimulate B cells, nude mouse splenic lymphocyte populations were supplemented with 20%  $\alpha$  enriched lymphocytes to improve the response of the B cells (Owens, 1981), and this population was stimulated with 3  $\mu$ g/mL con A and 25  $\mu$ g/mL lipopolysaccharide from Echerichia coli. Activation was measured by increase in cell size during stimulation and by  $^3\text{H}$ -thymidine incorporation at 48 h.

Increase in cell size during stimulation was monitored by measuring cell diameters with an ocular micrometer. Cell size profiles of resting and stimulated live cell populations were measured using a Coulter counter channelizer model Zf.

To assay mitogen induced DNA synthesis, after 48 h of culture, resting and stimulated cell populations were pulsed for 1 h with 2  $\mu\text{Ci/mL}$  of  $^3\text{H}$ -thymidine (New England Nuclear), harvested onto glass filters, washed with 5% TCA, then ethanol, and dried. TCA insoluble radioactivity was counted in a scintillation counter.

### 2.3 CAPPING

Following isolation, cells were cultured at 37°C in medium for 60 min. This allows the microtubules to recover from cold induced disassembly before capping (Togers et al., 1981). Cells were collected and resuspended to a density of  $10^7/\text{mL}$  in RPMI 1640.

For immunofluorescence studies, 45  $\mu$ L aliquots of cells, at 37°C, were induced to cap by the addition of 5  $\mu$ L of Rh-GaMIg in PBS to a final concentration of 200  $\mu$ g/mL. The cells were immediately settled onto poly-L-lysine coated coverslips, in a humidity chamber at 37°C. After incubation for specified times the cells, on the coverslips, were washed in PBS for 15 sec, fixed in 3% paraformaldehyde in PBS for 30 sec, given two 1 min PBS washes and then mounted on slides in 50% glycerol (V/V) in PBS at pH 7.8.

For electron microscopic studies, ferritin labelled GaMIg (F-GaMIg) was used to induce capping. Cells were at a density of  $10^7$ /mL in RPMI 1640, at 37°C. F-GaMIg was added to a final concentration of 200  $\mu$ g/mL. After a 10 min incubation at 37°C, cells were fixed with buffered glutaraldehyde and processed for electron microscopy.

The antisera used to induce capping contained NaN<sub>3</sub> as a preservative. The NaN<sub>3</sub> was removed by dialysing the antisera against PBS before use so that cellular metabolism would not be affected.

## 2.4 IMMUNOFLUORESCENT MICROTUBULE STAINING

### 2.4.1 Antibodies

The antisera to tubulin were a gift from Dr. V. I. Kalnins, Dr. J. Connolly and K. Rogers, of the Department of Anatomy, University of Toronto. They were prepared by in-

jecting electrophoretically pure porcine brain tubulin into rabbits as described by Connolly et al. (1978).

The secondary antibody was fluorescein conjugated goat anti-rabbit Ig (FITC-GaFig) from Cappel. The secondary antiserum was absorbed with mouse liver and spleen powders to reduce nonspecific binding to cellular determinants.

The powders were prepared by precipitating and washing homogenized mouse liver and spleen with 100% acetone. The precipitates were freeze dried, and the powders stored at  $-20^{\circ}\text{C}$ . Before being used to absorb the antiserum the powders were washed with PBS, until the high speed supernatant was clear. The antiserum was absorbed first with liver powder, then with spleen powder, by overnight incubation at  $4^{\circ}\text{C}$  with 250 mg/mL of washed powder. The antiserum was recovered by centrifugation of this mixture and aspirating off the high speed supernatant.

#### 2.4.2 Microtubule staining

To observe microtubules, cytoskeletons were prepared, and the microtubules were stained by indirect immunofluorescence. Samples of resting and stimulated populations, or cells induced to cap surface Ig were settled onto poly-L-lysine coated coverslips, washed for 15 sec in PBS, fixed for 30 sec in 3% paraformaldehyde in PBS and washed twice more for 30 sec in PBS. The cells were made permeable by a

60 min incubation in 1% Triton X-100 in a microtubule stabilizing buffer (SB: 50 mM imidazol, 50 mM KCl, 0.5 mM MgCl<sub>2</sub>, 1.0 mM ethyleneglycol-bis (B-amino-ethylether) N,N'-tetraacetic acid (EGTA), 0.1 mM disodium ethylene-diaminotetraacetic acid (EDTA), 1.0 mM 2-mercaptoethanol, 4 M glycerol pH 6.7) described by Bershadsky et al. (1978). The resulting cytoskeletons were washed twice for 30 sec in SB, and fixed for 10 min in 1% glutaraldehyde in SB. Free aldehyde groups were reduced by washing twice for 4 min in 1 mg/mL NaBH<sub>4</sub> in PBS. The cytoskeletons were washed twice for 3 min in PBS, then incubated with anti-tubulin antiserum diluted 1:30 in PBS for 45 min at room temperature in a humid chamber. The cytoskeletons were washed three times for 3, 5 and 4 min in PBS, and incubated with a 1:10 dilution of FITC-GaR1g for 45 min at room temperature, washed three times for 3, 5 and 4 min in PBS and mounted on a slide in 50% glycerol in PBS pH 7.8. Cells were examined using a Zeiss Universal Microscope equipped with epifluorescent and phase contrast optics, and photographed on Tri-X Pan film developed in Microdol X, or with Ilford XP1400 film processed with the the appropriate developer.

## 2.5 ELECTRON MICROSCOPY

Cells were collected by gentle centrifugation and fixed for 90 min in 4% glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.0 at room temperature. The cells were washed three times in a 0.05 M sodium phosphate buffer and postfix- ed in 1% OsO<sub>4</sub> in 0.05 M sodium phosphate buffer pH 7.0 at 4°C for 90 min. The cells were then washed three times in phosphate buffer, dehydrated in a graded acetone series and infiltrated with Spurr's hard resin mixture (Spurr, 1969) which was polymerized at 60°C for 18 h. Thin sections cut with a Dupont diamond knife on a Sorval Porter Blum MT-2B ultramicrotome were stained for 7 min with uranyl acetate and 5 min with lead citrate. Specimens were examined on a Philips 201C electron microscope. The relative numbers of microtubules in resting and stimulated populations, and in control and capped cells, were determined as follows. The centriolar regions of all cells containing a structure recognizable as part of the MTOC were photographed at 15,000X, and printed at a final magnification of 60,000X. The number of microtubules in a square (corresponding to 4 μm<sup>2</sup> area in the cell), centering on the MTOC, were counted.

## 2.6

PROTEIN DETERMINATIONS

Acetone powders of cell samples were prepared for the protein determinations. Cells were collected by centrifugation. The cells were precipitated by the addition of 15 volumes of ice cold acetone. The precipitates were washed twice with 100% acetone, and lyophilized.

2.6.1 Total cell protein determinations

Total cell protein contents of resting and stimulated cell populations were determined after hydrolysis in 6 N HCl at 106°C for 16 h, of known numbers ( $10^6 - 5 \times 10^6$ ) of live cells, to which norleucine (NRL) had been added as an internal standard. After removal of HCl under vacuum, amino acid analysis was carried out on a Technicon TSM Amino Acid Analyser. The protein content was determined by comparison of the ratios of GLX:NFL, ALA:NRL and LEU:NRL in the hydrolysates with the ratios produced when known amounts of bovine serum albumin were similarly treated.

2.6.2 Tubulin and actin determinations

Acetone powders of total cell protein were dissolved in 8 M urea containing 100 mM Tris-HCl, pH 8.0, and 2 mM dithiothreitol to a concentration of 10 mg/mL. Chemical modification was then carried out at room temperature for 45 min

in the dark after the addition of iodo[2-<sup>3</sup>H]acetic acid (Amersham-Searle) to a final concentration of 10 mM and a specific activity of 86 mCi/mmol. Under these conditions thiol groups are modified quantitatively. Reactions were stopped by the addition of excess 2-mercaptoethanol. After purification by dialysis against water, the chemically modified material was freeze dried and stored prior to use.

Samples of purified calf brain tubulin (Shelanski et al., 1973), and rabbit skeletal muscle actin (Spudich and Watts, 1971), were modified identically for use as known protein standards. Iodo[2-<sup>3</sup>H]acetic acid was used in some of the chemical modifications, and iodo[2-<sup>14</sup>C]acetic acid at a specific activity of 1 mCi/mmol was used in others.

Known amounts (measured by amino acid analysis) of <sup>3</sup>H-carboxymethylated lymphocyte total cell protein were mixed with known amounts of modified tubulin and actin standards. These mixtures were run on SDS polyacrylamide gels (Weber and Osborn, 1969). Approximately 100 ug of protein was applied to each gel tube (4x90 mm) and electrophoresis was carried out for 16 h at a current of 4 ma/tube. The gels were stained with Coomassie Brilliant Blue F and the protein bands with the mobilities of tubulin and actin were cut out of each gel. Tubulin bands and actin bands were each pooled, and extracted with three washes in 70% formic acid.

After removal of formic acid under vacuum, the dried extracts were washed once in 1 mL of ice cold trichloroacetic acid and twice in 1 mL of ice cold acetone to remove protein bound SDS. The protein was dried under vacuum, and resuspended in 250 uL of 0.5% ammonium bicarbonate.

Peptides were generated from the extracted proteins by digestion of tubulin with elastase and of actin with alpha-chymotrypsin. 2 ug of elastase and 1 ug of chymotrypsin were used per 100 ug of protein applied to the gels. Digestion was carried out in 0.5% ammonium bicarbonate buffer at 37°C for 16 h. Peptides were then separated by paper electrophoresis at pH 6.5 and at pH 2.1. <sup>14</sup>C-containing peptides were located by autoradiography. Tubulin and actin specific peptides were cut out and extracted from the chromatographic paper with 150 uL of 70% formic acid, and 100 uL of the extract was added to 9.9 mL of Aquasol 2, and the <sup>3</sup>H:<sup>14</sup>C ratio was measured in a scintillation counter with a double isotope program. To allow calculation of the tubulin and actin contents from <sup>3</sup>H/<sup>14</sup>C ratios, known amounts of <sup>3</sup>H and <sup>14</sup>C modified tubulin, and <sup>3</sup>H and <sup>14</sup>C modified actin were mixed together and electrophoresed. After generation and isolation of peptides, <sup>3</sup>H/<sup>14</sup>C ratios were determined and used as standards to calculate the amount of tubulin in lymphocyte samples.

To identify the  $^{14}\text{C}$ -containing peptides, peptides were produced from 40 mg of  $^{14}\text{C}$ -carboxymethylated calf brain tubulin by digestion with elastase under the identical conditions. After isolation the peptides obtained in high yield were identified.

Three tubulin specific carboxymethylcysteine (cmc) containing peptides which mapped away from the other peptides were used in determining the amount of tubulin in lymphocyte populations. Their positions in the protein were assigned from the sequence for alpha porcine brain tubulin (Ponstingl et al., 1981). Peptide A has the sequence cmc-cmc-leu. Peptide A accounts for cys 315 and cys 316 in the alpha chain and was isolated as the most acidic cmc containing peptide at pH 6.5. Peptide E was in the second most acidic group of cmc containing peptides at pH 6.5. At pH 2.1 peptide F was the most acidic peptide. Peptide E has the sequence glu-his-ser-asp-cmc-ala and accounts for the cys 200 in alpha tubulin. Peptide B has basic mobility at pH 6.5 and at pH 2.1. Peptide B has the sequence lys-cmc-asp-pro-arg-his-gly and accounts for cys 305 in the alpha chain. In some of the experiments a peptide derived from beta tubulin was isolated. It was present in equal amounts to the alpha tubulin peptides. This peptide was designated peptide N, and has the sequence val-cmc-asp-ile-pro-pro-arg-leu-gly and accounts for cys 354 of the beta tubulin chain (Krauks et al., 1981). Peptide N was neutral at pH 6.5, and was isolated as the most basic of these at pH 2.1.

The peptide used for actin determinations was the only cmc containing actin specific peptide with basic mobility at pH 6.5. This peptide was the carboxyl terminus, which had a sequence of arg-lys-cmc-phe, and accounted for cys 373 of muscle actin (Collins and Elzinga, 1975).

## section III

### RESULTS

#### 3.1 THE MICROTUBULE NETWORK OF B LYMPHOCYTES DURING CAPPING

##### 3.1.1 Capping on nude mouse B lymphocytes

To look for changes in the assembly of microtubules in lymphocytes which have been induced to cap surface Ig, splenic lymphocytes from nude mice were used because a large proportion (between 75% and 95%) are B lymphocytes. Capping of surface Ig on B cells is induced by incubating cells with goat anti-mouse Ig (GaMIg) antibody. Rhodamine labelled (Rh-), and ferritin labelled (F-) antibodies are used for light, and electron microscopic studies respectively.

Cells fixed with paraformaldehyde before incubation with anti-Ig, to prevent surface Ig redistribution, have a diffuse distribution of surface Ig (fig. 1A). Following binding of Rh-GaMIg to the cell surface Ig, the Ig anti-Ig complexes aggregate into patches (fig. 1B), which migrate to one pole of the cell to form a cap (fig. 1C-E). The cap is then internalized (fig. 1F) and appears as a group of fluorescent spots in the cytoplasm.

Figure 1. Immunofluorescent staining of nude mouse splenic B lymphocytes at different stages of the capping process. Cells fixed in paraformaldehyde before incubation with Ph-GaMIg show a diffuse staining pattern (A). Cells fixed following incubation at 37°C with 200 ug/mL Ph-GaMIg cells patch (B,C and D), cap (E), and internalize (F) the Ig anti-Ig complexes. All figures X2,000.

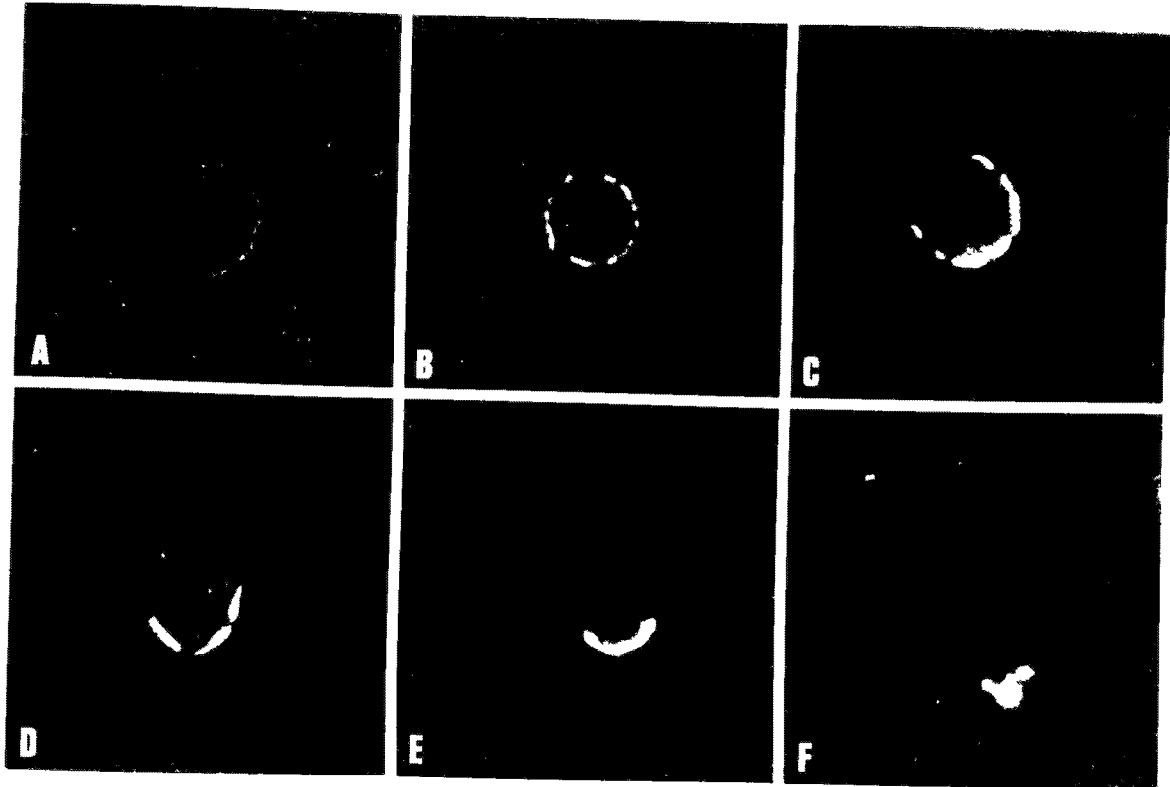
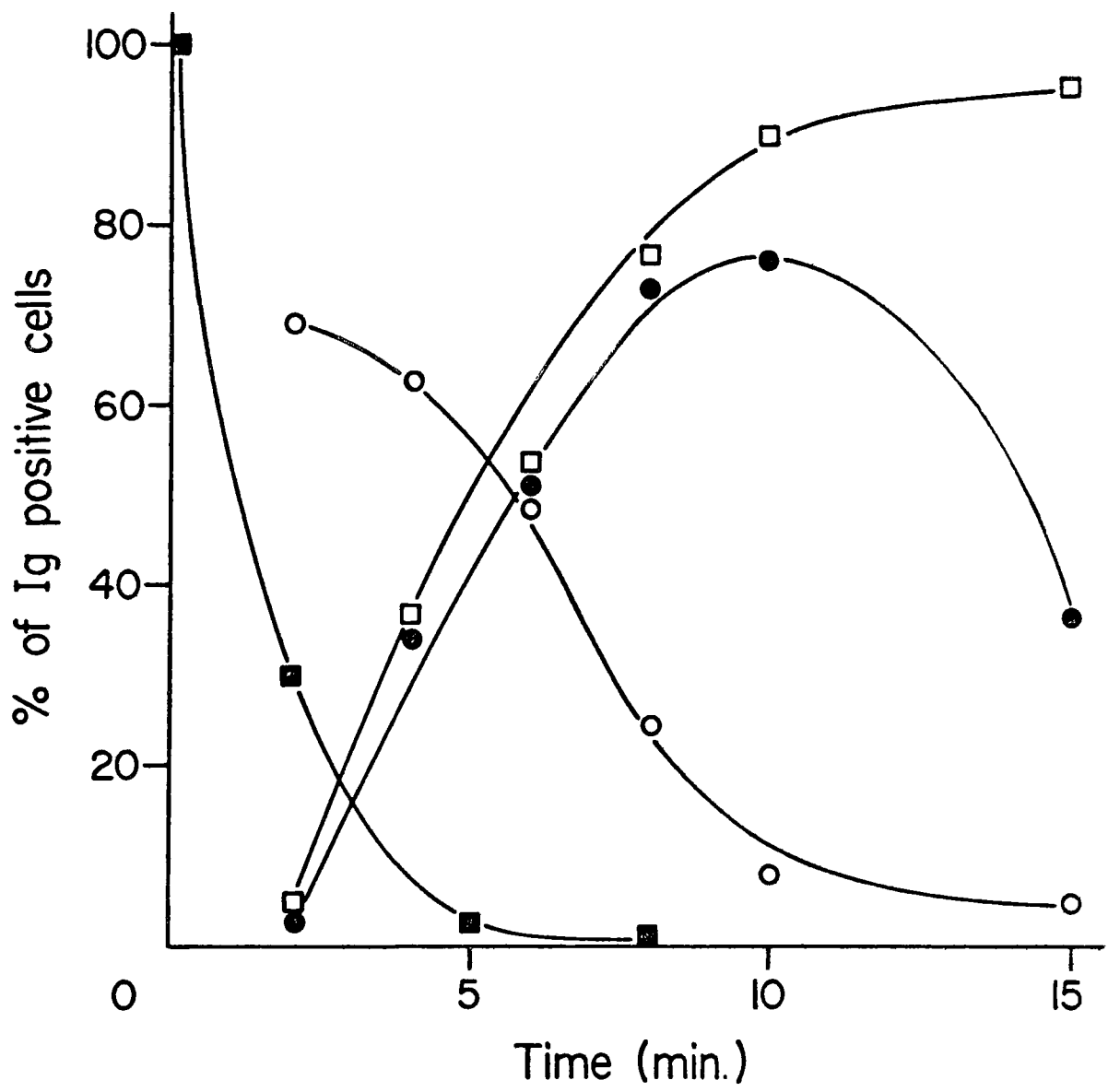


Figure 2. Time course of surface Ig redistribution on nude mouse B cells. After incubation for specific times at 37°C with 200 uq/mL Fh-GaMIg cells were fixed , washed and mounted for observation. The 0 time sample was fixed before surface labeling. 95% of all cells were Ig positive. At each time point the staining pattern of at least 100 Ig positive cells was scored as being either diffuse (\*), patched (o), capped (•), or internalized. The sum of capped and internalized cells is shown as (□).



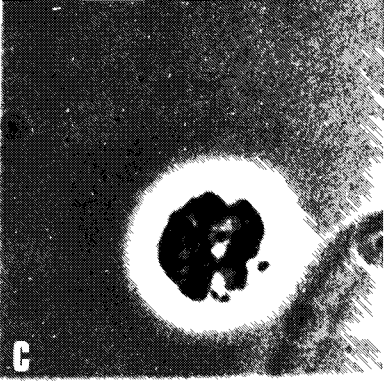
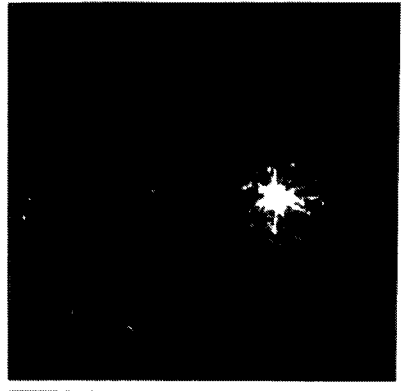
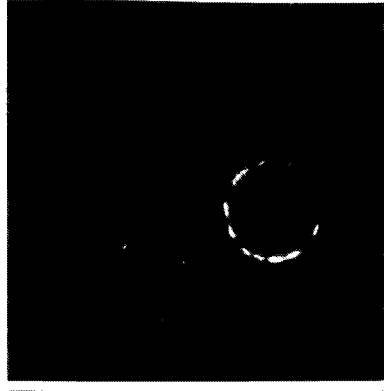
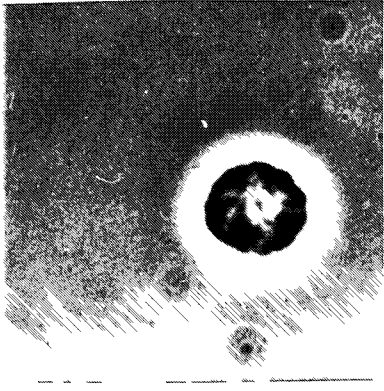
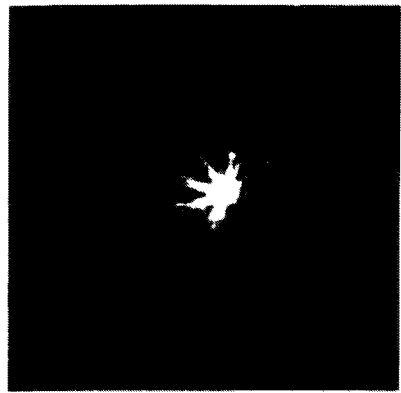
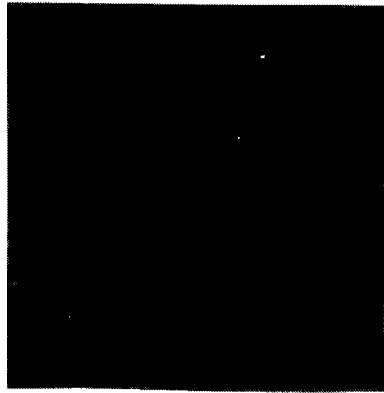
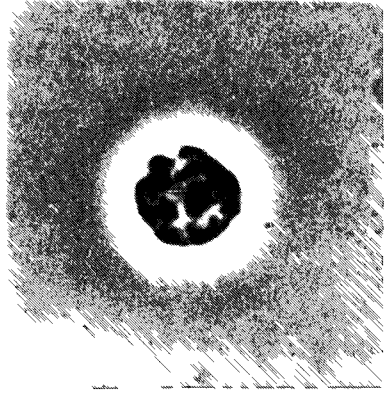
The time course of Ig redistribution was determined (fig. 2) in order to choose the best time to look at microtubule networks in capped cells. Cells are incubated at 37°C with Rh-GaMIg to induce capping, while settling on poly-L-lysine coated coverslips. Samples fixed at time points during incubation are scored for the percent of labelled cells with diffuse, patched, capped or internalized Ig anti-Ig distribution. Patching of surface Ig is a fast process. Only 30% of labelled cells have not patched the surface Ig after 3 min of incubation at 37°C. After 5 min of incubation, more than half of the labelled cells have capped their surface Ig. The maximum number of capped cells was seen at 10 min of incubation at 37°C. The caps continue to be internalized after this time.

### 3.1.2 Microtubule networks in resting and capped B cells

The microtubule networks of capped and control cells were examined by indirect immunofluorescence staining and electron microscopy to determine if there is a change in microtubule assembly during capping of surface Ig.

The immunofluorescent staining pattern of microtubule networks in control cells reveals stained fibers which converge on the single, brightly staining MTOC (fig. 3A). By focusing through the cell, the stained fibers can be seen to run from the MTOC to the other end of the cell.

Figure 3. Microtubule networks of nude mouse B lymphocytes at different stages of capping surface Ig. Pesting cells (A) were fixed before incubation with Rh-GaMIg. Cells fixed following incubation at 37°C with 200 ug/mL Rh-GaMIg had patched (B) or capped (C) surface Ig. The microtubules in these cells are stained with rabbit anti-tubulin primary antibody, and FITC-GaRIg secondary antibody. Cells are seen by phase contrast and epifluorescence optics. All figures X2,000.



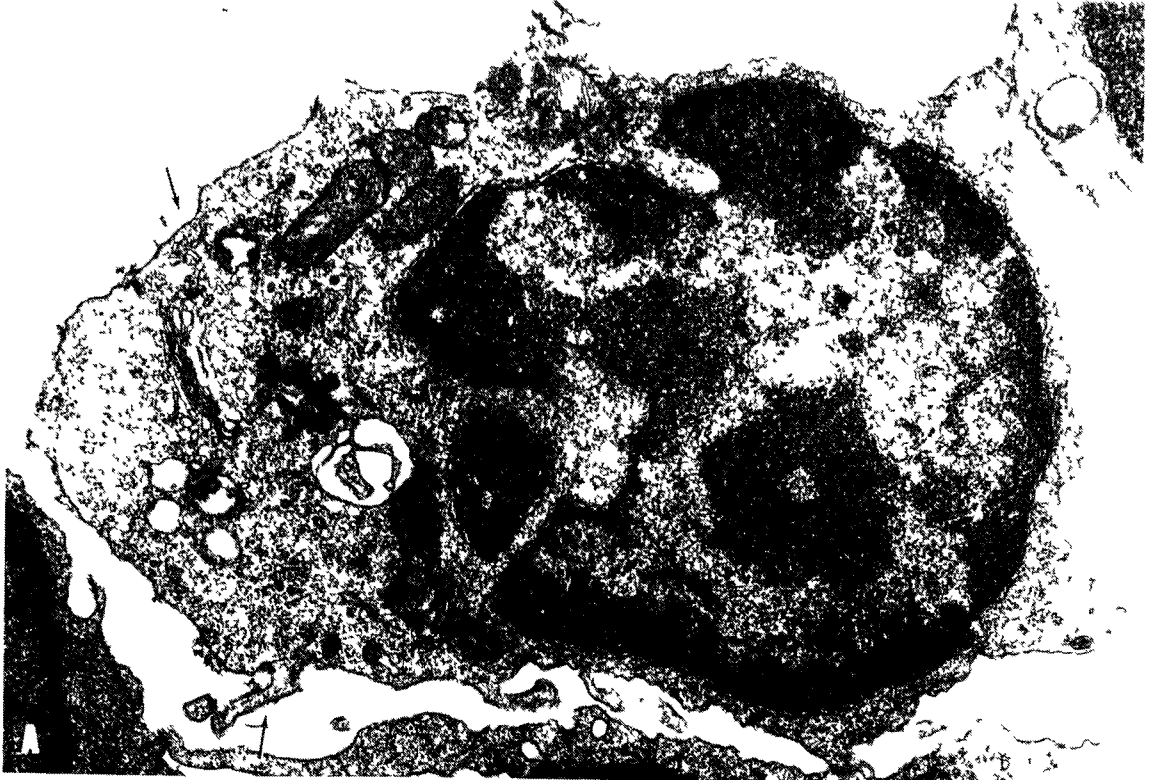
There is a change in the microtubule staining pattern in cells which have patched (fig. 3B) or capped (fig. 3C) surface Ig during incubation with Rh-GaMIg. There appear to be more fibers in these cells, and the staining pattern of the fibers is more delicate.

Electron microscopy was used to determine if the change in immunofluorescent staining pattern represents an increase in the number of microtubules during the capping process. Control cells, incubating in RPMI 1640 at 37°C, were fixed with buffered glutaraldehyde at 37°C. Capped cells were fixed after incubation in solution at 37°C with F-GaMIg. Cells were fixed after incubation for 10 min since this time point had the highest percentage of capped cells (fig. 2). This population was 95% Ig positive when stained with Rh-GaMIg.

Figure 4A is an electron micrograph of a cell which has capped the surface Ig over the MTOC region of the cell. The microtubules can be seen in a high magnification electron micrograph of the MTOC region of a capped B cell (fig. 4B).

Although it appeared from the immunofluorescent staining that more stained fibers (presumably microtubules) were present there is no significant difference in the microtubule content of capped cells. The number of microtubules counted in sections through the centriolar regions are  $14.6 \pm 5.1$  (n=30) in resting cells, and  $13.8 \pm 4.5$  (n=28) in the capped cell population.

Figure 4. Electron micrographs of capped B cells. The cap was induced by incubating cells at 37°C with 200 ug/mL of F-GaMIg for 10 min. In a whole cell view (A) the F-GaMIg cap is seen at one pole of the cell (between arrows), directly over the MTOC region (X21,000). At higher magnification (B) microtubules are seen in the MTOC region of the cell (X60,000). The ferritin labelled GaMIg is seen coating the outer surface of the plasma membrane.



### 3.2 INCREASE IN THE MICROTUBULE AND TUBULIN CONTENTS OF T CELLS DURING ACTIVATION

Several precautions were taken to ensure comparable results between experiments and between the microscopic and biochemical studies, T enriched lymphocyte populations were prepared so that the studies of microtubule and tubulin contents could be done on a more homogeneous population than is found in a total splenic lymphocyte population. For biochemical studies, live cell populations were prepared so that the protein content of dead cells would not contribute to protein determinations made on resting and stimulated populations. Con A mediated activation of T cell populations was monitored by measuring the increase in cell size and the incorporation of  $^3\text{H}$ -thymidine in 48 h stimulated populations.

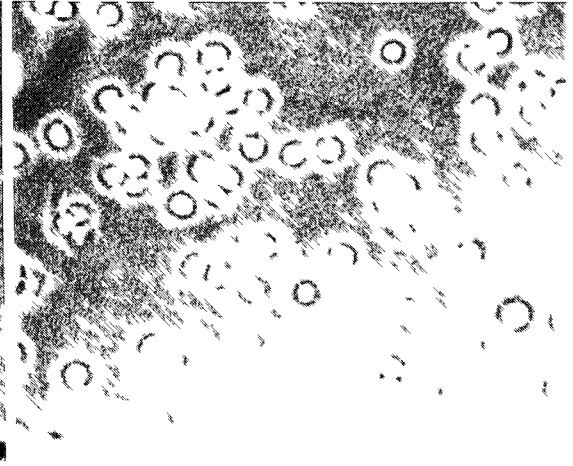
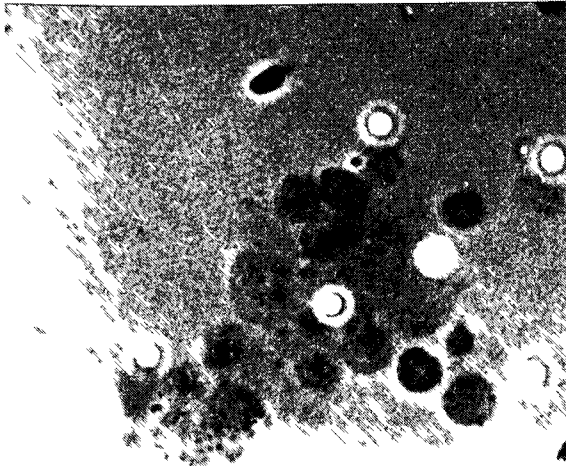
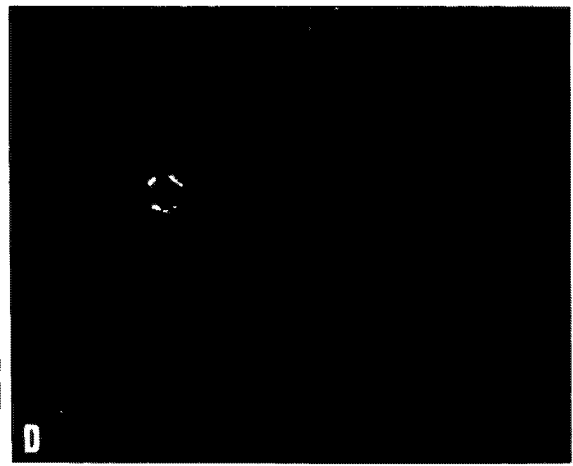
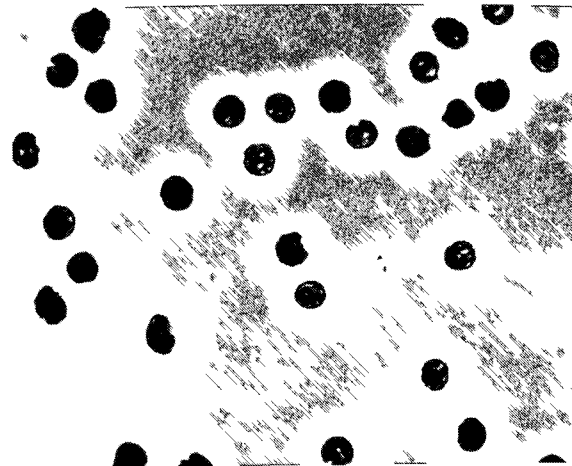
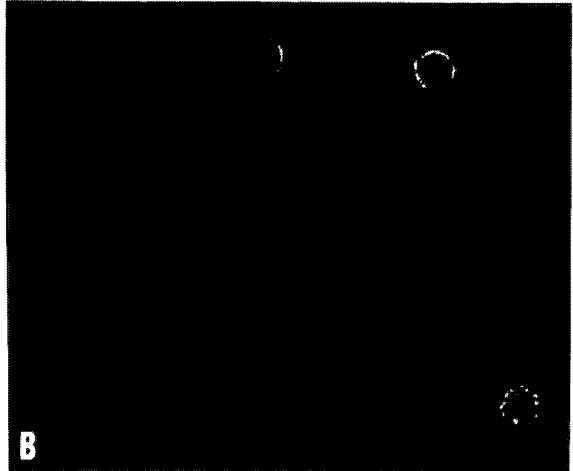
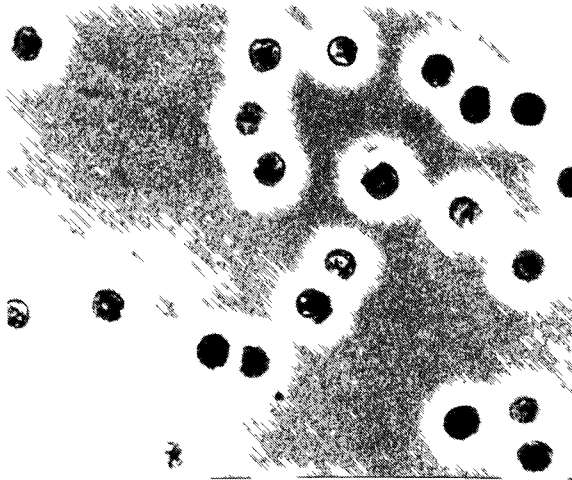
#### 3.2.1 Preparation of T lymphocyte populations

In a total splenic lymphocyte population, about 45% of the lymphocytes are Ig bearing (B) cells (fig. 5A). T enriched populations of lymphocytes, prepared by preferential agglutination of B cells with wheat germ agglutinin, have fewer than 5% Ig bearing cells (fig. 5B).

Dead cells are present immediately following isolation, and cell death occurs during long term cultures. In 48 h populations more than half the cells are dead. Live cell

Figure 5. Immunofluorescent staining of cell surface Ig. Mixed cell (A), and T cell enriched (C) populations were incubated with Rh-GaMIg to label surface Ig (B and D). The same populations (A and B; C and D) are seen by phase contrast and epifluorescent optics. In a mixed cell population 45% of the cells were Ig positive. In a T cell enriched population 4% of the cells were Ig positive. 400 cells were scored as Ig positive or negative. All figures X700.

Figure 6. Trypan blue staining of unfractionated and live cell populations. To prepare live cell populations cultured lymphocytes are suspended in a low ionic strength buffer. Dead cells stain darkly with trypan blue. The dead cells clump (A), and are filtered out over a nylon wool column to yield a live cell population (B) with fewer than 5% dead cells. 500 cells were counted. X500.



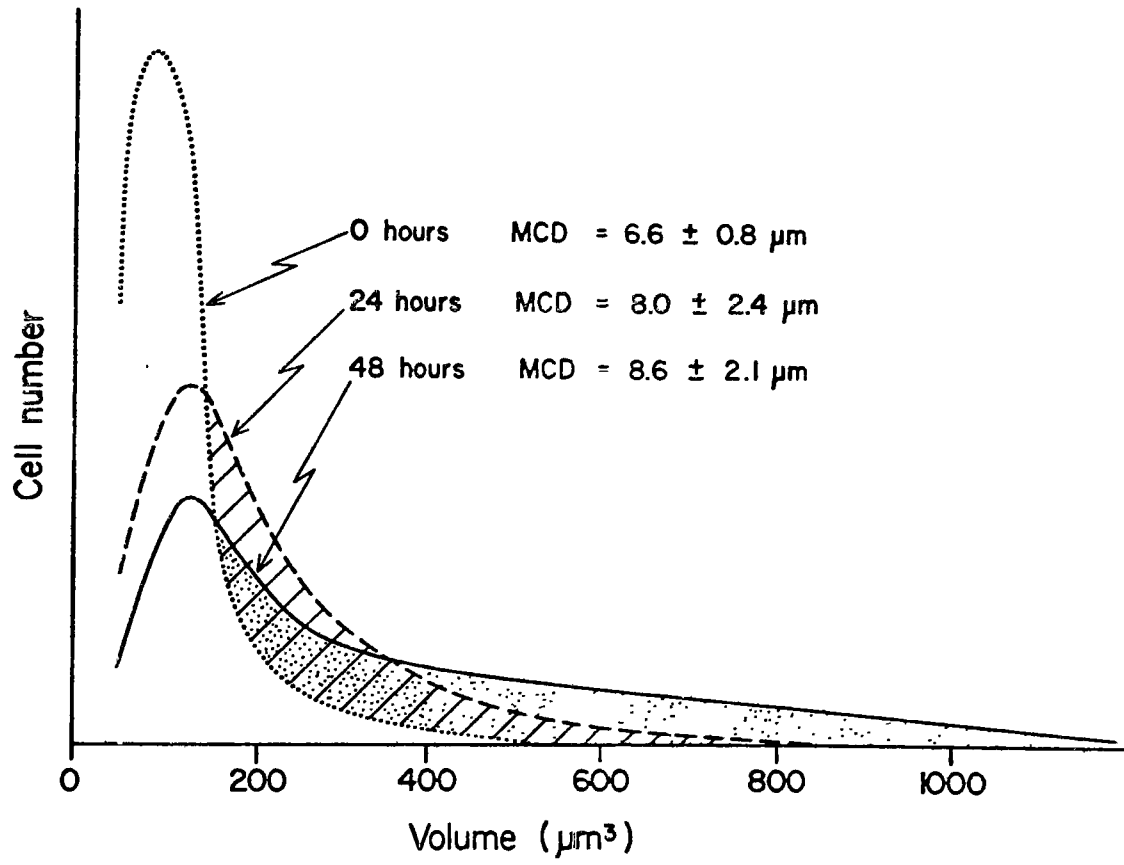
populations prepared by preferentially clumping out dead cells have 5% or fewer dead cells. Unfractionated, and live cell populations are shown in figure 6.

### 3.2.2 T cell activation

In all of the populations used the incorporation of  $^3\text{H}$ -thymidine into 48 h stimulated populations was 100-150 fold higher than in 48 h resting populations. Cell size changes were routinely determined by measuring the diameter of at least 70 cells with an ocular micrometer. The mean cell diameter increased from  $6.6 \pm 0.8$   $\mu\text{m}$  in a resting population to  $8.0 \pm 2.4$   $\mu\text{m}$  after 24 h of stimulation, and to  $8.6 \pm 2.1$   $\mu\text{m}$  after 48 h of stimulation. One third of the cells in a 48 h stimulated population have a diameter of 10  $\mu\text{m}$  or more and are considered to be blast cells.

Cell size profiles were obtained by measuring cell volumes with a Coulter counter channelizer. The cell size profiles obtained for 0, 24, and 48 h stimulated T cell populations are shown in figure 7. The majority of cells in a resting population fall within a narrow size range. After 24 h of stimulation, some of the cells have increased in size, as indicated by a shift of the curve to the right. In a 48 h stimulated population a large portion of the cells have increased in size. Approximately one third of the cells in a 48 h stimulated population have a volume greater than 500  $\mu\text{m}^3$ .

Figure 7. Cell size distribution of T lymphocyte populations stimulated for 0, 24, and 48 h. The cell size profiles were obtained by measuring the volumes of cells in live cell populations with a Coulter counter channelizer model Zf. The number of cells measured is proportional to the area under the curve. The area under each of the curves is the same. The mean cell diameters are calculated from measurements of at least 70 cells, made using an ocular micrometer, for each time point. The portion of the population which increased in size during stimulation is indicated by hatched lines for the 24 h stimulated population, and by stipple for the 48 h population.



### 3.2.3 The microtubule network

#### 3.2.3.1 Immunofluorescent staining

Indirect immunofluorescent staining was used to examine microtubule networks in T lymphocytes at various stages of stimulation (fig. 8). Staining shows a sparse microtubule network in resting cells which consists of fibers extending around the cell and converging on the single, brightly staining MTOC (fig. 8A). The microtubule network of a cell representative of the largest cells seen in a 24 h stimulated population is seen in figure 8B. Blast cells in a 48 h stimulated population have an extensive microtubule network (fig. 8C). Many stained fibers are seen to converge on the MTOC. The microtubules in both resting cells and blast cells can be seen to run from the MTOC to the other end of the cell by focusing up and down through the cell. Populations stimulated for 48 h contain cells at all stages of activation. Examination of the microtubule networks of cells in a 48 h stimulated population shows that the small cells have a microtubules network identical to that seen in 0 h resting cells.

#### 3.2.3.2 Electron microscopy

The major morphological differences between resting and stimulated cells are readily seen by electron microscopy. Resting cells (fig. 9A) have a scant layer of cytoplasm,

Figure 8. Indirect immunofluorescence staining of the microtubule networks of T cells at various stages of activation. Microtubules are stained with rabbit anti-tubulin primary antibody and FITC-GaR1g secondary antibody. The small resting lymphocytes have a sparse microtubule network which becomes more extensive during activation. The microtubule networks are seen in cells from resting (A), 24 h stimulated (B) and 48 h stimulated (C) T cell populations. Cells at all stages of activation are found in a 48 h population.

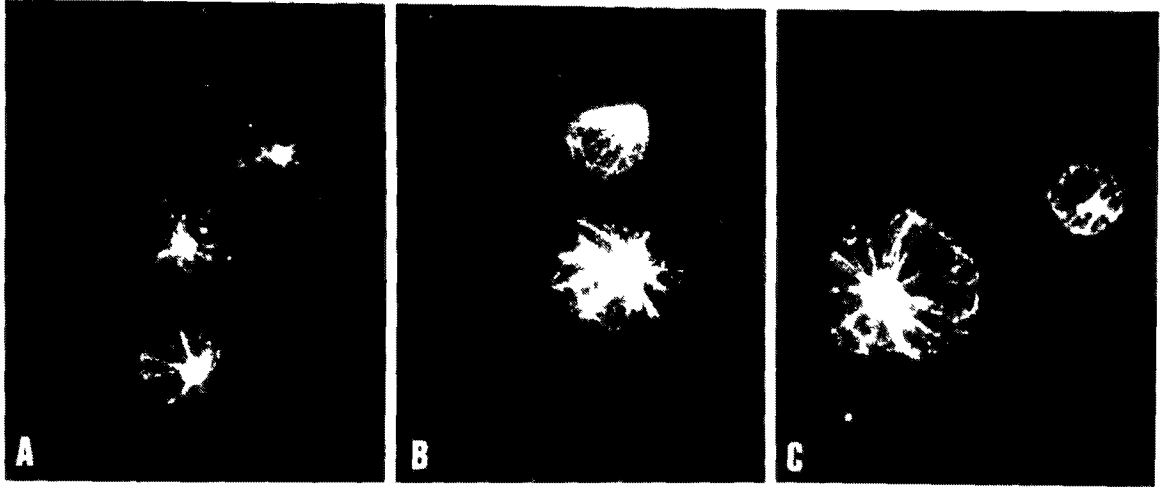
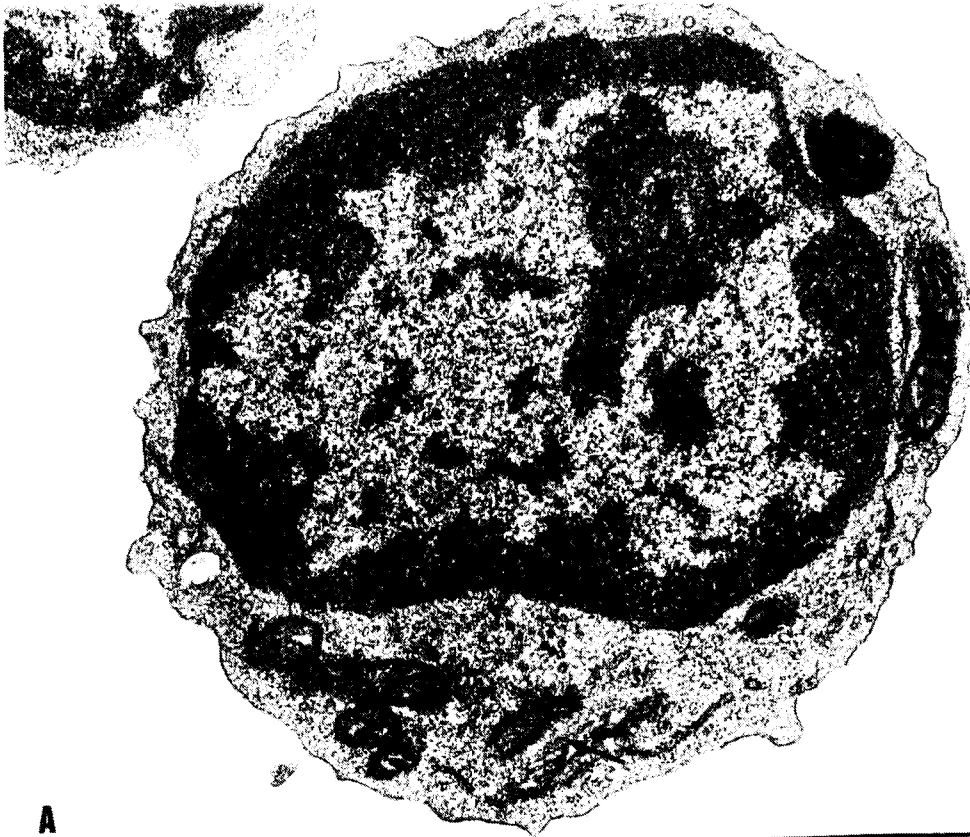


Figure 9. Electron micrographs of T lymphocytes. In resting cells (A, X25,000) there are few organelles in the scant layer of cytoplasm which surrounds the densely staining nucleus. The large increase in size of blast cells (B, X8,800) is due to increases in in both nuclear and cytoplasmic volumes. The MTOC regions are marked with arrows.



A



B

Figure 10. Electron micrographs of the centriolar regions of cells in a resting (A), 24 h stimulated (B) and 48 h stimulated (C) populations. X60,000. To determine the increase in number of microtubules, a random sample of MTOC regions from each of the populations were photographed, and the number of microtubules in a 4  $\mu\text{m}^2$  area centered around the MTOC were counted. The numbers are summarized in table 1.



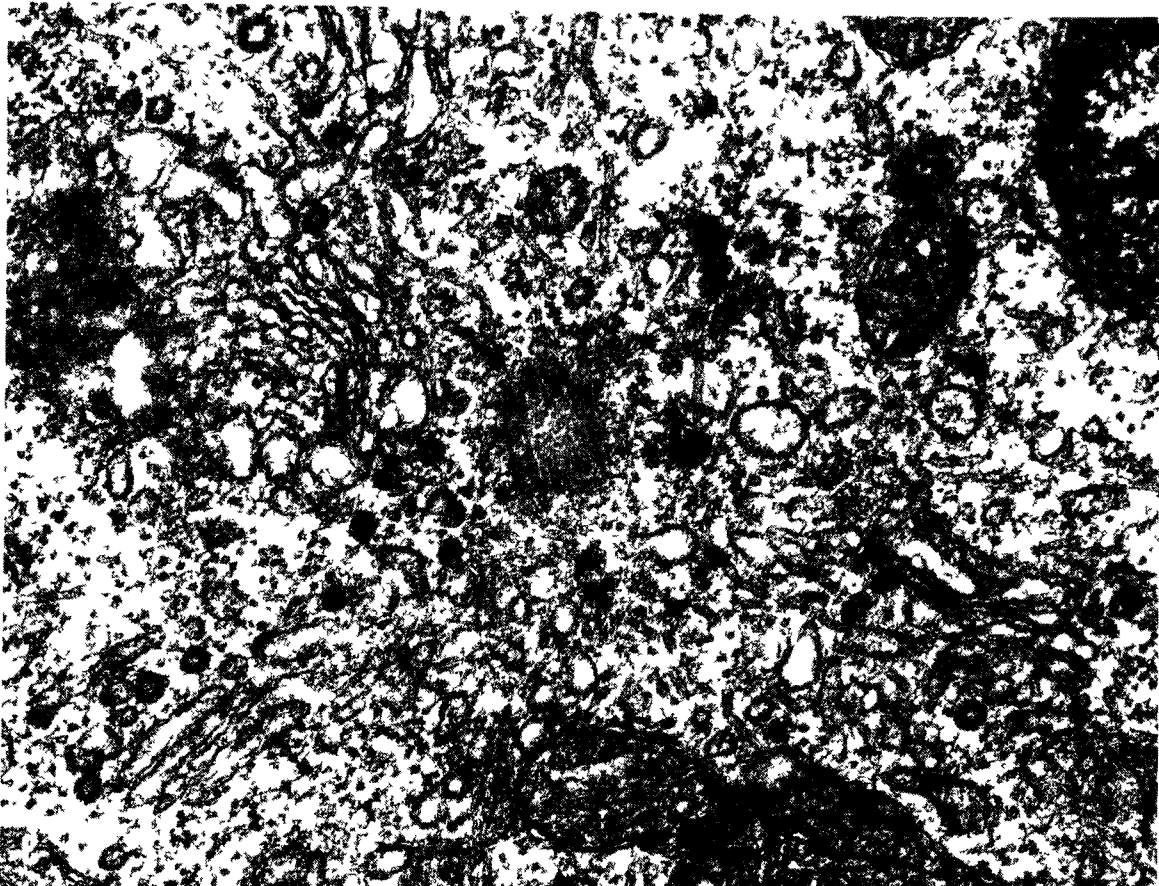


Table I. Microtubule numbers in resting and stimulated populations of T lymphocytes.

Microtubule numbers	Hours after stimulation of T lymphocytes.		
	0	24	48
Mean	15.7	26.3	35.1
S. D.	7.2	9.2	14.6

containing few organelles, surrounding the densely staining nucleus. Fully stimulated cells (fig. 9B) have undergone a large increase in size, due mostly to an increased cytoplasmic volume which contains many organelles. The chromatin in the nucleus is decondensed. The microtubules in these cells are most easily observed in high magnification views of the centriolar region, where the density of microtubules is the highest (fig. 10).

To determine the increase in microtubule content during stimulation, the numbers of microtubules in thin sections through the centriolar regions of cells in populations stimulated for 0, 24, and 48 h were counted (table 1). These counts show over a 2 fold increase in the mean number of microtubules in a 48 h stimulated population.

#### 3.2.4 Tubulin content

The double isotope labelling technique was used to directly measure the tubulin content, as a percent of total cell protein, in resting and stimulated T lymphocyte populations. Actin determinations were also made so that changes in the tubulin content could be compared to changes in another cytoskeletal protein. The samples of 0, 24 and 48 h control stimulated populations used for the tubulin and actin determinations consisted of live cells populations from three separate stimulation series which were pooled. Pool-

ing the stimulation series reduces the variability between stimulation runs, and by increasing the amount of protein improves the accuracy of the method. The tubulin and actin contents are an average of the three stimulation series.

The tubulin and actin contents of T lymphocytes during stimulation are summarized in table 2. The determinations show that the tubulin content increases from 0.8% in resting cells to 1.2% in a 24 h stimulated population, and to 1.3% in 48 h stimulated cells. The tubulin content per cell can be calculated from the tubulin as a percent of total cell protein, and the total cell protein values. The total cell protein, determined by amino acid analysis, increased from 25 ug/10<sup>6</sup> resting cells, to 29 ug/10<sup>6</sup> cells in a 24 h stimulated population, and to 39 ug/10<sup>6</sup> 48 h stimulated cells. These values are the averages of two determinations, made on separate stimulation series, which differ from the average by less than 4%. There is a 2.5 fold increase of the total tubulin content, from 0.20 ug/10<sup>6</sup> resting cells to 0.51 ug/10<sup>6</sup> cells in a 48 h stimulated population.

The actin content did not increase in the same manner as the tubulin content during stimulation. As a percent of total cell protein, the actin increased from 2.3% to 3.1% during the first 24 h of stimulation, but then dropped to 2.5% in 48 h stimulated cells. The total actin per cell, however, did increase throughout stimulation (table 2).

**Table 2.** Tubulin and actin contents in resting and stimulated populations of T lymphocytes.

Protein determinations		Hours after stimulation of T lymphocytes		
		0	24	48
Tubulin	as % total cell protein	0.8	1.2	1.3
Actin		2.3	3.1	2.5
Total cell protein	$\mu\text{g}/10^6$ cells	25	29	39
Tubulin	$\mu\text{g}/10^6$ cells	0.20	0.35	0.51
Actin		0.58	0.90	1.0

### 3.3 TUBULIN CONTENTS OF MIXED AND B LYMPHOCYTE POPULATIONS

Preliminary evidence showed higher tubulin contents, as a percent of total cell protein, in unfractionated, mixed lymphocyte populations than were found in T lymphocyte populations. The difference is presumably due to a higher tubulin content in the B cells present in the mixed population. The major differential increase in tubulin content is seen in the first 24 h of stimulation. The tubulin contents of resting and 24 h stimulated, mixed cell and nude mouse B cell populations were examined to determine if B cells undergo the same type of change.

Cell size profiles of resting and 24 h stimulated B lymphocyte and mixed lymphocyte populations are seen in figures 11 and 12 respectively. The resting B lymphocyte population has a wider cell size distribution, and a higher mean cell diameter, than resting mixed cell populations or resting T cell populations (fig. 7). An increase in cell size is seen during stimulation of both the B cell and the mixed cell populations.

Immunofluorescence staining of the microtubule networks in resting and stimulated B cells is seen in figure 13. The microtubule networks become more extensive as the cells increase in size. Cells at all stages of stimulation are found in the 24 h stimulated population.

Figure 11. Cell size profiles of resting and 24 h LPS stimulated B cell populations (see Materials and Methods), made with a Coulter counter channelizer. The number of cells measured is proportional to the area under the curve and the area under each of the curves is the same. The mean cell diameters are calculated from measurements of at least 70 cells, made using an ocular micrometer, for each time point.

Figure 12. Cell size profiles of resting and con A stimulated mixed cell populations. The cell size profiles and mean cell diameters calculations were made as described for figure 11.

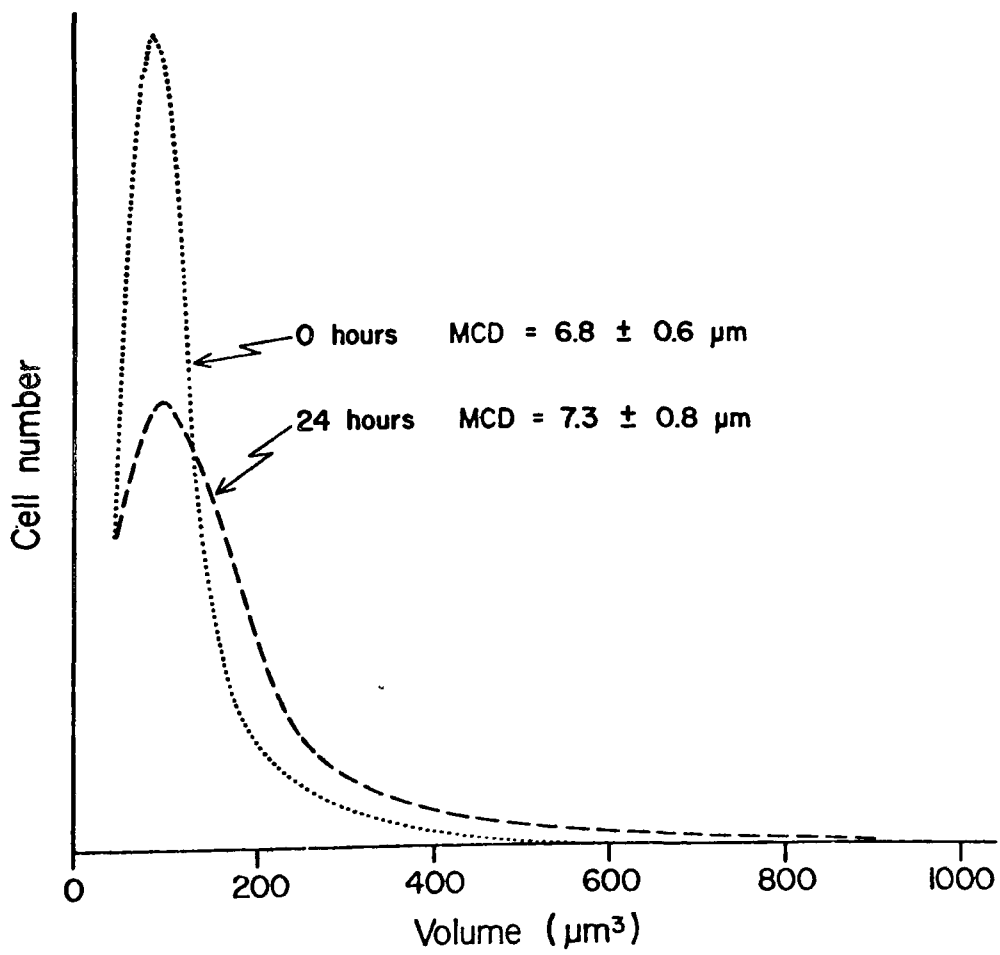
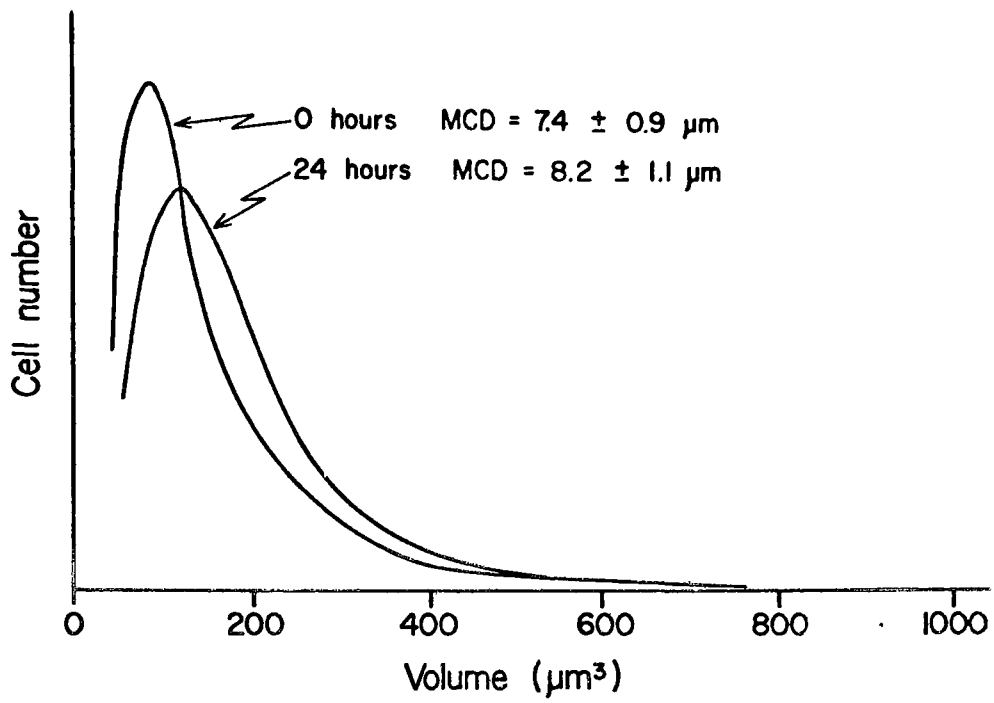
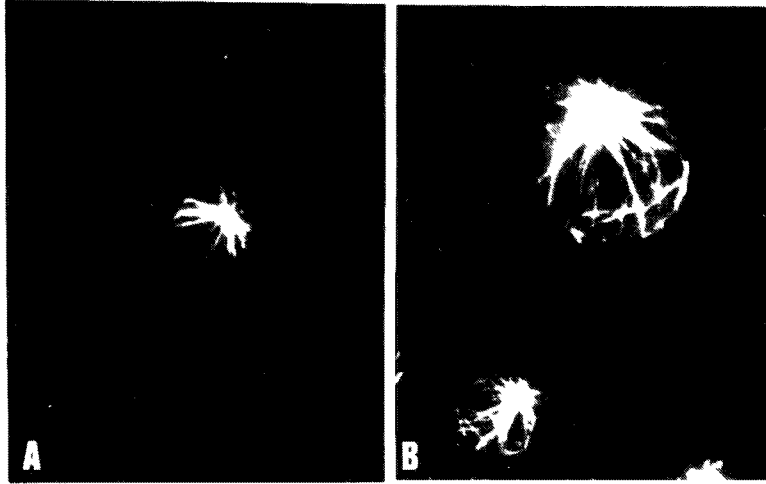


Figure 13. Immunofluorescent staining of the microtubule networks of B cells. A resting cell (A). Cells at all stages of stimulation are seen in the 24 h stimulated population (B). More extensive microtubule networks are seen in the larger cells.



The tubulin and actin contents of unfractionated (live and dead), and live B cell populations were measured to determine if dead cell in the lymphocyte population significantly altered the results. The unfractionated population had tubulin and actin contents of 4.4% and 3.0% respectively, as opposed to 2.9% and 2.4% in the live cell populations. The tubulin and actin contents of a second, independently prepared, live B cell preparation were lower, being 2.1% and 2.0% respectively.

The tubulin and actin determinations made on resting and stimulated B lymphocyte and mixed lymphocyte populations are shown in table 3. There is an increase in tubulin content during stimulation of both B cell and mixed cell populations. As a percent of total cell protein, there is a large difference in the tubulin contents of resting populations of mixed lymphocytes (3.6%) and T lymphocytes (0.8%, table 2). The higher tubulin content expected to be seen in a B cell population was not found in the nude mouse splenic lymphocytes, although the tubulin content of 2.0% was higher than the tubulin content of T cells.

The actin content increased during stimulation of the mixed cell population (table 3) as was seen in the T cell populations (table 2). In the B cell population there was a decrease in the actin content during stimulation (table 2).

**Table 3.** Tubulin and actin contents in resting and stimulated B lymphocyte and mixed lymphocyte populations.

Cell sample	Tubulin and actin contents as percent of total cell protein.	
	Tubulin	Actin
<b>B lymphocytes</b>		
0 h	2.9	2.4
24 h stimulated	3.2	1.9
0 h live and dead	4.4	3.0
*0 h	2.1	2.0
<b>Mixed lymphocytes</b>		
0 h	3.6	2.7
24 h stimulated	5.1	3.5

\* a separate cell preparation was used for this second determination on 0 h B cells.

## section IV

### DISCUSSION

It was proposed that patching and capping of surface Ig on lymphocytes resulted in disorganization of the microtubule network (Yahara and Kakimoto-Samishima, 1978). They could detect organized microtubules in resting lymphocytes by immunofluorescent staining, but not in cells which had patched or capped surface Ig. It was subsequently shown, by immunofluorescent staining and electron microscopy, that microtubule networks are present at all stages of the capping process (Pogers et al., 1981). These authors reported a change in the microtubule distribution, seen by immunofluorescent staining, in cells which had capped surface Ig and suggests that this could represent an increase in microtubule assembly in response to redistribution of cell surface Ig. Electron microscopy showed a four to five fold increase in the number of microtubules in the MTOC region of polymorphonuclear leukocytes following exposure to con A (Oliver et al., 1978).

I found no significant difference of the microtubule numbers in resting and capped cells using electron microscopy. The altered microtubule staining pattern seen in patched and capped cells (fig. 3B and C) might be due to a change in

spatial organization of the microtubules. Since this change occurs within minutes in a large percent of B lymphocytes induced to cap Iq, this would be an excellent model system to examine the regulation of rapid changes in microtubule networks in response to an external signal. If microtubules in control cells are in bundles, then individual microtubules within a bundle would not be resolved by immunofluorescence microscopy. A bundle would appear as a single staining fiber. This seems likely since only a few staining fibers are seen in resting cells and these are fewer than the numbers of microtubules determined by electron microscopy, and they appear thicker than the more numerous fibers seen in capped cells.

Movement of patched receptors into caps depends on microfilament contraction (Braun et al., 1978). Interaction of contracting microfilaments with microtubules in a bundle may result in the bundle being separated into individual tubules. This would cause the microtubule staining pattern to appear more delicate, as seen in figures 3B and C. Numerous interconnections of microfilaments inserting onto microtubules have been seen by electron microscopy (see for eg. Schliwa et al., 1981). Alternatively, the microtubules may redistribute first and then interact with microfilaments to direct the movement of patches to the pole of the cell where internalization occurs.

Changes in the spatial organization of the microtubule network during redistribution of surface Ig could be confirmed by making three dimensional models of the MTOC regions of resting and capped B cells, from serial sections. This type of analysis to determine the sequence of events during Ig redistribution, in combination with drug treatments which interfere with the microfilament or microtubule systems can be used to determine which of these models is correct.

Increases in the microtubule content during stimulation of mixed lymphocyte populations have been shown before by immunofluorescent staining (Rudd et al., 1979) and electron microscopy (Biberfeld, 1971; Pudd et al., 1979) of the microtubule networks. In selected lymphocyte populations, immunofluorescent staining showed increased microtubule networks in stimulated T cells (fig. 8B, C) and B cells (fig. 13B). The most extensive networks were seen in blast cells.

Microtubule numbers have been counted in the microtubule networks seen by immunofluorescent staining of well spread tissue culture cells. Averages of 135 and 178 microtubules per cell were reported for 3T3 cells (Hiller and Weber, 1978) and human fibroblast cells (Spiegelman et al., 1979) respectively. The microtubules are easy to count in these flat, well spread cells. A correspondence of one staining fiber to one microtubule in the margins of fibroblasts was

demonstrated by immunofluorescent staining and electron microscopy of the same cell (Weber and Osborn, 1976). This technique of counting microtubules cannot be used with accuracy for the smaller round lymphocytes, therefore, it was necessary to use electron microscopy.

The mean number of microtubules per section through the MTOC of T cells increased from 15.7 in resting cells to 35.1 in a 48 h con A stimulated population (table 1). In fully stimulated cells, which are recognized by increased cytoplasmic and nuclear volumes and a decondensed nucleus, up to five times as many microtubules were seen in sections through the MTOCs. The mean of 15.7 in resting cells is similar to the mean of 16.2 microtubules seen in sections through the MTOC regions of human peripheral blood T lymphocytes (Oliver et al., 1980).

The total number of microtubules in a resting cell and in a fully stimulated cell can be estimated. The estimates are made by multiplying the mean number of microtubules which terminate on the MTOC in a section, by the number of serial sections required to pass completely through the MTOC. Microtubule counts for this estimate are of microtubules which are seen to terminate on the MTOC in that particular section. Microtubules passing close by the MTOC, and terminating on the MTOC in another section, are not counted as they were for the determinations in table 1. The average number

of microtubules per section which terminated on the MTOC was 4 in resting cells and 11 in stimulated cells. The number of sections required to pass through the MTOC are 12 for a resting cell and 18 for a stimulated cell. The MTOC has the shape of an elongated sphere. These numbers are averages of the numbers of serial sections required to pass through the long dimension, and the short dimension of the MTOC. Estimates of the number of microtubules per cell are 48 microtubules in a resting T lymphocyte and 198 microtubules in a stimulated T lymphocyte.

198 microtubules in a fully stimulated T cell is a much higher microtubule content than 135 microtubules reported for a 3T3 cell (Hiller and Weber, 1978). These authors used a high triton concentration, and rapid permeabilization, to produce cytoskeletons of 3T3 cells for immunofluorescent staining. This rapid procedure may not adequately preserve the microtubules. More extensive microtubule networks could be seen in 3T3 cells stained by the immunofluorescence procedure I used than were shown by these authors (results not shown).

The tubulin contents, as a percent of total cell protein, increased during stimulation of each of the lymphocyte populations (tables 2 and 3). The increased tubulin contents coincided with the increased microtubule networks seen in stimulated cells (fig. 8C, 10C and 13B; table 1).

Differences in the tubulin contents of mixed, B cell, and T cell populations, and between cultured and purified live populations, emphasize the need to use selected populations to examine the microtubule and tubulin contents. T enriched populations were used for a careful comparison of the increases in microtubule and tubulin contents seen during stimulation. In the T enriched population, there was a 2.5 fold increase in the total tubulin content and a 2 fold increase in the number of microtubules during stimulation (tables 1 and 2).

The increase in tubulin and actin, as a percent of total cell protein, during activation may be a reflection of the increased ratio of cytoplasmic / nuclear protein mass seen during activation (Soren, 1970). All cytoplasmic proteins may increase in approximately the same proportions during stimulation. Tubulin and actin contents did not increase in the same manner throughout activation in T cell populations. Both showed large increases during the first 24 h. Between 24 and 48 h of activation the actin content dropped while the tubulin continued to increase.

A transient burst of actin synthesis was seen in quiescent 3T3 cells induced to proliferate by the addition of serum, but not in cycling 3T3 cells (Riddle and Pardee, 1979). 3T3 cells in G0 progress towards S when stimulated with growth factors, a response which is similar to resting lymphocytes stimulated with a mitogen such as concan A.

Increased tubulin contents were seen during PHA stimulation of mixed populations of human peripheral blood lymphocytes, although the values obtained were low (Pipeleers et al., 1977; Sherline and Mundy, 1977). The tubulin contents, determined by a  $^3\text{H}$ -colchicine binding assay, were 10 fold lower than tubulin determinations made on fresh human peripheral blood lymphocytes by the double isotope labelling procedure (Anderson, personal communication). The double isotope labelling procedure found that the tubulin content of lymphocytes from individual donors ranged from 3% to 6% of total cell protein, with a mean value of  $4.1 \pm 1.8\%$ .

The double isotope labelling procedure directly measures tubulin specific peptides. The  $^3\text{H}$ -colchicine binding assay measures the ability of tubulin to bind colchicine. Tubulin determinations of 3T3 cells made using  $^3\text{H}$ -colchicine binding assay (Eichorn and Peterkofsky, 1979) were more than 10 fold lower than the determinations of 3.5% and 3.3% of total cell protein made by double isotope labelling (Anderson, 1979) and radioimmunoassay (Hiller and Weber, 1978) respectively.

It could be that not all of the tubulin in these cells is competent to bind colchicine, or that tubulin is being degraded when cell homogenates are incubated at  $37^\circ\text{C}$  with labelled colchicine. These problems may be particular to the cell type being used. Borisy's group found a tubulin content of 4.6% of total cell protein, using a  $^3\text{H}$ -colchicine

binding assay, which was comparable to determinations made using other techniques (Bulinski et al., 1980).

The tubulin content of 0.51 pg/cell in the 48 h stimulated T cell population (table 2) was an average of all the cells in the population. The increased tubulin content during stimulation was due to a much larger increase in the fully stimulated cells. An estimate of the tubulin content in fully stimulated cells can be made. From the cell size profiles, 40% of the cells in the 24 h population increased in size (hatched area in fig. 7). If the increased tubulin content is due to the cells which increased in size, then the partially stimulated cells in the 24 h population have a tubulin content of 0.43 pg/cell. In the 48 h stimulated population, nonstimulated cells represent 40% of the area under the curve in figure 7 (the non stippled area). 30% of the cells are partially stimulated (stippled and hatched area), and 30% of the cells are fully stimulated (stippled and not hatched area). If nonstimulated and partially stimulated cells have tubulin contents of 0.20 pg/cell and 0.43 pg/cell respectively, then the fully stimulated cells must have a tubulin content of 1.00 pg/cell to make the average of 0.51 pg/cell in the population.

The amount of tubulin in polymer form in resting and stimulated cells can be calculated in order to determine if there is a change in the polymer / soluble tubulin ratio.

The mass of tubulin per unit length of tubule is 0.27 fg/ $\mu\text{m}$ , based on a MW of 100,000 for a tubulin dimer (Valenzuela et al., 1981) and 1625 dimers per micrometer of tubule (Amos, 1977). The 48 microtubules in a resting cell (average diameter 6.6  $\mu\text{m}$ , circumference of 20.7  $\mu\text{m}$ ), running under the cell surface from the MTOC to the other end of the cell, have a total length of 494  $\mu\text{m}$  of microtubule and a tubulin content of 0.13 pg. A blast cell with a diameter of 11  $\mu\text{m}$  and 198 microtubules, each 17.3  $\mu\text{m}$  long, has 3426  $\mu\text{m}$  of tubule which contains 0.93 pg of tubulin. Using these estimates, the percent of tubulin in polymer form is 65% in resting cells and 93% in a blast cell. The percent of tubulin in polymer form in human peripheral blood lymphocytes, estimated by  $^3\text{H}$ -colchicine binding assay of total and sedimentable tubulin contents, was 50% in resting cells and increased to 75% after 72 h of PHA stimulation (Pipeleers et al., 1977). The tubulin content in polymer form was estimated to be 40% in 3T3 cells (Hiller and Weber, 1978) and 60% in human fibroblasts (Spiegelman et al., 1979B). The respective authors considered these to be underestimates, because of the methods used to determine the polymer content. During differentiation of neuroblastoma the percent of tubulin in polymer form increased from 11% to 55% of the total tubulin content (Climstead, 1981).

The microtubules in a 3T3 cell were estimated to contain 2 pg of tubulin (Hiller and Weber, 1978); much more than the

polymer content of a stimulated T cell. A 3T3 cell was estimated to have fewer microtubules than a stimulated T cell but they are much longer, having an average length of 50  $\mu\text{m}$  and extending from the MTOC to the cell margins. The soluble tubulin concentration in a 3T3 cell was estimated to be 1.2 mg/mL (Hiller and Weber, 1978). During differentiation of neuroblastoma, the concentration of soluble tubulin decreased from 1.6 mg/mL to 0.8 mg/mL (Olmstead, 1981). These concentrations are much higher than the critical concentration for in vitro assembly of 0.3 mg/mL (Bergan and Borisy, 1980). This suggests that in these cells not all of the soluble tubulin is available for assembly.

A resting T lymphocyte with 70 fg of unpolymerized tubulin and a cell volume of 95 fL (fig. 7) would have a soluble tubulin concentration of 0.74 mg/mL. A blast cell with a soluble tubulin content of 70 fg and a volume of 600 fL would have a soluble tubulin content of 0.12 mg/mL. This low concentration of soluble tubulin suggests that the tubulin content may be limiting microtubule assembly in blast cells.

All of these estimates of soluble tubulin assume that there is no partitioning of soluble cytoplasmic proteins across the nuclear membrane. Goldstein and Ko (1981), using Amoeba proteus,

showed that  $^{35}\text{S}$ -labelled cytoplasmic proteins readily diffused into unlabelled nuclei which were transplanted into the labelled cytoplasm. If soluble tubulin were excluded from the nucleus then the tubulin concentration in the cytoplasm would be higher than the estimates, particularly in resting cells which have a large nucleus / cytoplasm ratio.

In neuroblastoma there was a large increase in the microtubule content, but no increase in the total tubulin content during differentiation (Olmstead, 1981). The increased microtubule assembly and concomitant drop in concentration of soluble tubulin may have been due to the synthesis of a new MAP which promotes microtubule assembly (Olmstead and Lyon, 1981). MAP synthesis could also play a role in regulation of microtubule assembly during lymphocyte activation. Synthesis of more, or new species of MAPs may contribute to more efficient microtubule assembly resulting in the lowered concentration of soluble tubulin. No one has looked at MAPs in resting or stimulated lymphocytes.

Unlike the change in microtubule assembly seen during differentiation of neuroblastoma, the increase in microtubule content during lymphocyte activation was accompanied by a large increase in tubulin content, due to *de novo* tubulin synthesis. Rates of tubulin synthesis and degradation could regulate microtubule assembly by determining the availability of assembly competent tubulin.

Microtubule assembly could be altered by the synthesis of different tubulins during activation. The appearance of isotubulins with different assembly properties could account for the increase in polymer / soluble tubulin ratio and the drop in concentration of soluble tubulin during activation.

Isotubulins may have different functional properties. Analysis of tubulin isolated from flagellar or cytoplasmic microtubules from Polytomella showed that different isotubulins were assembled preferentially into one or the other class of microtubule (McKeithan and Rosenbaum, 1981). It wasn't shown if these functionally different isotubulins were coded for by different mRNAs, or resulted from post-translational modification.

Changes in the pattern of isotubulin synthesis were seen during early brain development (Dahl and Weibel, 1979). Changes were also seen in isotubulins translated in vitro from mRNAs isolated from the brain (Gozes et al., 1980). Recently, eight isotubulins were isolated from a single differentiated neuroblastoma cell (Gozes et al., 1981). These authors suggested that in neuroblastoma, different isotubulins may be incorporated preferentially into either axonal or neurite microtubules.

Multiple alpha and beta tubulin genes were found in several species (see for example Cleveland et al., 1980). Different restriction maps were shown for the multiple alpha

(Kalfayan and Weinsink, 1981) and the multiple beta (Cowan et al., 1981) tubulin genes, suggesting that the appearance of isotubulins is regulated, at least in part, at the level of gene expression.

The increased microtubule network seen during lymphocyte stimulation appears to be due to a coordinated increase of the MTOC initiation capacity (Schweitzer and Brown, 1981), the tubulin content, and probably also of the MAP content. The increased efficiency of microtubule assembly during activation, seen as a drop in the soluble tubulin concentration, may also involve the synthesis of isotubulins with different assembly properties, and/or the synthesis of new species of MAPs. Analysis of the assembly properties of tubulin and MAPs from resting and stimulated lymphocytes should reveal the contribution of each to the increased efficiency of microtubule assembly during activation.

The time course of tubulin and MAP synthesis should also be carefully examined, using selected lymphocyte populations. These studies must be accompanied by an ultrastructural and biochemical examination of the MTOCs of resting and stimulated cells to determine how closely changes in tubulin and MAPs are coordinated with changes which result in the increased initiation capacity of the MTOC.

Previous studies (Ben-Ze'ev et al., 1979; Cleveland et al., 1981) of the changes in tubulin synthesis in response

to a change in the polymerization state of the microtubule system have relied on the use of drugs which disrupt the microtubule network, but may also have a number of secondary effects on cellular metabolism. I have established that during lymphocyte activation, there is both a change in the polymerization state of the microtubule system, and a large increase in the tubulin content due to de novo tubulin synthesis. These co-ordinated increases in microtubule and tubulin contents are not seen in the other well studied tissue culture systems discussed in this thesis. This suggests that the activating lymphocyte may be an excellent model system to study the regulation of tubulin synthesis in response to a change in the polymerization state of the microtubule system, without the use of drugs. If tubulin synthesis in lymphocytes is responsive to the soluble tubulin pool size as has been suggested in 3T6 cells (Ben-Ze'ev et al., 1979; Cleveland et al., 1981), then one could propose that the increase in tubulin synthesis during activation might be preceded by an increase in the MTOC activity. The resultant increase in polymer would deplete the pool of soluble tubulin and could induce the increase in tubulin synthesis.

The inhibitory effect of antimicrotubule drugs on several aspects of lymphocyte activation have indicated the potential importance of the microtubule network in lymphocyte activation. The work described in this thesis has clearly established that there is a progressive increase in the

microtubule network and tubulin content of lymphocytes during the activation process. With this basis, studies can be designed to determine which of the events of lymphocyte activation coincide with, and which are dependent upon, the increase in microtubule assembly.

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