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Changes in the Organization and Activity of Lymphocyte Centrosomes During Stimulation

by

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ABSTRACT

Changes in the organization of lymphocyte microtubule organizing centers (centrosomes) were studied by electron microscopy using serial sectioned intact cells. Computer-generated three dimensional reconstructions (Hoens and Hoens, 1981) were made to examine the arrangements of the centrosome components: a pair of centrioles, pericentriolar material and dense aggregates called satellite bodies which are bounded by the pericentriolar material.

The reconstructions show that the satellite bodies of the resting cell centrosomes are preferentially associated with one centriole of the centrosome. Electron micrographs of the centrosomes show that these satellite bodies are connected to the centriole by short stalks. The majority of satellite bodies of stimulated cell centrosomes are situated between the two centricles without any direct connection to either. This centrosome also has more, and somewhat larger satellite bodies which may account in part, for the increase in microtubule content in stimulated cells. The stereo pairs also indicate that both centrosome types are ovoid in shape. The stimulated cell centrosome is slightly larger due to the beginning of centriole pair separation.

Changes in centrosome microtubule initiation activity were tested *in vitro* using Triton X-100 permeabilized cells, pretreated to remove endogenous microtubules, and phosphocellulose-purified bovine brain tubulin and assayed by electron microscopy. Resting and stimulated cells pretreated with cold to disassemble microtubules also lacked satellite bodies which reformed upon incubation at 37°C only if microtubule assembly occurred. In cells pretreated with colchicine to disassemble microtubules the satellite bodies were preserved. At 1mg/ml and 2mg/ml of tubulin in the reaction mixtures the centrosomes of stimulated cells assembled more microtubules than resting cell centrosomes paralleling the increase in microtubule content during stimulation in intact cells. As well, more microtubules were assembled *in vitro* than *in vivo* suggesting that there are more initiation sites than are used in intact cells. This observation, along with the tubulin content determinations of Waterhouse *et al.*, (1983) further suggest that tubulin may be limiting in intact cells. More microtubules were assembled *in vitro* using cold-treated cells suggesting that disaggregation and reformation of the satellite bodies may expose or activate additional microtubule initiating sites.

The results suggest that the change in centrosome organization and the increase in microtubule initiation capacity are related events important in regulating the development of the microtubule network during lymphocyte stimulation.

RESUME

Nous avons étudié les changements dans l'arrangement du centre organisateur des microtubules dans les lymphocytes, par microscopie électronique au moyen de coupes minces en série de cellules intactes. Des reconstructions tridimensionelles ont été réalisées par ordinateur (Hoens et Hoens, 1981) afin d'étudier l'organisation des composantes du centrosome: un paire de centrioles, le matériel péricentriolaire et les corps satellites, agrégats denses entourés par le matériel péricentriolaire.

Les reconstructions montrent que les corps satellites du centrosome des cellules au repos sont préferentiellement associés à l'un des centricles du centrosome. Les micrographies électroniques du centrosome démontrent que les corps satellites sont reliés au centriole par de petits bras. Dans les cellules stimulées, la plupart des corps satellites du centrosome se retrouvent entre les deux centricles sans association directe avec l'un ou l'autre. Ces corps satellites sont également plus nombreux et un peu plus volumineux: cela pourrait, en partie, expliquer l'augmentation du nombre de microtubules dans les cellules stimulées. Les reconstructions en stéréo montrent, de plus, que les deux types de centrosome ont un forme ovoide. Le centrosome des cellules

stimulées, est légèrement plus gros parce que les deux centrioles commencent à se séparer.

Nous avons étudié par microscopie électronique, les changements dans l'activité du centrosome à initier les microtubules *in vitro*. Nous avons utilisé des cellules perméabilisées avec le triton X-100, prétraitées pour éliminer les microtubules endogènes, et de la tubuline isolée à partir du cerveau de boeuf, purifiée sur phosphocellulose. Les cellules au repos et les cellules stimulées qui ont subi un traitement "au froid" pour désassembler les microtubules sont dépourvues de corps satellites: ceux-ci se reforment durant une incubation à 37°C seulement s'il y a assemblage des microtubules. Dans les cellules prétraitées avec la colchicine pour désassembler les microtubules, les corps satellites sont préservés. A 1mg/ml et 2mg/ml de tubuline dans les solutions de réactions, le centrosome des cellules stimulées assemble plus de microtubules que celui des cellules au repos: cela corrobore l'augmentation dans le contenu en microtubules durant la stimulation de cellules intactes. Egalement, plus de microtubules ont été assemblés *in vitro* que *in vivo*: cela suggère qu'il y a plus de sites d'initiation que ceux utilisés dans les cellules intactes. Cette observation, de même que les déterminations du contenu en tubuline effectuées par WaterHouse et collab. (1983) suggère de plus que la tubuline peut être un facteur limitant dans les cellules intactes. Plus de microtubules ont

été assemblés in vitro dans les cellules traitées au froid: il semble ainsi que la désagrégation et la réformation des corps satellites peuvent exposer ou activer des sites additionels initiant les microtubules.

Les résultats suggèrent que les changements dans l'organisation du centrosome et l'augmentation de sa capacité à initier les microtubules soient des processus reliés, importants dans la régulation du développement du réseau de microtubules durant la stimulation des lymphocytes.

ABBREVIATIONS

MTOC	Microtubule Organizing Centre
MT	Microtubule
SB	Satellite Body
PCM	Pericentriclar Material
PIPES	Piperazine-N-N'-bis[2-ethane sulfonic acid]
EB	Bershadsky's Buffer
GTP	Guanosine 5' triphosphate
con A	Concanavalin A

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N-acetylglucosamine	Sigma
PIPES	Sigma
Rhodamine goat anti-mouse IgG	Cappell
Colchicine	Baker Chemical Co.
Phosphocellulose P-11	Whatman
Other chemicals	reagent grade or better

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CONTENTS

Chapter I

INTRODUCTION

In his 1966 review Porter suggested the existence of "foci" capable of initiating microtubules. Pickett-Heaps (1969) later suggested the term microtubule organizing center (MTOC) for these foci. He advanced the idea that the MTOC would control microtubule assembly spatially and temporally.

Much research has been done on microtubules because they are involved with chromosome movement (reviewed by Pickett-Heaps, Tippit and Porter, 1982), cytoplasmic transport (reviewed by Hyams and Stebbings, 1979), maintenance of cell shape (reviewed by; Tucker, 1979; Brown, Stearns and MacRae, 1983) and flagellar and ciliary motion (reviewed by Warner, 1979)

The ability of microtubules to carry out these functions depends on how they are organized into specific arrays. Accordingly much research has involved investigating MTOCs in order to determine the role they have in governing microtubule organization (reviewed by; Raff, 1979; Tucker, 1979; Peterson and Ferns, 1980; Brown et al., 1983).

1.1 THE DIVERSITY OF MTOCS

Can the structure of an MTOC regulate the spatial organization of microtubules? The MTOCs of lower eukaryotes exhibit varying degrees of structure and thus serve as good systems in which to study the contribution of the MTOC to spatial control of microtubule assembly.

Brown and Bouck (1973, 1974) showed that there are two MTOCs that organize precisely arranged sets of cytoplasmic microtubules in Ochromonas danica. One is called the kinetobeak and the other is known as the rhizoplast. The cytoplasmic microtubules emanating from the MTOCs were shown to have differential, and reversible, sensitivities to the anti-microtubule agents colchicine, high hydrostatic pressure, and the herbicide isopropyl N-phenylcarbamate. After loss of the normal fusiform cell shape due to treatment with such agents, cells were allowed to recover in fresh medium. Microtubules initially reappeared from the MTOCs in the pattern seen prior to the disassembly treatment suggesting that the MT pattern was imposed by the MTOC.

Another highly structured MTOC is found in the quadriflagellate alga Polytomella agilis. Eight rootlets of the basal body complex initiate the assembly of precisely ordered sets of cytoplasmic microtubules. Microtubule reassembly after various microtubule disrupting treatments duplicated the original pattern (Stearns and Brown, 1981). Stearns and Brown also showed (1981) that these complexes, devoid of

original microtubules, could assemble microtubules from purified brain tubulin *in vitro* in a pattern that closely resembled that seen *in vivo*.

Tucker (1982) compared the cytopharyngeal basket MTOC of *Nassula* and the axopodial MTOC of *Echinosphaerium*. The basket MTOC is composed of two parallel dense layers attached to basal bodies. Arrays of parallel microtubules emanate from the lower dense layer. Cross sectional views of the microtubule arrays close to the MTOC reveal the presence of dense intermicrotubular material. Microtubule disassembly/regrowth experiments showed that the MTOC initiated microtubules in the same regular pattern seen in untreated cells.

In *Echinosphaerium* the axopodial MTOC is not structured. Instead, amorphous blobs initiate the assembly of microtubules in an apparently random fashion. The blobs come into close association and the appearance of intermicrotubule linkers imposes a pattern on the microtubules after their initiation. Tucker has called the basket MTOC of *Nassula* a nucleating template. That of *Echinosphaerium* could be considered solely as an initiating MTOC.

The [redacted]s of *Dictyostelium discoideum* and *Polysphondilium violac[redacted]* (also known as Nucleus Associated Bodies; NABs) are made [redacted] of a layered discoidal core, a fibrillar matrix and nodules (Roos, 1982). Roos considers the NABs to be of the nucleating template type because of the spatial arrangement of the nodules, each of which initiates one or two mi-

crotubules, on the core. Microtubule disassembly/regrowth experiments show that some of the microtubules contribute to maintenance of the nucleus' pear shape, however the the rest of the microtubule distribution is not as regular as that seen in Ochromonas, Polytomella, or Nassula.

1.2 MTOCS IN MAMMALIAN CELLS

MTOCs by definition organize microtubules. Therefore both centrosomes and kinetochores are MTOCs. While it is well accepted that centrosomes are MTOCs that initiate microtubule assembly, there is debate when considering kinetochores. The next three sections will illustrate; 1) the commonly held view on centrosome function, 2) some of the *in vivo* data regarding microtubule initiation by kinetochores and, 3) the lack of agreement between the *in vivo* and *in vitro* data as it relates to the microtubule initiating capacity of kinetochores.

1.2.1 Centrosomes as MTOCs

Immunofluorescence microscopy using antibodies to tubulin and a wide variety of cell lines (Osborn, Webster and Weber, 1978; Watt and Harris, 1980; Brinkley *et al.*, 1981; and many others) as test systems have shown extensive networks of microtubules associated with a brightly fluorescing spot. In disassembly and regrowth experiments *in vivo* Osborn and Weber (1976) showed that microtubules emanate from these bright spots which likely correspond to the centrosomes.

Centrosomal MTOCs in animal cells are composed of a pair of centricles and amorphous, osmicphilic pericentriolar material. Pericentriolar satellites have also been detected in some cell types. Centrosomes are less structured than the MTOCs of lower eukaryotes (see section 1.1) and consequently, it is difficult to imagine how the MTOC could exercise spatial control over precisely patterned arrays of microtubules.

One of the earliest electron microscopic studies of centrosomes was carried out by Stubblefield and Brinkley (1967). They described the structure of centricles from Chinese Hamster Don-C cells and suggested that the centricle was the initiator of microtubules. Pericentriolar satellites were also described in these cells and were proposed to anchor microtubules and allow their elongation after initiation by the centricles. More recent experimental studies have shown that this is not the case.

Berns et al., (1977) carried out a series of experiments using acridine orange-treated mitotic PtK2 cells to determine what components of the centrosome were necessary to have proper spindle function. Even in mitosis these large cells remain fairly flat making the spindle easy to see by phase contrast microscopy. The centrosome, which is duplicated prior to mitosis, is seen as two dots at the ends of the spindles. By prophase the dots representing the duplicated centrosome have separated far enough so that one can

be irradiated with a laser microbeam. The dye absorbs maximally at the wavelength used and the heat resulting from the irradiation damages a highly localized region of the cell, in this case the spindle pole. The cell is then cultured to see if it will proceed through mitosis before being prepared for electron microscopy. Electron micrographs of the irradiated spindle pole region showed that pericentriolar material was disrupted and that microtubules were absent when compared to the nonirradiated spindle pole. Even though the centrosomes had separated only a half spindle was left and anaphase movement had ceased.

In a second paper Berns and Richardson (1977) reported that the specific irradiation of centricles in acridine orange-treated PtK2 cells at prophase caused their displacement from the poles of the spindle but that mitosis proceeded normally. This was taken to mean that the pericentriolar material is the true microtubule initiating structure of the mitotic centrosomal MTOC. This interpretation has been supported by in vitro assembly studies (see below).

1.2.2 Kinetochores as MTOCs

Kinetochores of mammalian cells are trilaminar structures found at the constriction of condensed chromosomes. Telzer, Moses and Rosenbaum (1975) reported that chromosomes isolated from mitotic HeLa cells could be used in in vitro microtubule assembly assays with chick brain tubulin. Whole mount

electron microscopy of chromosomes isolated at low temperatures showed the kinetochores as differentially staining structures. When the chromosomes were incubated with the chick brain tubulin microtubules were seen radiating from the constricted region of the chromosome, presumably attached to the kinetochores.

Nocodazole, an antimicrotubule drug whose depolymerization activity is more easily reversible than colchicine or colcemid (De Brabander et al., 1976) was used by De Brabander and his colleagues (1980) in experiments investigating kinetochore function in living mitotic PtK2 cells. Microtubule reassembly was detected by immunocytochemistry and electron microscopy at spindle poles and in the vicinity of kinetochores facing spindle poles shortly after removal of nocodazole. If reassembly occurred in the presence of ATP inhibitors kinetochore microtubule assembly was prevented. Instead of a spindle, only large centrosomal asters formed. They concluded that inhibition of kinetochore initiated microtubules prevented the interaction between spindle poles and kinetochores and resulted in failure to form a spindle.

In order to test if kinetochores could initiate microtubule assembly in vivo Witt, Ris and Borisy (1980) used colcemid-treated CHO cells to collect mitotic cells whose chromosomal kinetochores had never been exposed to microtubules. After the colcemid was removed, microtubule growth occurred and was assayed at various times by high voltage electron

microscopy. Spindles were reconstructed from serial thick sections and the kinetochores of chromosomes that were far away from the centrosomes were examined. It was reasoned that any microtubules associated with these kinetochores would not have been initiated by the centrosome. Their results showed that in this system kinetochores would serve as initiating MTOCs.

Results related to those of De Brabander et al., (1980, 1981) were reported by Rieder and Borisy (1981) for mitotic PtK1 cells recovering from cold treatment. In the early stages of recovery microtubules were associated only with the spindle pole. A bit later microtubules were associated with the kinetochores closest to the poles. The other kinetochores of the same chromosomes were not as close to the poles and had microtubules attached to them only at a later time. It was suggested that the sequence of microtubule association events could be explained most readily by assuming that centrosome-initiated microtubules interacted either directly with the kinetochores or with small microtubules initiated by the kinetochores.

The protocol used by Witt et al., (1980) allowed microtubule assembly from both kinetochores of the chromosomes without any time delay as noted by De Brabander et al., (1981) and by Rieder and Borisy (1981). This difference could be attributed to the different microtubule disassembly treatments that were used.

1.2.3 Do Kinetochores Initiate Microtubules?

There is no question in the literature that kinetochores can initiate microtubule assembly *in vitro* (McGill and Brinkley, 1975; Snyder and McIntosh, 1975; Pepper and Brinkley, 1979). There is also evidence to support kinetochore-initiated microtubule assembly *in vivo* (Witt *et al.*, 1980; De Brabander *et al.*, 1981; Rieder and Borisy, 1981). If this is the case then one would expect the structural polarity of microtubules of the mitotic half-spindle to be antiparallel. However this does not appear to be the case.

Two techniques have been developed that decorate microtubules and by doing so indicate their structural polarity. The tubulin hook-decorating technique (Heidemann and McIntosh, 1980) and the dynein decorating technique (Telzer and Haimo, 1981; Haimo, 1982) have shown that microtubules of the mitotic half spindle of PtK2 cells (Euteneuer and McIntosh, 1981) and of the meiotic half spindle of surf clam eggs (Telzer and Haimo, 1981) show a single polarity. This suggests that kinetochores do not initiate microtubules but may anchor centrosome-initiated ones. If they do initiate microtubules it is such that the growth polarity of kinetochore microtubules is opposite to that of centrosomal microtubules. This second view is favored by De Brabander (1982).

1.3 CHANGES IN CENTROSOME STRUCTURE AND ACTIVITY

1.3.1 Relationships of Structural Changes to Mitosis

An early study concerned with changes occurring in the centrosome during the cell cycle was reported by Robbins, Jentzsch and Micali (1968). Using synchronized HeLa cells centrosomes were observed during different phases of the cell cycle by electron microscopy. They found that the parent centrioles commenced replication sometime in S phase and terminated before the end of G2. As the cells proceeded through G2 to prophase the pericentriolar material became more prominent. Robbins and coworkers suggested that changes in the pericentriolar material were causally related to initiation of spindle microtubules.

Weisenberg and Rosenfeld (1975) carried out the first *in vitro* assembly experiments using MTOCs with homogenates of activated and unactivated surf clam eggs. They examined the development of the MTOC as well as increases in initiation capacity. Pellet and supernatant fractions from the two cell preparations were made and mixing experiments were done. They found that the pellet fraction (which contained the MTOC) from homogenates of activated eggs initiated the assembly of microtubules to form asters when mixed with either supernatant fraction. If the pellet from the unactivated egg fraction was used microtubules did not assemble. This demonstrated that the supernatants were functionally the same and led to the conclusion that MTOC activation was one of the

results of egg activation. Thin-section electron microscopy of asters prepared from eggs homogenized 2.5 minutes after activation showed few microtubules and little pericentriolar material; nor were the centrioles obvious. Samples prepared 4.5 minutes after activation showed prominent centrioles surrounded by more pericentriolar material. Many microtubules were seen emanating from the pericentriolar material.

Studies using microtuble-free centrosomes in in vitro assembly assays have shown that the pericentriolar material initiates microtubule assembly in mitotic and interphase cells. If the pericentriolar material of mitotic CHO cells was removed only a few microtubules assembled from the ends of the centrioles during in vitro assembly assays (Gould and Borisy, 1977). The isolated pericentriolar material, which could be identified by the presence of virus-like particles in negatively stained electron microscope preparations, still initiated microtubule assembly in vitro.

Electron microscopic observations of centrosomes from lysed mitotic HeLa cells showed that they were capable of in vitro microtubule assembly whereas those from interphase cells weren't (Telzer and Rosenbaum, 1979). These researchers concluded that the pericentriolar material became competent to assemble microtubules at mitosis. The inability of interphase centrosomes to assemble microtubules in their in vitro assay system seems odd since interphase cells do contain microtubules. They suggested that interphase centro-

somes of HeLa cells retain a minimal initiating capacity that may be completely abolished during the centrosome isolation procedure.

The close association between the centrosome and the nucleus has been noted by a number of researchers (Bornens, 1977; Maro and Bornens, 1980; Nelson and Traub, 1982) and has suggested that centriolar and nuclear replication might be linked. To test this possibility Kuriyama and Borisy (1981a) devised a gentle cell lysis procedure that allowed them to collect centrosomes that were still associated with nuclei. Centriolar changes, as a function of the cell cycle, were studied by whole mount electron microscopy of negatively stained preparations. The centrosomes were classified into six categories based on centriole orientation and the length ratio between daughter and parent centrioles and these classes were related to the different phases of the cell cycle. They found that the addition of thymidine to synchronize the cells had no effect on daughter centriole initiation, elongation or disorientation. If cytoplasts containing centrosomes were prepared and followed through the cell cycle it was found that elongation and disorientation were unaffected but that daughter centriole initiation was inhibited. Kuriyama and Borisy suggested that the nucleus may be needed to signal centriole replication.

To investigate changes in centrosome microtubule initiating capacity during the cell cycle Kuriyama and Borisy

(1981b) prepared cytoplasts from CHO cells and treated them with colcemid to disassemble microtubules. The cytoplasts were lysed to free the centrosomes and used to assemble microtubules *in vitro* using purified brain tubulin. Quantification of microtubules at different stages of the cell cycle showed no significant changes until mitosis when they recorded a five fold increase.

A very detailed study of the changes in the structure of the centrosome during the cell cycle was carried out by Vorobjev and Chentsov (1982) through electron microscopic observations of nonsynchronized pig kidney embryo cells. These cells possess primary cilia attached to one of the centricles. The presence of a primary cilium has been shown to serve as a morphological marker for cells that are in Go of the cell cycle (Tucker, Pardee and Fujiwara, 1979). Vorobjev and Chentsov showed that pericentriolar satellites are associated with the parent centriole in G1 cells. Markham rotation micrographs of thin sections of parental and daughter centrioles showed appendages on the parent and none on the daughter. The satellites, which have microtubules radiating from them, disappeared in G2 to be replaced by fibrillar material surrounding the parent centricles at each pole as the spindle formed. It was determined that the spindle microtubules converge on this material during mitosis. From their observations of centrioles, Vorobjev and Chentsov concluded that a daughter centriole matures fully and becomes a

parent centricle only one and a half cycles after its replication. Very similar findings on the asymmetric distribution of pericentriclar material in mitotic PtK2 centrosomes were reported by Rieder and Borisy (1982) after serial thick and thin sectioning electron microscope studies of interphase and mitotic cells.

Recently it was shown that changes in the MT initiation activity of mitotic centrosomes could by pinpointed to the metaphase/anaphase transition (Snyder, Hamilton and Mullins, 1982). Snyder and her coworkers used PtK1 cells because they remain relatively flat during mitosis. Careful examination of the cells by phase contrast microscopy allowed them to determine that anaphase movement started 30±2 minutes after nuclear membrane breakdown. The transition of late metaphase to early anaphase was separated by only one to two minutes.

Cells were treated with nocodazole *in vivo* prior to anaphase onset or at metaphase/anaphase transition. Microtubule regrowth from the centrosomes was initiated by removing nocodazole. They found that a complete spindle could form and mitosis could be successfully completed. Regrowth of microtubules in cells treated with nocodazole at the onset of sister chromatid separation in anaphase, however, was reduced and the cells were unable to complete mitosis. Snyder *et al.*, (1982) also demonstrated this decrease in MT initiation capacity at the metaphase/anaphase transition in *in vitro* studies and concluded that once the cells had passed

anaphase the centrosomes were committed to a microtubule initiation capacity typical of interphase cells, even if mitosis was aborted.

1.3.2 Rapid Nonmitotic Changes

The changes that occur in MTOC-microtubule complexes demonstrate that the system is dynamic yet little is known about the triggers that cause such changes in the cytoskeleton. The triggers, or signals could be inside or outside the cell. Internal triggers may come from the cytoplasm or the nucleus. External triggers may be due to changes in culture media for tissue culture cells *in vitro*, the association of one cell type with another *in vivo*, or the presence or absence of hormones, among other factors.

Studies of angelfish melanophores have shown that dramatic changes in centrosome activity can take place within seconds. Schliwa *et al.*, (1979) showed that pigment distribution was a function of the state of the central apparatus (the MTOC of this cell). When the pigment was dispersed about 2400 microtubules radiating from a large diameter pericentriolar cloud could be counted in electron micrographs. Aggregated pigment was correlated with a much reduced microtubule number and a smaller pericentriolar cloud. The aggregation and dispersal of pigment takes place in less than than a minute as does microtubule disassembly and reassembly.

Cells with aggregated and dispersed pigment were treated with colcemid or cold temperature to disassemble microtubules and then lysed into purified brain tubulin solutions to test *in vitro* MTOC assembly capacities. MTOCs of cells with dispersed pigment assembled more microtubules *in vitro* than the MTOCs of cells with aggregated pigment. It was concluded that variations of the ability of the central apparatus to regulate microtubule assembly are a function of the size of the pericentriolar cloud which is correlated with changes in pigment distribution.

The role of microtubules in pigment movement was also studied by Porter and McNiven (1982). Pigment granule movement was followed in living squirrelfish erthyrophores through cycles of aggregation and dispersion and demonstrated that each granule dispersed to the same position in the cytoplast (their term for the structure consisting of pigment, microtubules and a fibrous network called the microtrabecular lattice). They found that pigment aggregation (and cytoplast contraction) was most likely due to shortening of the microtrabeculae. Dispersed granules returned to the same location because of lengthening of the microtrabeculae. The microtubules, which did not appear to undergo major changes, served as guides for the radial expansion of the cytoplasts. Porter and McNiven suggest that the discrepancy between their results and those of Schliwa's group could be due to observations of "a relatively small popula-

tion of microtubules central to the cytoplast that is less stable and does depolymerize as the pigment aggregates." The disparity between the two sets of results indicates that more work could be done to determine if there are different sets of microtubules or just species differences.

Undifferentiated neuroblastoma cells are round cells that can be induced to differentiate by the removal of fetal calf serum (external trigger) from the medium. Immunostaining of undifferentiated cells (Spiegelman, Lopata and Kirschner, 1979) with antitubulin antibodies have shown multiple flucrescent spots with associated microtubules. On the basis of disassembly/regrowth experiments Spiegelman and coworkers identified these spots as MTOCs. Upon differentiation these spots migrate and coalesce into one large spot prior to the extension of a microtubule-filled neurite. Disassembly/regrowth experiments showed that this large spot served as an MTOC. They suggested that this large spot represented an aggregate of all the initiation sites of the cell. Sharp, Osborn and Weber (1981) later showed by immunoflucrescence microscopy and electron microscopy of the same cell that the multiple initiation sites in undifferentiated cells were multiple centricles. The large fluorescent spot was shown to be an aggregation of the multiple centricles.

A different type of external signal was demonstrated by the immunostaining of scleroblasts detached from, or attached to fish scales from squirrelfish (Byers, Fujiwara and

Porter, 1980). If the cells were stained while still attached to the scales the microtubules in adjacent cells exhibited a pattern of linear microtubules radiating from an MTOC. Byers et al., (1980) claimed that the pattern was superimposable from one cell to the next with good matches in about 90% of the cells. However, if the scleroblasts were removed from the scales and cultured before immunostaining the linear pattern of microctubules was not observed. In its place they saw microtubule loops running throughout the cell and the pattern was variable. The results suggest that the arrangement of scleroblasts in situ serves as an external signal that may be required by the MTOC to maintain the regular radial microtubule pattern.

Immunofluorescence microscopy, phase contrast microscopy, and cinemicrophotography were used by Gotlieb et al., (1981) to study MTOC behavior in wounded cultures of porcine aortic endothelial cells. An external signal was introduced by removing a portion of cells from the substrate (wounding). Immunofluorescence and phase contrast time lapse records show that the cells closest to the wound began to migrate into it about four hours later with the centrosome between the leading edge and the nucleus of these cells. In comparison, immunofluorescent staining of confluent layers showed microtubules radiating from MTOCs that were randomly positioned with respect to the nucleus. The centrosome reorientation was most obvious in the cells closest to the wound. Treat-

ment of the cells with cytochalasin B to inhibit cell migration did not prevent MTOC reorientation, but colcemid treatment did (Gotlieb, Subrahmanyan and Kalnins, 1982). They concluded that intact microtubules were required for MTOC reorientation but that reorientation could occur independently of cell migration.

The existence of internal signals was suggested by Solomon from his studies of neuroblastoma cell morphology. He proposed that the relatedness of sister cell morphologies (1979) and the recapitulation of neurite morphology after microtubule disassembly (1980) was controlled by a "heritable endogenous determinant" and suggested that the MTOC was a likely candidate.

1.4 COMPOSITION OF MTOCS

In addition to tubulin in basal bodies (Anderson and Floyd, 1980), which are equivalent to centrioles, other components such as calmodulin, nucleic acids and trypsin-sensitive proteins (reviewed by Brown et al., 1983) have been detected in MTOCs using various techniques. These components have been suggested to function in microtubule disassembly (calmodulin), function and/or replication of centioles and kinetochores (nucleic acids) and microtubule initiation (trypsin-sensitive proteins). However, the localization of the components or precise definition of their proposed function(s) have not yet been firmly established.

1.5 THE MICROTUBULE SYSTEM OF LYMPHOCYTES

Lymphocytes can be used as a model system to study changes in centrosome organization and activity because they undergo blastogenesis in response to the addition of mitogenic lectins. Resting splenic lymphocytes are in the Go stage of the cell cycle. Addition of concanavalin A results in increases in DNA, RNA and protein synthesis (Ling and Kay, 1975). Increases in microtubule content also have been described for PHA-stimulated human peripheral blood lymphocytes (Biberfeld, 1971) and for con A-stimulated mouse mixed splenic lymphocyte populations (Rudd et al., 1979).

The contribution of microtubules in lymphocytes to the redistribution of cell surface receptors (patching and capping) has been investigated extensively by Edelman and coworkers (Edelman, Yahara and Wang, 1973; Yahara and Edelman, 1973a; Yahara and Edelman, 1973b). Treating the cells with high concentrations of con A inhibits capping of surface immunoglobulin. If the cells were treated with colchicine the con A inhibition was relieved and the cells were able to cap. They suggested that the state of assembly of the microtubules modulated cell surface receptor redistribution (Edelman et al., 1973). Yahara and Kakimoto-Sameshima (1978) later suggested that patching and capping disassembled microtubules. Their conclusion was based on immunofluorescence micrographs in which they were unable to detect microtubules in capped cells.

Using an improved fixation technique Rogers, Khoshbaf and Brown (1981), demonstrated by immunostaining that microtubules were present at all stages of the capping process and that there were only subtle changes in their organization. Cells with intact microtubules always had caps located over the region of the cell where the centrosome was located. Colchicine disassembly of microtubules resulted in random cap formation.

Recently, the microtubule assembly-promoting drug Taxol was also shown to affect capping (Paatero and Brown, 1982). Treating lymphocytes with taxol caused an extensive reorganization of microtubules into bundles radiating from the centrosome, and random cap formation with respect to the centrosome. In addition, Con A inhibition of surface immunoglobulin capping was also relieved by taxol.

The taxol results along with the colchicine results of Rogers et al., (1981) led Paatero and Brown to suggest that the normal spatial organization of cytoplasmic microtubules in untreated lymphocytes is required to get cap formation consistently occurring over the region of the cell containing the centrosome.

Increases in microtubule content, seen by electron microscopy, in PHA-stimulated human peripheral blood lymphocytes (Eiberfeld, 1971) and in con A-stimulated mouse splenic lymphocytes (Rudd et al., 1979) appears to be dependent, in part, on increases in the amount of tubulin. Using a

^{3}H-colchicine binding assay Sherline and Mundy (1977) showed that there was an increase in tubulin content, relative to total cell protein, in PHA-stimulated human peripheral blood lymphocytes. Recently Waterhouse, Anderson and Brown (1983) accurately determined a differential increase in tubulin content for con A-stimulated mouse splenic T lymphocytes. They also showed, by direct microtubule counts from electron micrographs, that there was about a 2-fold increase in microtubules in stimulated cell populations and suggested that this increase was dependent on an increase in the amount of tubulin.

Kocsksrethey and Schafer (1982) measured the amounts of translatable tubulin, calmodulin and actin messages, isolated from resting and stimulated bovine lymph node lymphocytes, in cell-free translation systems. They showed that the tubulin message level increased significantly as a result of stimulation. Actin message, present in large amounts in resting cells, showed a five fold increase during stimulation while calmodulin message levels did not change.

The observation that the tubulin message level rose dramatically from nearly undetectable amounts in resting cells lends support to the suggestion of Waterhouse and coworkers that tubulin content is a limiting factor in the increase in microtubule content.

Given that microtubules radiate from the centrosome and that stimulation of lymphocytes is accompanied by an in-

crease in microtubule content, I decided to investigate the effects of stimulation on the organization and activity of the centrosomal microtubule organizing center.

There are three main purposes to the research presented in this thesis:

1) To describe the structure of the centrosome in resting lymphocytes, and the structural changes that occur as a result of stimulation.

2) To identify those structural changes which are correlated with the increase in microtubule content of stimulated cells.

3) To develop an *in vitro* microtubule assembly assay for the microtubule initiation capacity of the lymphocyte centrosome in order to test for changes resulting from stimulation.

Chapter II

MATERIALS AND METHODS

2.1 CELL PREPARATION

Male Balb/c mice, eight to fourteen weeks old, were killed by cervical dislocation and their spleens removed. In order to make a crude suspension in medium (RPMI 1640 + 10% fetal bovine serum; FBS) the spleens were minced through a stainless steel screen. The crude splenocyte suspension was then passaged through a 20 gauge needle several times in order to break up cell clumps.

2.2 T CELL ENRICHMENT

To enrich the culture for T lymphocytes the wheat germ agglutinin (WGA) method of Bourguignon, Poder and McMahon (1979) was used. Splenocyte cultures were treated with 100ug/ml WGA at a density of approximately 4×10^6 cells/ml for thirty minutes on ice and the suspensions were overlayed on a 90% calf serum cushion. The B lymphocytes clump, ~~along~~ with most of the red blood cells, and settle to the bottom of the cushion. After thirty minutes on ice the presumptive T cell layer was removed from the top of the cushion and centrifuged at 450xg for 5 minutes in an IEC clinical centrifuge. The pellet was resuspended twice in 0.2M N-acetyl-glucosamine to wash out remaining WGA.

Remaining red blood cells were lysed by resuspending the pellet in 0.16M NH_4Cl for seven minutes. The suspension was underlayed with 100% calf serum and then centrifuged at 450xg. The supernatant containing red cell membranes was aspirated and the pellet of T cells was resuspended in medium and cultured at a density of $2x10^6$cells/ml.

The degree of enrichment was determined by incubating an aliquot of cells with Rhodamine-labeled goat anti-mouse IgG for ten minutes at 37°C. The percentage of Ig positive cells was determined by fluorescence microscopy (Rogers et al., 1981). Using the WGA enrichment method it is possible to obtain populations of the cells that are 93%-97% T cell enriched.

2.3 CON A STIMULATION

If T cells were to be stimulated, concanavalin A (con A) was added to the culture at a final concentration of 4ug/ml. The cells were cultured in the continuous presence of con A for 48 hours with supplements of con A-free medium (10% of original volume) added at 24 hours in order to increase cell viability.

The extent of stimulation was determined by ^3H-thymidine (1 hour pulse, 2uCi/ml; Rudd et al., 1979) uptake at 48 hours as well as by increases in cell diameter as determined with an ocular micrometer.

2.4 CELL PERMEABILIZATION

Resting and stimulated cells were permeabilized in the microtubule stabilizing buffer described by Bershadsky et al., (1976) containing; 4M glycerol, 50mM Imidazol, 50mM KCl, 0.5mM MgCl2, 1mM EGTA, 0.1mM EDTA, 1mM 2-mercaptoethanol, pH 6.7 (BB) with 0.005% Triton X-100 for thirty minutes. If the cells were to be used in in vitro assembly assays the microtubules were disassembled in one of two ways; 1)cell cultures were incubated on ice for two hours and then permeabilized in BB for thirty minutes on ice. 2) cell cultures were treated with 10^{-6}M colchicine for six hours and then permeabilized in BB at room temperature for thirty minutes.

2.5 TUBULIN PREPARATION

Fresh bovine brains were purchased from a local slaughterhouse (Crabtree Meatpackers) and used as the source material for microtubule proteins. The temperature dependent assembly/disassembly method of Borisy et al., (1974) was used to isolate microtubule proteins from the brain tissue.

After four cycles of assembly/disassembly, the protein was loaded onto a P11 phosphocellulose column (Weingarten et al., 1978), equilibrated with PIPES buffer (100mM PIPES (Piperazine-N-N'-bis(2-ethane sulfonic acid)), 1mM MgCl2, 1mM EGTA (ethyleneglycol-bis-(beta-aminoethyl ether) N,N' tetraacetic acid) and fractions eluted with PIPES were collect-

ed at 4°C. Tubulin does not bind to the column and is eluted while microtubule associated proteins (MAPs) remain bound to the column. The A280 of the fractions were monitored using a Pye Unicam SP8-100 spectrophotometer. Those fractions with an A280 greater than or equal to 1 were pooled for overnight dialysis against PIPES/8M glycerol.

The following morning the contents of the dialysis bag were diluted 1:1 with PIPES without glycerol and processed through one more cycle of assembly/disassembly by the addition of MgCl2 to 10mM and of GTP to 1.8mM. The concentration of tubulin (6S) was determined by the lowry method and stored at -70°C at a minimum concentration of 10 mg/ml in PIPES without glycerol.

Prior to use in <u>in vitro</u> assays the tubulin was checked for the inability to self-assemble by diluting an aliquot of the protein to the desired final concentration with PIPES and 1.8mM GTP in a final volume of 50ul. After the appropriate incubation time at 37°C the reaction was stopped by the addition of an equal volume of 4% glutaraldehyde in PIPES. A sample of this mixture was then put on a formvar-coated copper grid, negatively stained with uranyl acetate and viewed in a Philips EM201C. Only those tubulin preparations that did not self-assemble were used in <u>in vitro</u> assembly experiments.

2.6 IN VITRO ASSEMBLY CONDITIONS

Resting and stimulated cells pretreated by both microtubule disassembly procedures were permeabilized as described above. At the end of the permeabilization step the cells were centrifuged at 450xg for 5 minutes and the buffer aspirated. The permeabilized cells were then gently resuspended in 0.5 ml of reaction mixture containing self-assembly incompetent tubulin at 1 mg/ml or 2 mg/ml, PIPES and 1.0mM GTP, and incubated at 37°C for 15 minutes or five minutes, respectively. Mock assembly controls were run using the same reaction mixture, but excluding tubulin, at 37supOC for 15 minutes. The reaction was stopped by the addition of an equal volume of prewarmed 4% glutaraldehyde/PIPES and the samples were processed for electron microscopy.

2.7 ELECTRON MICROSCOPY

The experimental samples described in the previous section as well as controls were centrifuged and the cell pellets were resuspended in 4% glutaraldehyde buffered with 0.1M sodium phosphate (pH 7.0) and fixed at room temperature for ninety minutes. After a series of washes (3x10 minutes) in the buffer, the cells were postfixed in 1% OsO4 buffered with 0.05M sodium phosphate on ice for another ninety minutes. This was followed by another series of washes in 0.05M buffer and then by dehydration in a graded acetone series (10 minutes each step). Infiltration was done overnight in

Spurr's hard resin mixture (Spurr, 1969). The following day the resin was replaced with a fresh volume and six more hours of infiltration was done at room temperature. The samples were placed in BEEM capsules and the resin was polymerized at 60°C overnight.

Silver sections (60-70nm thick) were cut using a Dupont diamond knife and a Sorvall Porter-Blum MT2-P ultramicrotome. For serial sectioning ribbons of silver sections were picked up on formvar-coated slotted copper grids with the aid of a Nikon microscope modified for use as a "third hand" as suggested by Fieder (1981). All sections were stained with 2% uranyl acetate and lead citrate (Reynolds, 1963), and examined in a Philips EM201C.

2.8 MICROTUBULE COUNTS

Micrographs were taken of centrosomes in intact cells and in *in vitro* assembly samples at a magnification of x20000. They were enlarged and printed at a final magnification of x60000. The centrosome was centered in a square that corresponded to 1.21μm^2 in the cell. Only microtubules that were bounded by this square were counted.

2.9 COMPUTER RECONSTRUCTIONS FROM SERIAL SECTIONS

The centrosome regions of resting and stimulated cells were traced onto acetate sheets along with the outlines of cell membranes, mitochondria and nuclei. Once a series was completely traced, four fiducial marks in a rectangle were introduced on the last acetate of the series. The next to last acetate was laid on top of the last one. After achieving a "best fit" (Fieder, 1981) according to the cellular landmarks, the fiducial marks were then traced over. In this manner the fiducial marks were transferred up through the entire acetate series. This procedure was repeated for each series.

These acetate sheets were then used to enter the data into the computer that would do the stereo reconstructions. The equipment used for the stereo reconstructions is in the lab of Dr. F. Moens of the Department of Biology, York University. It consists of a Numonics 1224 digitizer, an Ohio Scientific Challenger 3 microcomputer and a DECwriter II terminal (Moens and Moens, 1981). The programs used to do the reconstructions were cowritten by Dr. F. Moens and Mr. T. Moens.

The display unit of the digitizer shows cartesian co-ordinates as determined by the position of the cursor. Once the cursor is aligned and calibrated the first acetate is laid down with its fiducial marks superimposed on the reference marks on the work table. A program called "TUESAVE" is

called up from disk storage and prompts the user for a name for the FILE (the acetate series) as well as the current section number. The cursor is moved to the end of a microtubule (for example) and that end's coordinates are entered onto the computer disk by touching the "enter" button on the digitizer keypad. The cursor is then moved further along the length of the microtubule and the process is repeated. Once all the pertinent information (i.e. microtubules, centricles, Pericentriolar Material (PCM), Satellite Bodies (SBs)) in that section is entered onto the computer disk the process is repeated for following sections. The only change is to increment the section number by one.

In order to get a printout a program called "SMCTPEN" is called up from disk storage. The program requests information on the file name, the number of sections in the file, section thickness (in nm) and the number of different structures to be traced out in different colored inks. Once the information is supplied the program is run and the results are traced using a Calcomp 81 plotter.

The second half of the stereo pair is made by calling up a program called "TUPN" from disk storage. This program prompts the user to supply information on the original file name, a file name for the tilted data and the number of degrees that the data should be tilted either about the X or the Y axis of the cartesian graph. The program then searches the disk for the original data, tilts it the desired num-

ber of degrees and stores the tilted data back on disk under the tilted data file name (the second half of the stereo pairs in this thesis were tilted 10° about the X axis).

To get a printout of the tilted data the "SMOTPEN" program is used again, this time supplying the new file name when prompted by the computer. The stereo effect is achieved by aligning the two printouts side by side and viewing them through stereo glasses. The data can also be called up in various combinations (eg. microtubules and SBs; see figure (5)), using the "SMOTPEN" program, to investigate relationships between the components of the centrosome.

Chapter III

RESULTS

3.1 DESCRIPTION OF CELL SYSTEM

Populations of T lymphocytes, enriched to a maximum of 97% (as judged by immunofluorescence microscopy) and stimulated 80-110 fold (measured at 48 hours by ^{3}H-thymidine uptake) by con A addition were used to ensure that the results of these experiments, previous research (Waterhouse et al., 1983) and ongoing work would be comparable.

Figures 1 and 2 show the major structural changes that occur during con A stimulation of lymphocytes. Resting splenic mouse T lymphocytes are uniformly small with a diameter of 5-7um. Few organelles are found in the thin layer of granular cytoplasm surrounding the nucleus, which is composed largely of condensed chromatin (figure 1).

The addition of the T cell mitogen con A induces the lymphocytes to undergo blastogenesis. There is an increase in cell diameter and at forty eight hours, at least one third of a stimulated cell population is fully stimulated with a minimum diameter of 10um. The amount of cytoplasm and the number of organelles have increased while the density of the cytoplasm is reduced. The nucleus is also less dense due to the dispersion of the condensed chromatin (figure 2).

Figure 1. Resting splenic T lymphocyte. Longitudinal view of centricle (arrow) shows its location in the nuclear cleft and its associated satellite bodies (arrowheads). The nucleus contains a lot of condensed chromatin and the cytoplasm is very dense. Magnification; x32500. The centrosome of this cell is shown in series in figure 3, in stereo in figures 5a and 5b and in model form in figure 6a.

Figure 2. Stimulated splenic T lymphocyte. A centricle is visible in cross section (arrow) located in the nuclear cleft. The condensed chromatin is dispersed throughout the nucleus and the cytoplasm is less dense. The cytoplasmic volume has also increased; magnification x25000. The centrosome of this cell is shown in series in figure 4, in stereo in figures 5c and 5d and in model form in figure 6b.

(2)

3.2 CENTROSOME STRUCTURE

Resting and stimulated cells have one centrosome usually located in the nuclear cleft (figures 1 & 2). At the centre of the centrosome is a pair of centrioles. In lymphocytes centrioles are approximately 0.5um long (figure 3g) and 0.2um in outer diameter (figure 4). The centrioles are surrounded by amorphous, electron dense PCM (figure 4). Within the PCM dense aggregates called SBs can be seen (figures 3 & 4). Microtubules can be seen radiating from the SBs of both cell types; more MTs radiate from the SBs of stimulated cells.

The SBs in resting and stimulated cells lack definite shape although those that are associated with centrioles appear attached to them by short stalks (for example figure 3b). Occasionally, those SBs attached to centrioles have a striated appearance which persists through permeabilization and *in vitro* assembly assays (see for example figures 12b and 13c).

The distal end of the centriole was originally defined from electron microscope observations of cells that have primary cilia growing from one end of the centriole (Wheatley, 1982). This end was also found to have an electron dense material in the lumen (Wheatley, 1982). In longitudinal sections of lymphocyte centrioles (figure 3g), the distal ends are recognized by the presence of the electron dense material. This material extends about halfway down the length of the centriole.

Viewed in cross section (figure 4a-b) starting at the distal end, the electron dense material in the centriole lumen is prominent. Proceeding proximally, the diameter of the centriole increases slightly and at the same time there is a lessening in the amount of the electron dense material in the centriole core.

The orthogonal configuration (i.e. one centriole perpendicular to the other) was not seen for any of the resting or stimulated cell centrosomes that were serial sectioned. Nor did this arrangement appear common in random views of resting and stimulated cells.

The positioning of the centrioles relative to the nucleus is such that the distal end of one of the centrioles is close to the nucleus. The centriole arrangement with respect to each other has the distal ends of the centrioles at opposite ends of the centrosome. This feature was found in resting and stimulated cells.

3.3 CHANGES IN CENTROSOME ORGANIZATION DURING STIMULATION

Serial sectioning of resting and stimulated cells makes it possible to see the relationships between the different substructures of the centrosome.

The centrosomes of three resting cells and three stimulated cells were serial sectioned and three dimensional reconstructions were made. The resting cell series (figure 3)

Figure 3. Centrosome series from resting cell shown in figure 1. Satellite bodies (arrows) appear closely associated with one centriole of the centrosome. Microtubules (arrowheads) are distinguishable radiating from the satellite bodies. A stalk (curved arrow) can be seen showing the attachment of a satellite body to the centriole. Magnification; all figures x60000.

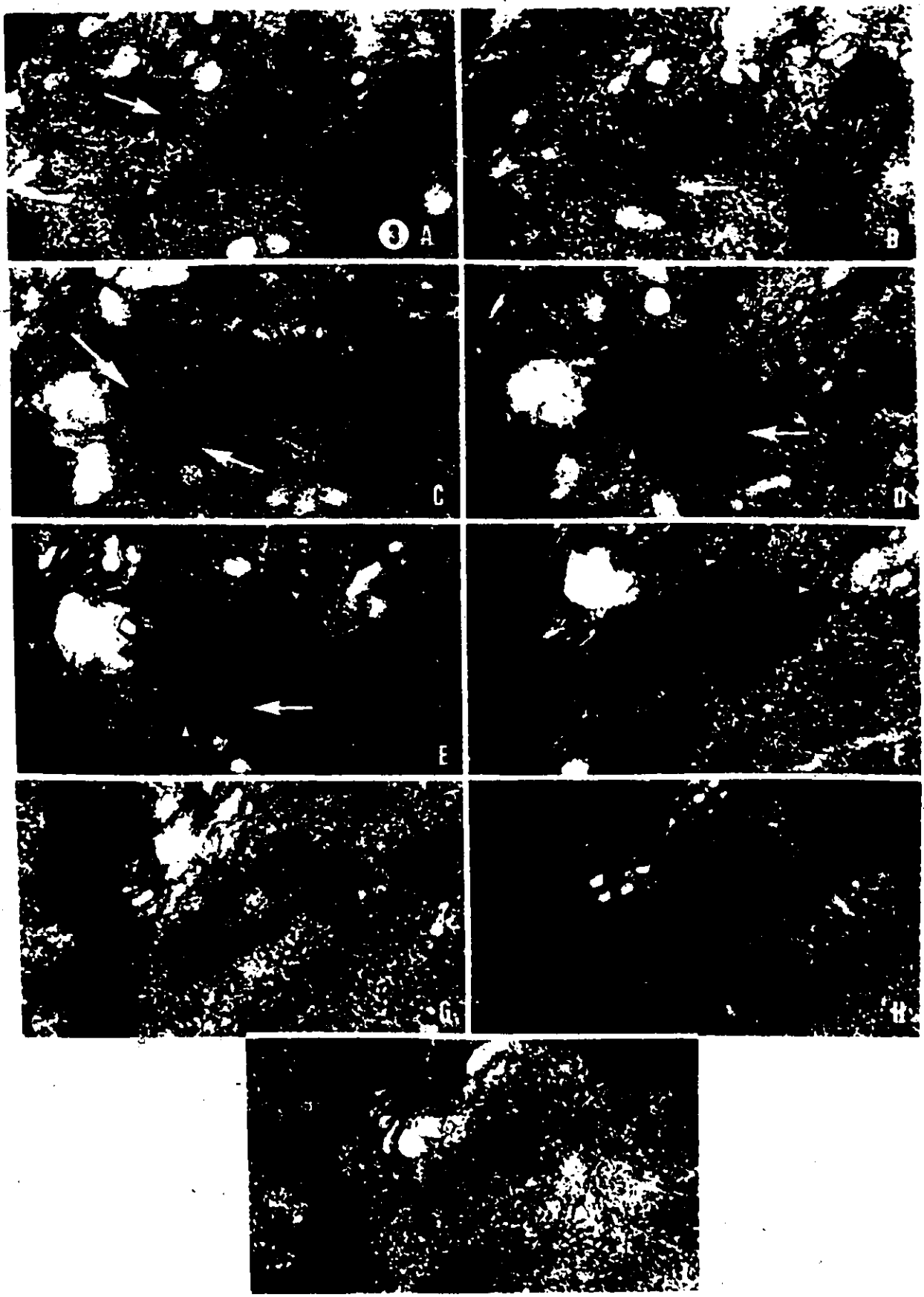
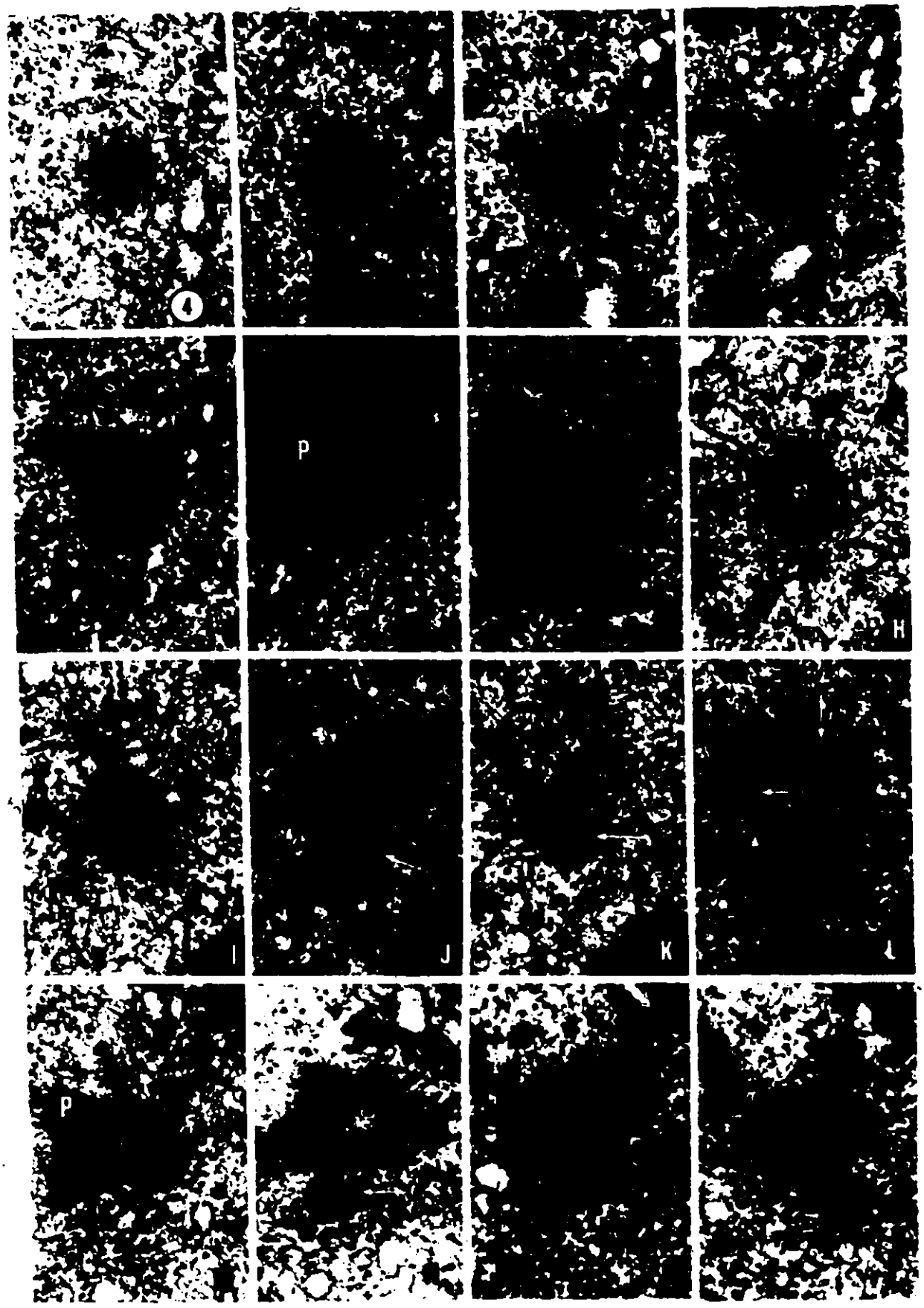

3
A
B
C
D
E
F
G
H

Figure 4. Centrosome series from stimulated cell shown in figure 2. Satellite bodies (arrows) are largely collected between the two centrioles. Many microtubules (arrowheads) can be seen radiating from the satellite bodies. Procentricles (P) (figures 4f and 4m) indicate that this cell is in some stage of S phase. Magnification; all figures x6CCOC.


P
H
I
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K
L
P

and the stimulated cell series (figure 4) are represented in model form in figures 6a and 6b, respectively. These cells were chosen because their centriole orientations were representative of resting cell and stimulated cell centrosomes, respectively. PT counts were done, on a per section basis, for the two representative centrosome models. A mean of 11.5 was calculated for the resting cell model while the stimulated cell model had a mean of 26.6.

The PCM of the resting cell centrosome is noticeably different from the surrounding cytoplasm (this is particularly evident in figures 3c-e) in that it appears finer due to the apparent exclusion of ribosomes. In the model (figure 6a), this difference is shown by the separation of the large and small stippling. The SBs, which are electron dense aggregates bounded by the PCM, exhibit a preferential association with one centriole of the pair and appear attached by short stalks along the length of the centriole. Microtubules are seen radiating from the SBs.

The organization of the stimulated cell centrosome is different from that of the resting cell. PCM is present and is especially prominent in figures 4e and 4f. Unlike the SBs of the resting cell centrosome, those of the stimulated cell are collected in the region between the two centrioles (figures 4i-l). The relationship between microtubules and SBs is clearly evident if the first row of figure 4 is compared to the third. As more SBs appear more microtubules are also

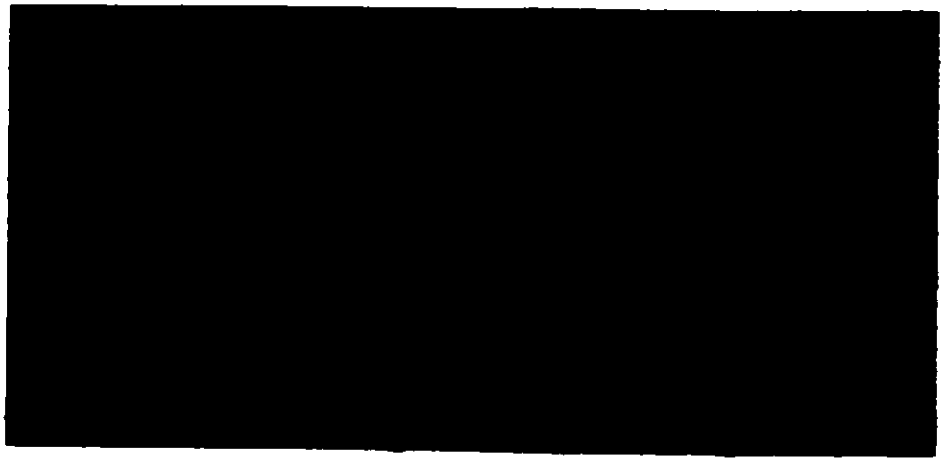
present; however, where the SBs appear to contact each other it is difficult to determine if there are MTs. The SBs of the stimulated cell centrosome also appear larger and more numerous.

The stimulated cell centrosome is presented in model form in figure 6b. This view shows the collection of the majority of SBs in the space between the two centricles. As with the resting cell, the smaller stippling represents the PCM and excludes ribosomes, which are represented by the larger stippling. The centrosome of the stimulated cell is slightly larger than that of the resting cell because the centricles have started to move apart. Small procentrioles (figures 4f and 4m) indicate that this cell has entered the S phase of the cell cycle (Tokuyasu, 1972).

Figures 5a and 5b are stereo views of the resting cell centrosome shown in figures 3 and 6a. Viewed through stereo glasses the ovoid shape of the centrosome can be perceived. It is also possible to see that the microtubules radiate from the centrosome at different planes. By selectively calling up the data from computer disk storage a view as given in figure 5b can be created. Here it is seen that microtubules (black lines) radiate primarily from the SBs (green lines).

Figures 5c and 5d are stereo representations of the stimulated cell centrosome shown in figures 4 and 6b. The most

Figure 5. Stereo pairs showing the centrosomes of a resting cell (figure 5a) and a stimulated cell (figure 5c). Microtubules are represented by black lines, centrioles are in red, satellite bodies are green and pericentriolar material is represented by blue lines. Figures 5b and 5d show the relationships between the microtubules and satellite bodies in the resting (figure 5b) and in the stimulated (figure 5b) cell centrosomes.



obvious difference is the increase in the number of microtubules. Figure 5b shows how these parts of the SEs (green lines) that are touching, or are closest to the centrioles, do not have microtubules radiating from them. The stimulated cell centrosome is also ovoid.

3.4 CENTROSOME MODELS

To determine if the centrosomes of cells that were serial sectioned are representative of the majority of centrosomes, the 3-D reconstructions were compared against micrographs of random single sections through the centrosomes of cells in resting and stimulated populations.

Centrosomes could be identified in resting and stimulated cells by the presence of at least one centriole, or in some instances, by the presence of PCM or SEs. Inspection of the serial sections and many single sections from resting and stimulated cells show that the angle between the centrioles of both cell types is fairly constant. ~60° (9 of 15) of the centrosomes from a resting cell population showed a centriole arrangement that could be found in either a resting or a stimulated cell population. SEs were used as markers to distinguish resting cell centrosomes from those in stimulated cells. Using this additional criterion 67% of the resting cell sample showing centrioles also showed SEs in a centrosome configuration that is consistent with the resting cell model (figure 6a).

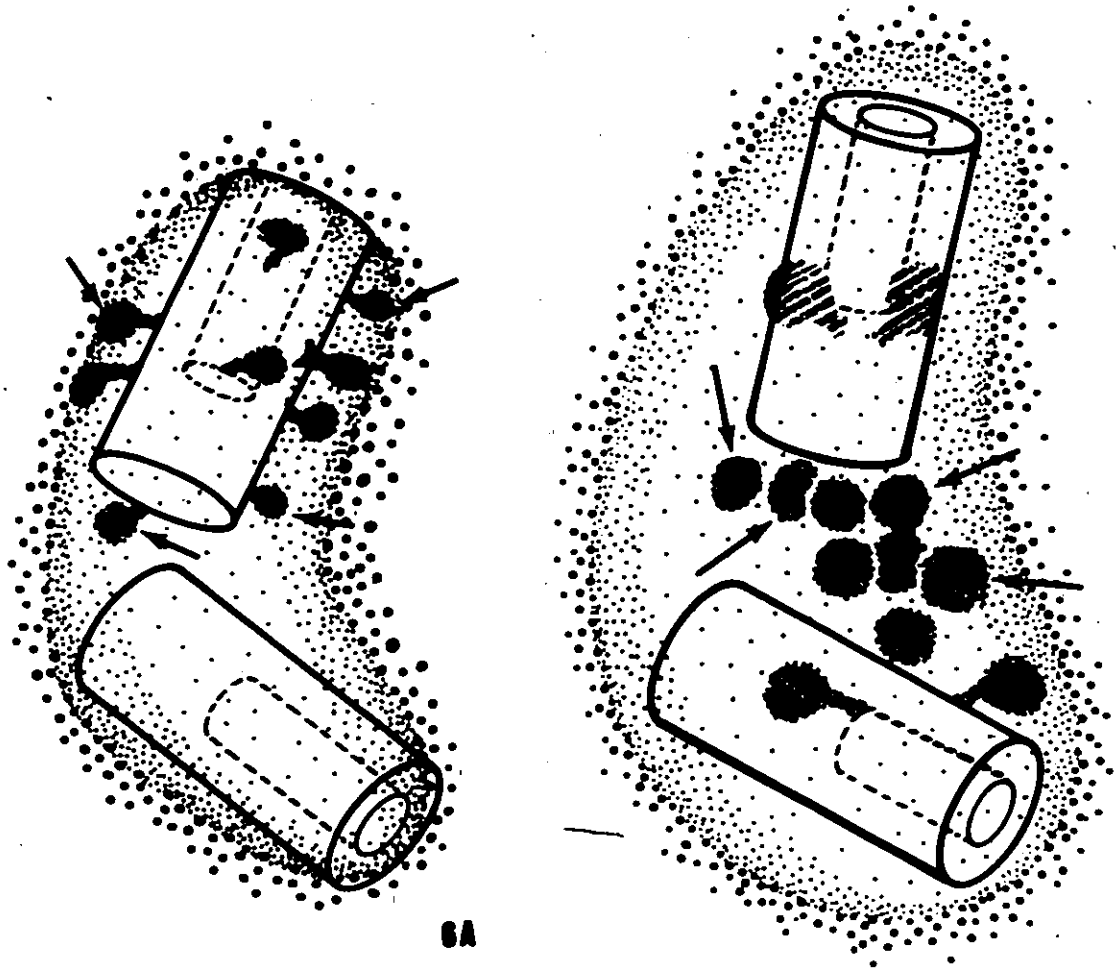
By following the same centriole/centrosome identification procedure 67% (50 of 74) of the random single sections through the centrosomes of resting B lymphocytes resembled the resting cell model.

A forty eight hour stimulated cell population was analyzed in the same manner and it was found that of a sample in which centrioles were present, the presence and location of SBs indicated that 75% (21 of 28) of the sample resembled the stimulated cell model.

3.5 DISASSEMBLY OF MICROTUBULES

Populations of resting and stimulated T cells were prepared and placed on ice for two hours to disassemble microtubules. After this treatment the cells were immediately fixed by the addition of 4% ice cold glutaraldehyde in 0.1M phosphate buffer (pH 7.0). Figure (7a) shows a resting cell treated in such a manner. The cytoplasm is very dense and makes detection of microtubule fragments difficult. The PCM still retains its fine appearance and ribosomes are still excluded from the region. SBs are not present and appear to have been disaggregated as a result of the cold treatment. The observation that cold treatment caused SB disaggregation

Figure 6. Models derived from serial sections of the resting cell (figure 6a) presented in figures 1, 3 and 5a-b, and of the stimulated cell (figure 6b) presented in figures 2, 4 and 5c-d. Large stipple in both models represent ribosomes which appear to be excluded from the pericentriclar material which is shown by the the small stipple. The satellite bodies (arrows) appear attached to one centricle of the centrosome in the resting cell and are collected in the intercentriclar space in the stimulated cell centrosome. The dashed cylinders in the centricles represent the electron dense material in the lumens of the centricles. The material starts at the distal ends of the centrioles and extends proximally.


6A
B

was unexpected and also a very interesting one since SB rearrangement may be an important factor when assaying changes in the centrosomes' microtubule initiating capacity.

To determine if the disaggregation of SBs was due to the removal of MTs or to some other effect of low temperature, colchicine was used as an alternative method to disassemble MTs. Resting and stimulated cells were treated with 10^{-6}M colchicine for 6 hours at 37°C, a treatment shown by Rudd et al., (1979) to effectively disassemble MTs. The effects of colchicine on intact resting cell centrosomes and on stimulated cell centrosomes are shown in figures 7b and 7c, respectively. The centrosome's position, in the nuclear cleft, is unaffected by the colchicine treatment. This is particularly evident in figure 7b. Both resting and stimulated cell centrosomes do not show any microtubules but SBs are still present and in the arrangements seen in intact cells.

3.6 IN VITRO ASSAYS

3.6.1 Permeabilization Procedures

In order to study changes in the initiating capacity of lymphocyte centrosomes, it was necessary to develop a procedure which allowed for the preservation of MTOCs. Permeabilization of cells using concentrations of Triton X-100 ranging from 0.05% to 0.25% in PIPES not containing glycerol have been reported effective in producing MTOCs for use in

Figure 7. The effects of cold or colchicine on microtubule disassembly in intact resting and stimulated mouse splenic lymphocytes. Figure 7a shows the centrosome of a resting, cold-treated cell. Centrioles (curved arrows) and pericentriolar material (asterisks) are visible but there are no microtubules or satellite bodies. If resting (figure 7b) or stimulated (figure 7c) cells are treated with 10^{-6}M colchicine for six hours at 37°C microtubules are disassembled but satellite bodies persist. Magnification; figure 7a x70000; figures 7b and 7c x60000.


7A
B

in vitro assembly assays (Pepper and Brinkley, 1979; Gould and Borisy, 1977). Permeabilization of lymphocytes in PIPES buffer, with or without glycerol, containing 0.05% or 0.25% Triton X-100 resulted in complete lysis of the cells. I reasoned that using a buffer system which preserves the MTs (Bershadsky *et al.*, 1978) might also preserve the centrosomes. When these concentrations of Triton X-100 were tried in BB there was extensive damage to the cells. In many instances all that remained were small blebs of cytoplasm attached to swollen nuclei (data not shown). Reducing the Triton X-100 concentration to 0.005% in BB and permeabilizing the cells for 30 minutes removed most of the cytoplasm in the cells and did not appear to greatly affect the structure of the centrosome; SBs were still located in their usual locations with MTs radiating from them. In all experiments reported here, cells were permeabilized using BB/0.05% Triton X-100.

Cold treatment of cells, followed by permeabilization in BB/0.05% Triton X-100 for 30 minutes at room temperature was not sufficient to completely disassemble all the microtubules (data not shown) so permeabilization was carried out on ice for 30 minutes. Figure (8) shows a resting cell centrosome (figure 8a) and a stimulated cell centrosome (figure 8b) after cold treatment and cold permeabilization. SB disaggregation and the lack of microtubules are consistent features of cells treated in this way.

Figure (9) shows a resting, colchicine-treated cell permeabilized at room temperature in EB/0.005% Triton X-100. The cell membrane, while still present, is not intact and much of the cytoplasm has been removed. The nucleus appears unaffected but the mitochondria, recognized by the presence of double membranes and residual cristae, are swollen. The centrosome is still present and located in the nuclear cleft. It should be noted that cells that have been cold treated and cold permeabilized have the same appearance. The major difference is the lack of SBs in the cold treated cells. The inset shows that in colchicine-treated cells SBs are still present. When stimulated colchicine-treated cells are permeabilized in EB/0.005% Triton X-100 it is clear that microtubules are no longer radiating from the SBs, nor have the SBs been noticeably affected (figure 10).

3.6.2 In Vitro Microtubule Assembly

One purpose of the *in vitro* assembly experiments was to see if the increase in microtubule content *in vivo* was correlated with an increase in MT initiation capacity of the MTOC.

Figure 6. The effects of cold disassembly and cold permeabilization on resting and stimulated mouse splenic lymphocytes. After cold disassembly and cold permeabilization the resting cell centrosome (figure 6a) and the stimulated cell centrosome (figure 6b) are devoid of microtubules and satellite bodies. Magnification: x96500.

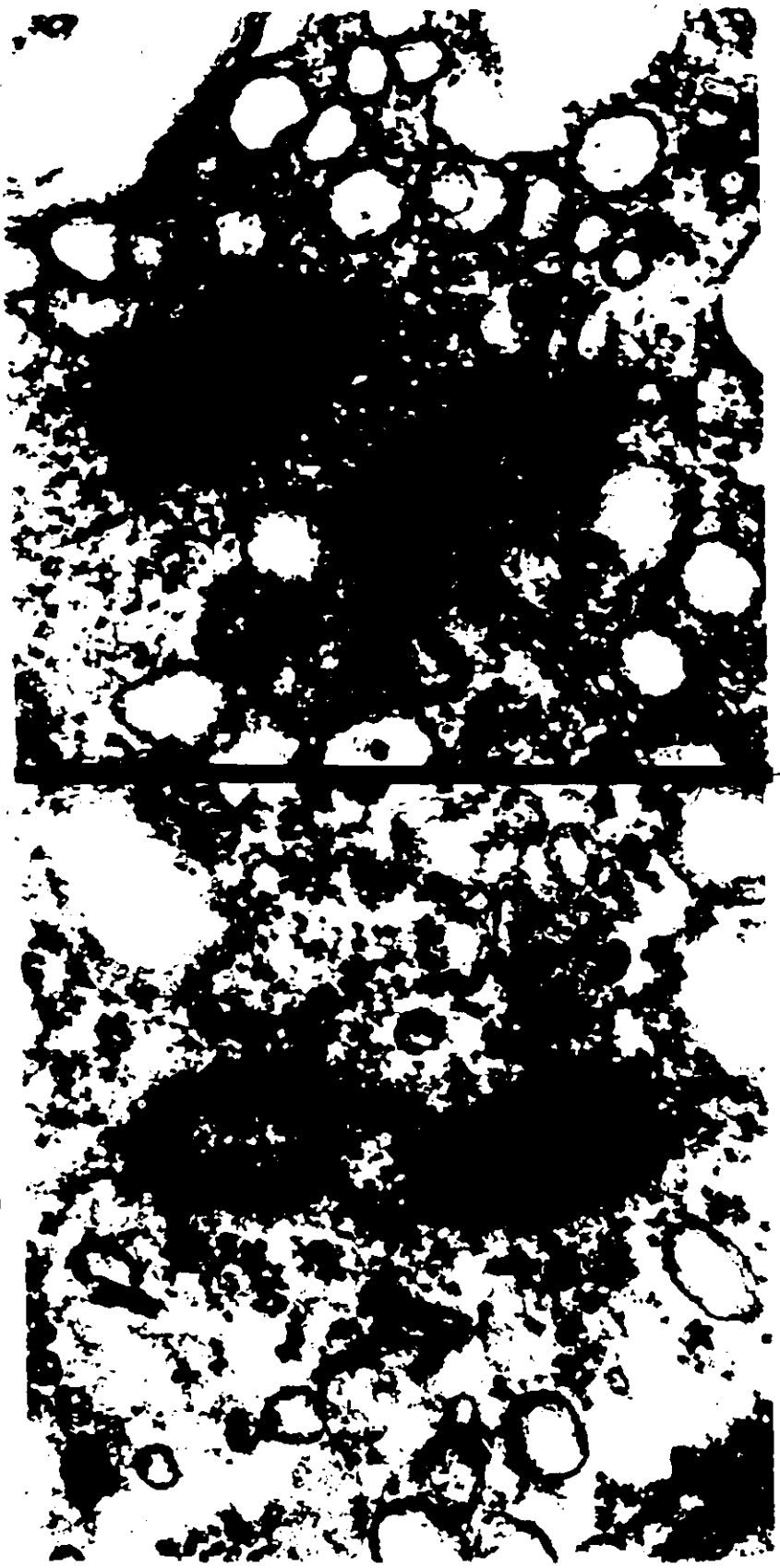
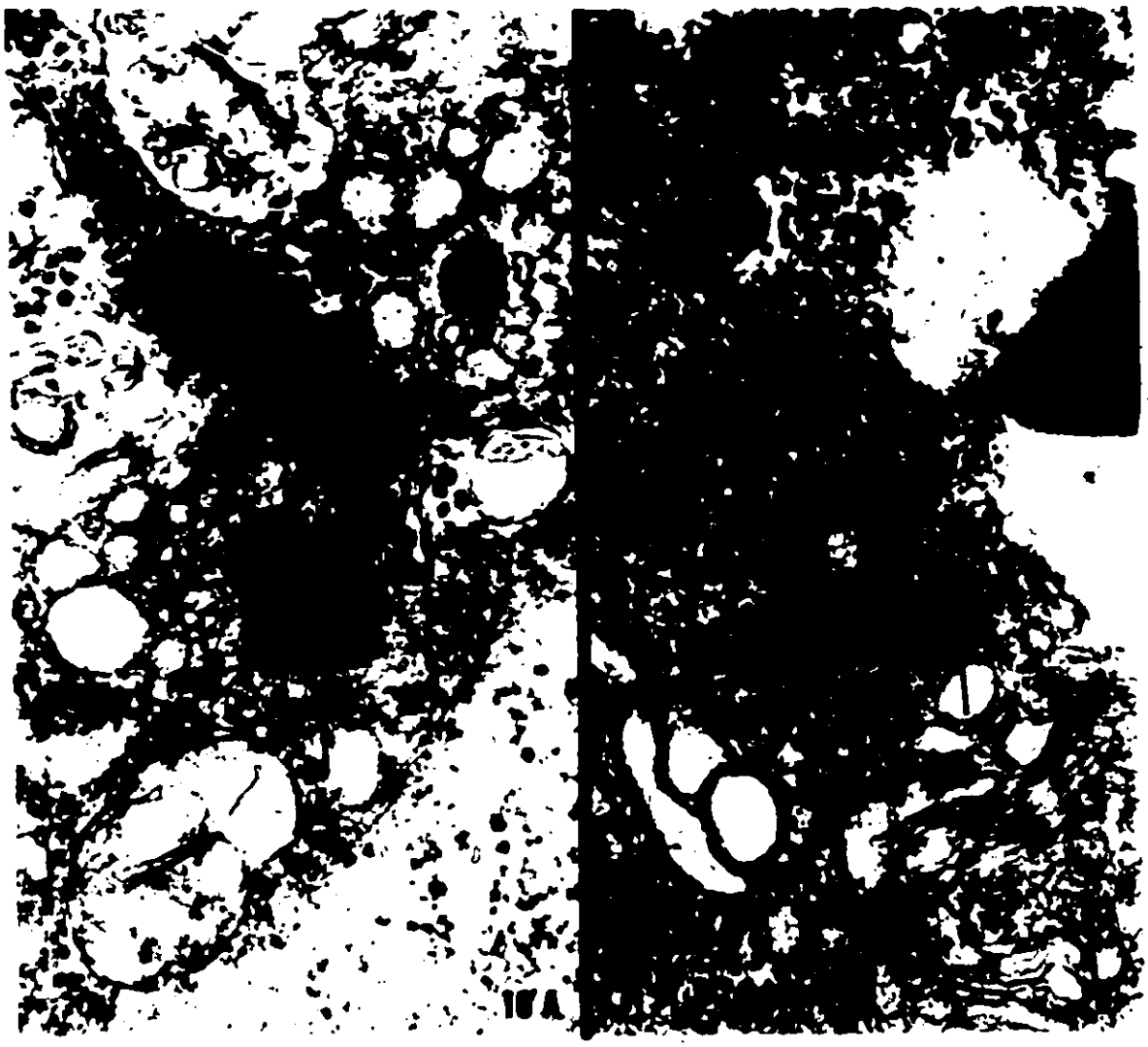


Figure 9. The effects of permeabilization on resting colchicine-treated lymphocytes. In this resting cell, permeabilized at room temperature, mitochondria (arrows) are swollen and a lot of cytoplasm has been removed. The nucleus, however, appears unaffected. The centrosome (double arrows) remains in the nuclear cleft. The inset shows that the satellite bodies (arrowheads) are still present. Magnification: x33750, inset; x60000.

Figure 10. The effects of permeabilization on stimulated colchicine-treated lymphocytes. In these cells, permeabilized at room temperature, satellite bodies are still present (arrows) even though microtubules are not. Magnification; x72500.


10 A

Mock assembly controls were run using resting and stimulated cells treated with cold or colchicine procedures described in section 2.4 to test if the structure of the centrosomes would be preserved during the assembly assays and determine the extent, if any, of MT reassembly from endogenous tubulin still present following the permeabilization. After permeabilization the cells were resuspended in 0.5 ml of reaction mixture that did not contain phosphocellulose-purified tubulin.

Figure (11) shows resting (figures 11a and 11b) and stimulated (figures 11c and 11d) cold-treated cells. These views show that the centrioles are still located close to the nucleus and that almost all of the cytoplasm has been removed. Centrioles remain structurally intact with PCM apposed to their sides while SBs have not reformed. One or two short MTs can be seen (figure 11).

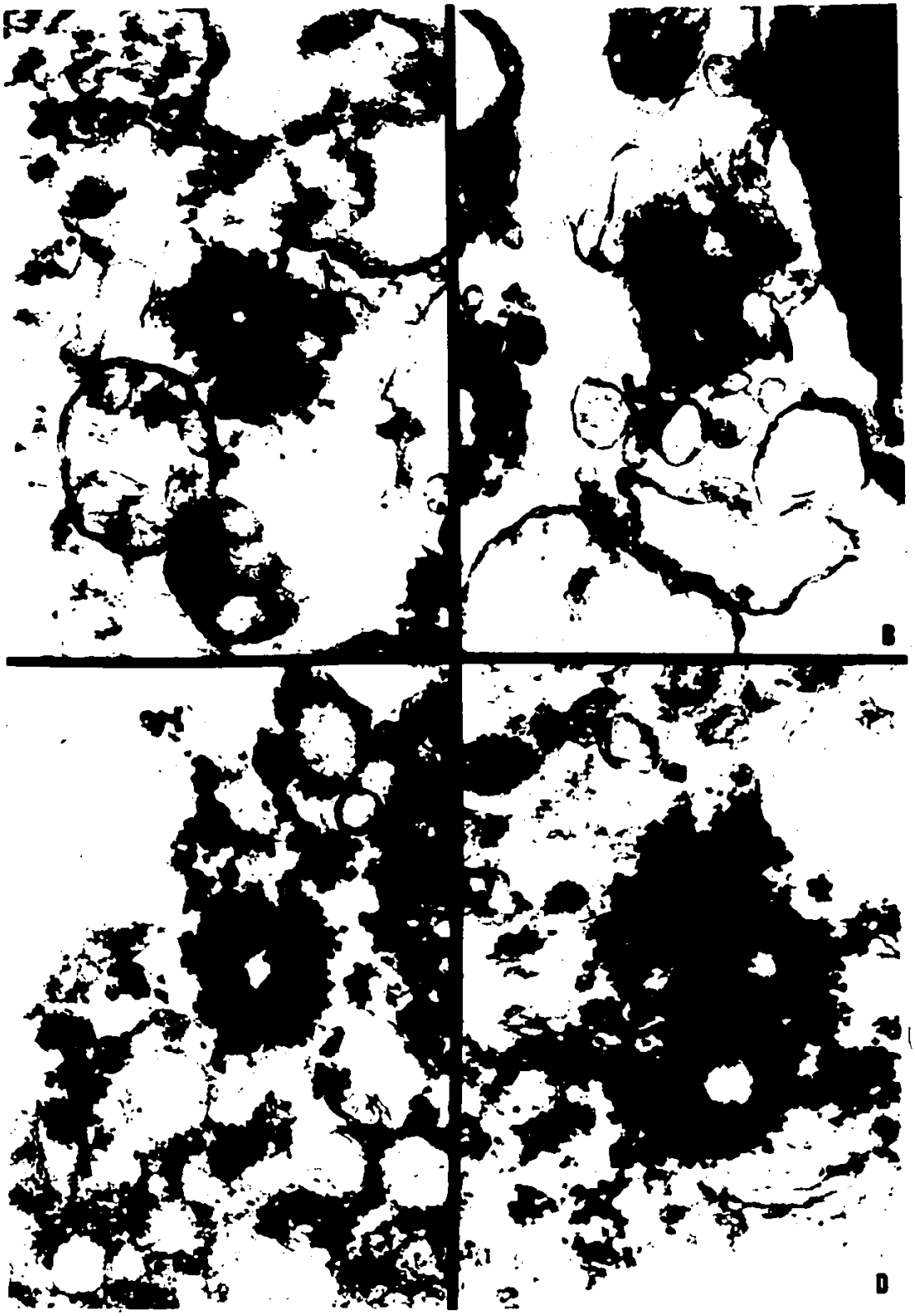
Figure (12) shows centrosomes from colchicine-treated resting (figures 12a and 12b) and stimulated (figures 12c and 12d) cells used in mock assembly assays. Here, too, most of the cytoplasm has been removed by the permeabilization procedure without noticeably affecting the centrioles' structure or their perinuclear position. PCM is also present and, unlike the situation in the cold-treated cells, so are the SBs.

Phosphocellulose-purified tubulin was used at 1mg/ml for 15 minutes or 2mg/ml for 5 minutes in the reaction mixture

Figure 11. Cold disassembled and cold permeabilized resting (figures 11a and 11b) and stimulated (figures 11c and 11d) cells used in *in vitro* rock assembly assays. One or two short microtubule fragments (arrows) can be seen near the centrioles. Satellite bodies do not appear to have aggregated. Magnification: x70000.

C
D

Figure 12. Resting (figures 12a and 12b) and stimulated (figures 12c and 12d) colchicine-treated cells used in *in vitro* rock assembly assays. Microtubules are not present but satellite bodies (arrows) persist. Magnification; x80000.


B
D

for in vitro assembly assays. Two concentrations were tested to see if there might be a difference in the number of microtubules initiated by the centrosome. Short incubation times were used in order to avoid damage to the centrosomes due to the absence of glycerol in the reaction mixtures. In addition, it was found that at the higher concentration long microtubules were assembled and when two cells were close together it was difficult to determine which centrosome initiated those MTs.

Figure (13) shows resting, cold-treated cells incubated with 1mg/ml (figures 13a and 13b) or 2mg/ml (figures 13c and 13d) of phosphocellulose-purified tubulin for 15 minutes and 5 minutes, respectively. The centrioles are close to the nuclei and are structurally intact. Microtubules radiate from the CPs which have reaggregated. When the concentration of tubulin was increased to 2mg/ml, even as the incubation time was reduced by two thirds, it appeared that more microtubules were initiated by the reaggregated CPs.

The same incubation conditions were used when stimulated cells (figure 14) were tested. At 1mg/ml (figures 14a and 14b) it appeared that microtubules radiated from the reaggregated CPs. Figures 14c and 14d show two different views of stimulated cell centrosomes incubated with 2mg/ml of tubulin for 5 minutes. Figure (14c) is a view of the intercentriolar region of a stimulated cell centrosome comparable to that of the intact cell shown in figures 4i-l.

Figure 13. Cold disassembled and cold permeabilized resting cells used in *in vitro* assembly assays at 1mg/ml (figures 13a and 13b) or 2mg/ml (figures 13c and 13d) of phosphocellulose-purified tubulin. The centrioles are still located in their perinuclear position and the reaggregated satellite bodies are apposed to, or very near, the centrioles. Microtubules (arrows) can be seen radiating from the reformed satellite bodies (double arrows) in all four views. Magnification; x67500.

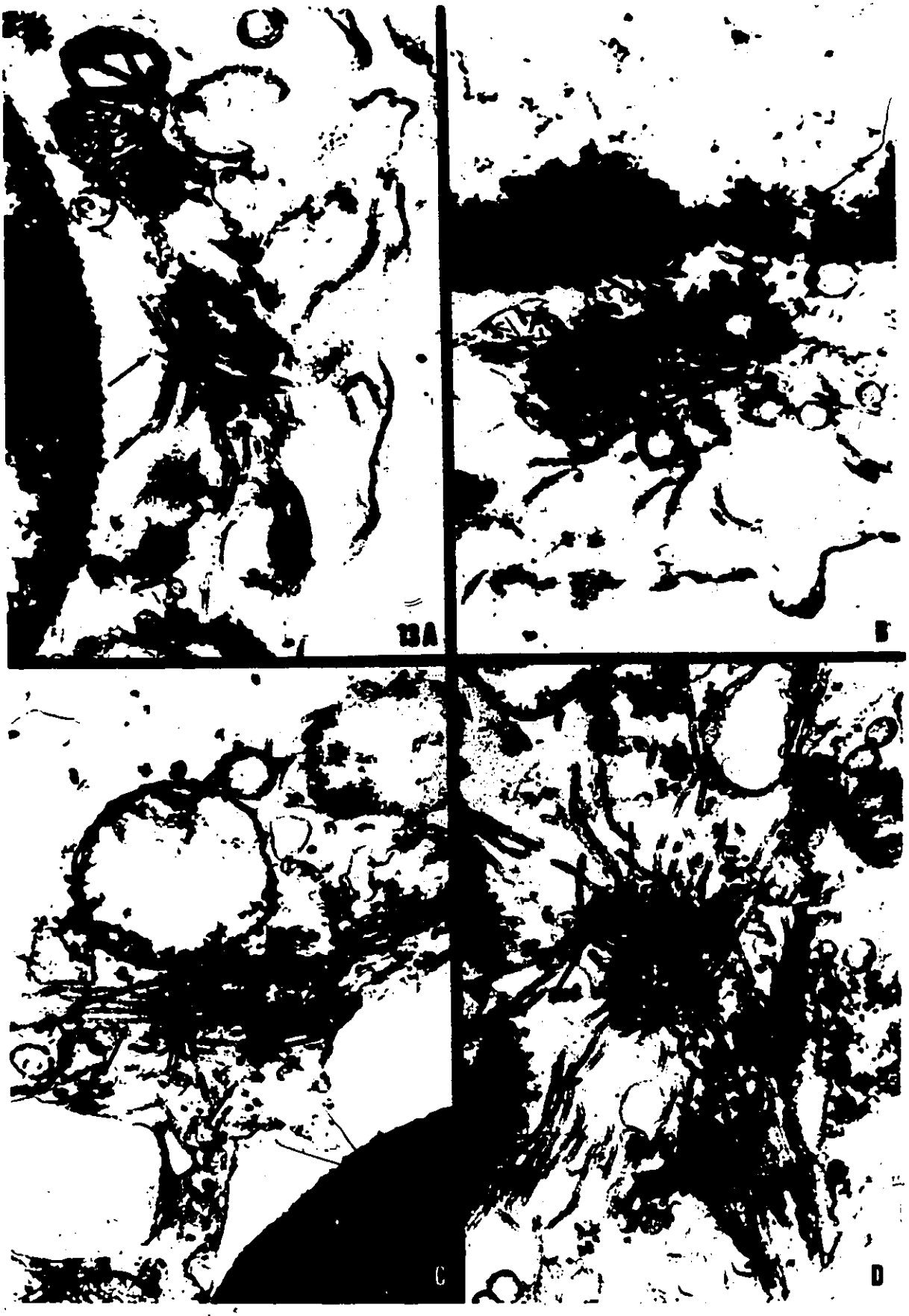

BA
B
C
D

Figure 14. Cold disassembled and cold permeabilized stimulated cells used in *in vitro* assembly assays at 1mg/ml (figures 14a and 14b) or 2mg/ml (figures 14c and 14d) of phosphocellulose-purified tubulin. The centrioles are found in the nuclear cleft with the reaggregated satellite bodies at a slight distance from them. The satellite bodies appear slightly larger and more numerous than in resting cells. Figure 14c shows the intercentriolar collection of satellite bodies and associated microtubules which is similar to that seen in intact stimulated cells. Many microtubules are seen radiating from the reformed satellite bodies (double arrows) in all four views. Magnification; x67500.

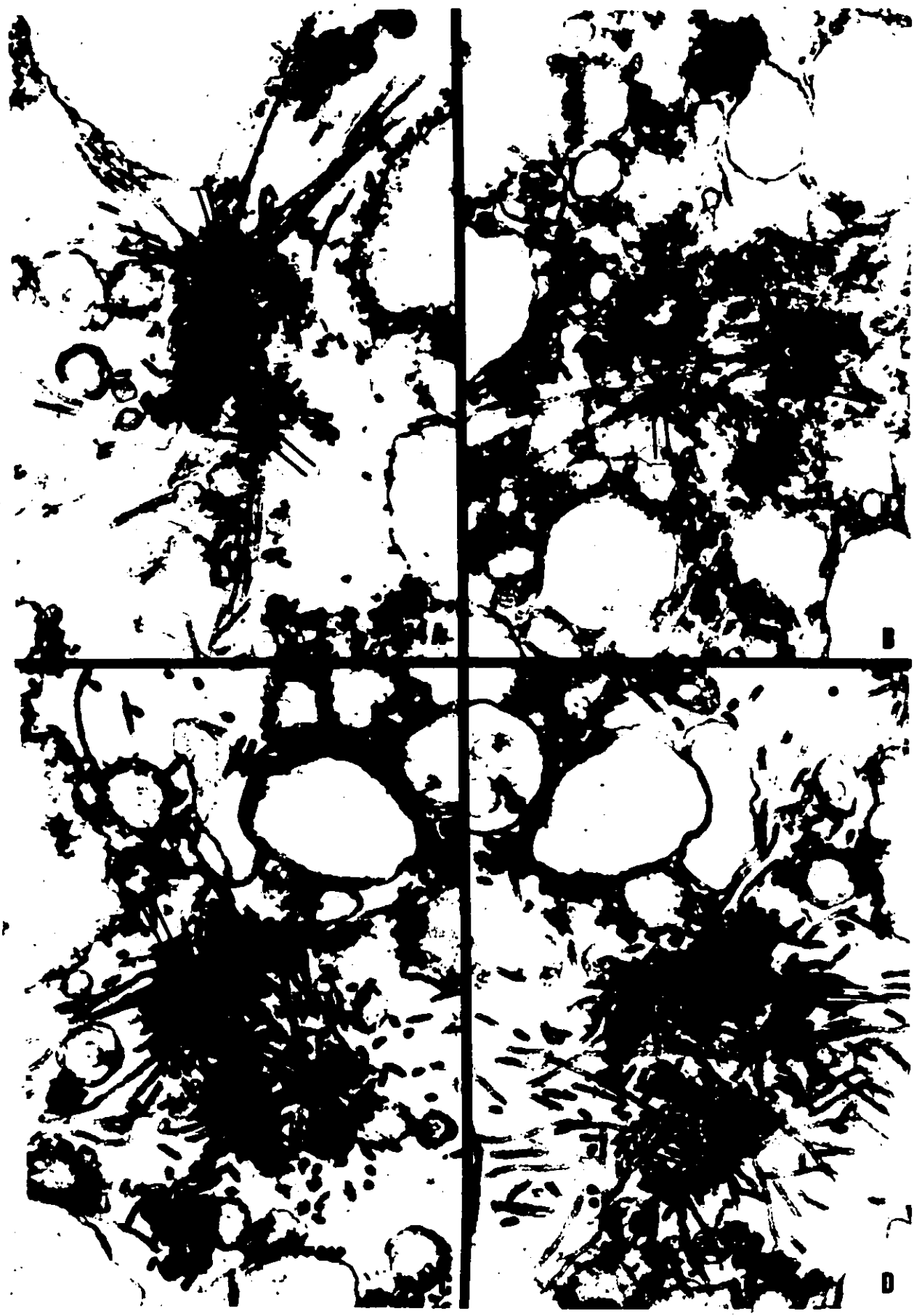

B
D

Figure 15. Resting (figures 15a-d) and stimulated (figures 15e-g) colchicine-treated cells used in *in vitro* assembly assays at 1mg/ml (figures 15a,b and 15e) or 2mg/ml (figures 15c,d and 15f,g) of phosphocellulose-purified tubulin. Microtubules (arrows) radiate primarily from the satellite bodies (double arrows). Magnification: all figures except 15c x66250; figure 15c x50000.

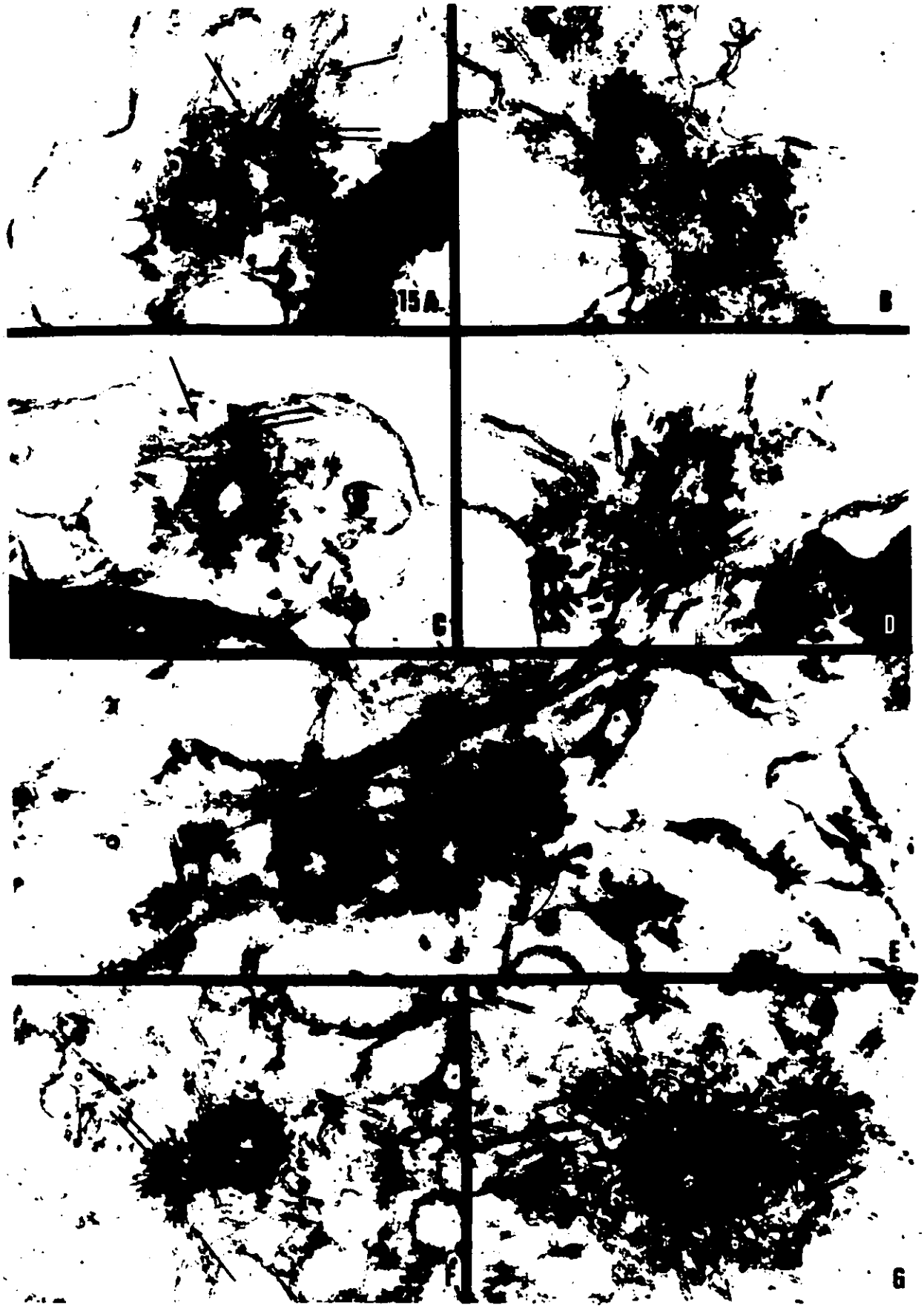

15A.
B
C
D
E
F
G

Figure (15) shows resting and stimulated colchicine-treated cells used in in vitro assembly assays. Neither the perinuclear position of the centrioles nor their structure appear to have been affected by the colchicine treatment, the permeabilization procedure or the incubation in the reaction mixture. The SFs, which persisted throughout the centrosome preparation protocol still serve as the primary microtubule initiating structure of the centrosome. As with cold-treated cells used in the in vitro assays, there appeared to be an increase in the number of microtubules initiated when the tubulin concentration was increased.

The comparison of resting and stimulated cells, incubated with 1mg/ml tubulin or 2mg/ml tubulin, showed that stimulated cell centrosomes initiated more MTs.

3.7 COUNTS OF IN VITRO ASSEMBLED MICROTUBULES

Analysis of changes in the microtubule initiating capacity of the centrosome was carried out by direct counting of microtubules on electron micrographs, enlarged to x40000. Counts were made in a 1.21um^2 area centered on the centrosomes of permeabilized cells and tested for significance using two way Student's T tests. The counts for cell populations are summarized in Table I for cold-treated cells and in Table II for colchicine-treated cells.

The first line of both tables shows microtubule counts per section in intact, untreated resting and stimulated

cells. These values demonstrate that there is about a two fold increase in microtubule content in populations of stimulated cells without selection for fully stimulated cells. Up to a five fold increase can be counted for fully stimulated cells.

The efficiency of the buffer in stabilizing microtubules and centrosomes was tested by permeabilizing cells and then counting microtubules. This is shown in the second line of both tables. Using Student's two way T test to compare the microtubule counts of intact resting cells with their permeabilized counterparts showed no statistically significant difference ($p > .05$) indicating that all the microtubules seen in intact cells were preserved in the permeabilized cells. Furthermore, electron micrographs of permeabilized cells showed that the centrosome structure appears unaffected. Intact stimulated cells were then tested against permeabilized stimulated cells and again no statistically significant difference was found ($p > .05$).

The effectiveness of both disassembly treatments is shown in line 3 of the tables. Both colo (Table I) and colchicine (Table II) treatment disassemble almost all microtubules in resting and stimulated cells. The values in the last lines of both tables reflect the fact that very little tubulin is likely to remain in the permeablized cells. Any amount that might remain probably doesn't contribute much to the *in vitro* microtubule assembly since phosphocellulose-purified tubulin is supplied in vast excess.

3.7.1 Use of Cold-Treated Cells in In Vitro MT Assembly

At both concentrations tested more MTs were assembled *in vitro* using stimulated cell centrosomes. This parallels the results seen for intact cells and shows that the difference in MT initiation capacity between resting cell centrosomes and stimulated cell centrosomes is retained.

When the permeabilized cells used in the *in vitro* assembly assays are compared with the intact controls the most obvious difference is the apparent increase in the numbers of assembled microtubules. The mean microtubule number for the 1mg/ml test increased from 15.7±7.2 to 28.8±15.2 for the resting cells. The values for stimulated cells rose from 35.1±14.6 to 57.5±17. When the tubulin concentration was increased to 2mg/ml the microtubule content increased to 70.5±22.1 for resting cells and 111.9±24.7 for stimulated cells. In all cases these increases were statistically significant ($p < .001$) and represented a minimum 1.6 fold increase.

3.7.2 Use of Colchicine-Treated Cells in In Vitro MT Assembly

Table II summarizes the MT counts made using colchicine-treated cells in *in vitro* assembly assays. In almost all cases the stimulated cell centrosomes assembled more MTs at both concentrations than did the resting cell centrosomes. However, in one case the mean for the *in vitro* sample (stimulated cells, 1mg/ml) was less than its control. In some

instances the standard deviations are high indicating great variability in the counts. This variability may be due to the colchicine treatment since the incubation procedure did not vary from that used on cold-treated cells.

A comparison of MT disassembly procedures by two way T tests shows that both are equally effective; however, there is a significant difference when considering initiation capacity. The means of the in vitro assembly assays using cold-treated cells are higher than those using colchicine-treated cells.

Table I. Microtubule counts for cold-treated cells.

Column one describes how the cells were treated (room temp in BE means permeabilization at room temperature for 30 minutes in Pershadsky's buffer containing 0.005% Triton X-100; cold diss & cold perm means microtubule disassembly on ice for two hours followed by permeabilization on ice for 30 minutes in BE/0.005% Triton X-100.

Column two describes incubation conditions. The assembly buffer contains PIPES, tubulin (at the indicated concentration) and 1.0mM GTP. The stock assembly buffer did not contain tubulin.

The values in the tables are presented as; mean±standard deviation with the sample sizes in parentheses.

TABLE I. MICROTUBULE COUNTS FOR COLD-TREATED CELLS

TREATMENT	INCUBATION	RESTING CELLS	STIMULATED CELLS
NONE	NONE	15.7+7.2 (N=26)	35.1+14.6 (N=25)
ROOM TEMP PERM IN B.B.	NONE	15.4+8.5 (N=11)	27+18.5 (N=8)
COLD DISS & COLD PERM	NONE	.62+.77 (N=13)	.9+.94 (N=11)
"	1MG/ML FOR 15' @ 37°C	28.8+15.2 (N=14)	57.5+17 (N=15)
"	2MG/ML FOR 5' @ 37°C	70.5+22.1 (N=14)	111.9+24.7 (N=14)
"	MOCK ASSEMBLY 15' @ 37°C	5.8+2.6 (N=14)	1.3+4.2 (N=16)

Table II. Microtubule counts for colchicine-treated cells.

Column one describes how the cells were treated (colchicine diss and P.T. perm means disassembly of microtubules by the addition of 10^{-6} M colchicine for six hours at 37°C for six hours followed by permeabilization of the cells at room temperature in PF/G.G65% Triton X-100 for 30 minutes). See legend for Table I for details described in column two.

TABLE II. MICROTUBULE COUNTS FOR COLCHICINE-TREATED CELLS

TREATMENT	INCUBATION	RESTING CELLS	STIMULATED CELLS
NONE	NONE	15.7+7.2 (N=26)	35.1+14.6 (N=25)
ROOM TEMP PERM IN B.B.	NONE	15.4+8.5 (N=11)	27+18.5 (N=8)
COLCHICINE DISS & R.T. PERM	NONE	.79+1.3 (N=14)	.3+.8 (N=14)
"	1MG/ML FOR 15' @ 37°C	19.2+5.7 (N=18)	31.1+20.5 (N=16)
"	2MG/ML FOR 5' @ 37°C	34.8+20.2 (N=16)	61.7+32.7 (N=15)
"	MOCK ASSEMBLY 15' @ 37°C	.38+.6 (N=16)	1.2+1.3 (N=13)

Chapter IV

DISCUSSION

It has been suggested by a number of researchers (Robbins et al., 1968; Rieder and Borisy, 1982; Vorobjev and Chentsov, 1982) that ultrastructural changes in the centrosomes of eukaryotic cells may be causally related to changes in microtubule initiation capacity during the cell cycle. Their studies described the changes in the organization and distribution of pericentriolar material which were correlated with increased MT assembly in cells entering mitosis. A decrease in the initiation capacity of the centrosomes of mitotic PtK1 cells was pinpointed to the metaphase/anaphase transition (Snyder et al., 1982) however, structural changes correlated with this transition were not reported.

Pericentriolar material and additional appendages were shown to be preferentially associated with the parent centrioles of PtK2 cells (Rieder and Borisy, 1982) and pig embryo kidney cells (Vorobjev and Chentsov, 1982). Parent centrioles can be distinguished from daughter centrioles by the presence of a cilium at the distal end of the parent while the cell is in interphase (Wheatley, 1982). The parent is also the only centriole to have appendages. These two studies showed that daughter centrioles can serve as parent cen-

tricles nc earlier than cne cycle after their replicaticn. Robbins and Gcnatas (1964), Rieder and Bcrisy (1982) and Vcrobjev and Chentscv (1982) present micrographs and/cr diagrams tc claim that satellites and pericentricler material are interccnvertible fcrms with the satellites being absent during mitosis.

The terminclcgy used tc describe centrcsomes is not ccnsistent. For example, the satellites described by Rieder and Borisy (1982) in PtK2 cells are different frcm thcse described fcr pig kidney embryo cells (Vorobjev and Chentscv, 1982) and spermatocytes cf the jellyfish Phialidium gregarium (Szollcsi, 1964). These latter twc different cell types have striated satellites attached tc cne centriole of the pair with MTs radiating frcm thes. The parent centricle cf pig kidney embryc cell centrcsomes alsc has cther projecticns called appendages at the distal end. These appendages did nct have MTs associated with thes. Albrecht-Buehler and Bushnell (1980) described the same striated satellites attached tc the ciliated parent centriole of quiescent 3T3 cells but called them basal feet. They used the term alar sheet fcr the additional projecticns. In contrast, the satellites of PtK2 cells are not striated ncr are they directly attached to the parent centriole. They are, however, associated with the parent (Rieder and Borisy, 1982).

Striated SBs were occasionally seen in resting lymphcytes. When they were present they were directly attached tc

a centriole and would persist through permeabilization (figures 12b, 13c). Thus, these SBs resemble the ones described by Szollosi (1964), Albrecht-Buehler and Bushnell (1980) and Vorobjev and Chentsov (1982). Most of the SBs are not obviously striated leading to the possibility that there are at least two types of SBs in resting lymphocytes, perhaps with different MT initiating capacities. It may be more likely, however, that all the resting cell SBs are striated but because of their small size the striations are only seen in perfect longitudinal sections. Striated SBs were never seen in stimulated cell centrosomes indicating that these SBs are of a different structure.

The first class of Kuriyama's and Borisy's six class scheme to describe centriole orientations during the cell cycle (1981a) takes into account variations of 0°-180° of the angle between the two centrioles. This class is representative of centricle orientations in late M phase to early G1 phase cells. The representative resting cell centrosome could be placed in this class. Only one instance of an extreme variation of the angle between centrioles was noted and this was for one of the three serial sectioned resting cell centrosomes. Among the random single sections there was little variation. Classes two and three are denoted by the formation of procentrioles and are found in S phase cells. The representative stimulated cell centrosome would fall into either of these classes. There was little variation in

the sample of random single sections from a stimulated cell population.

During con A stimulation there are changes in centrosome organization. The most obvious change is in the location of the satellite bodies. Resting cell centrosomes have their SBs associated with one centriole of the centricle pair while fully stimulated cells have most of their SBs clustered between the two centrioles. The SBs may also be larger and more numerous in stimulated cells; serial sectioning of more stimulated cells would There are no results in the literature that can be indicate if this is the case. compared directly to these, although related results on increases in the diameter of the pericentriolar cloud have been reported for Hela cells as they enter mitosis (Robbins and Gonatas, 1964). There are at least 2 possible explanations for the SF redistribution. Firstly, and most simply, they may just move from their resting cell configuration to their stimulated cell configuration. This is unlikely if there are two structural classes of SBs (see above). Alternatively, the SBs that are attached to the centriole in the resting cell centrosome may disaggregate and the material may reaggregate in a new position in the stimulated cell centrosome. This might involve an interconversion between SBs and PCM as suggested by Rieder and Borisy (1982) and Vorobjev and Chentsov (1982) who observed that SBs were absent in mitotic cells when the PCM was most evident and reap-

peared in interphase cells coincident with a decrease in size of the pericentriolar cloud.

The satellites and PCM of PtK2 cells and of pig kidney embryo cells always appear associated with the parent centriole (see for example, figure 30 in Vorobjev and Chentsov, 1982). A preferential association of SBs with one centriole of resting lymphocyte centrosomes, therefore, would be in agreement with the observations of Rieder and Borisy (1982) and Vorobjev and Chentsov (1982). As stated by Rieder and Borisy and shown by the diagrams and electron micrographs of Vorobjev and Chentsov, the satellite material is not partitioned equally between the duplicated centrosomes. The majority of the PCM is associated with the centrosome containing the parent centriole that had striated SBs attached during interphase. The redistribution of SBs to the intercentriolar region of stimulated cell centrosomes may indicate a different partitioning of SBs to both centrosomes in lymphocytes.

The attachment of SBs to one centriole of resting cell centrosomes may impose a certain level of spatial organization or directionality on the MTs. The MT initiation sites may not exist or may be unavailable in those parts of the SBs that are closest to the centrioles so that MTs are only assembled radiating away from the SBs. A similar situation may exist in other types of MTOCs. For example, the basal body rootlets of Polytomella are ocated only along the sides

by a layer of dense material. MTs are initiated *in vivo* and *in vitro* from this dense material and the MTs emerge from the rootlet MTOCs in a precise, ordered array (Stearns and Brown, 1981). The striated SBs attached to the centrioles of resting cell centrosomes may be analogous to the rootlets that are attached to the basal bodies in Polytrella.

The overall pattern of MTs in lymphocytes is not easily detected at the electron microscopic level but is seen by immunofluorescence microscopy (Rogers *et al.*, 1981). The pattern is stellate in resting cells and is made of few staining fibers, which may represent MT bundles, radiating from the single, brightly fluorescent centrosome. The association of MTs to form bundles is seen in the reconstructions from the serial sections. In the stimulated cells MT bundles are seen and the spaces between the bundles are filled with more MTs giving a finer stellate pattern which is seen by immunofluorescence microscopy (Waterhouse *et al.*, 1983) and in the reconstructions. Changes in MT distribution in stimulated cells may result, in part, from the SP redistribution to the intercentriolar region.

Analysis of MT number by direct counting in random sections of resting and stimulated cells gives an initial estimate of the changes in MT number in the cell populations. The average number of MTs per section is 15.7 for the resting cell population which compares favorably with the mean

for the serial sectioned resting cell (11.9) presented in figure (1). This mean also is in good agreement with the mean value of 16.2 determined by Oliver et al., (1980) using a similar counting procedure for resting human peripheral blood lymphocytes. The mean for stimulated cell populations rises to 35.1 representing about a 2 fold increase in MT content in the stimulated cell population. In the large, fully stimulated cells in this population there is a 4-5 fold increase in MT content.

By counting microtubules in serial sections, it is possible to determine more accurately the total number of microtubules in resting and stimulated cells. Immunofluorescence microscopy has shown that microtubules radiate from a single brightly fluorescent spot that corresponds to the centrosome (Rogers et al., 1981) and serial sectioning did not reveal other foci for MTs. Therefore, it was sufficient to consider only those sections that show the centrosome and only count these MTs clearly radiating from the SBs or PCM. Longitudinally sectioned MTs ending on SBs are unlikely to be counted more than once since the sections are thicker than MTs. Considerable care was taken to avoid counting cross-sectioned or obliquely-sectioned MTs more than once by tracing them back through the series of sections to their origin.

From this counting procedure it was determined that there is an average of four microtubules that terminate on each of the nine SBs in the serial sectioned resting cell centrosome

presented in figure (3). In addition, there are about 4-9 PCM-initiated MTs giving a total of about 40-45 microtubules per resting cell. This is very likely an underestimate since the density of the cytoplasm could obscure some short, SF- and PCM-associated MTs.

From the 4-5 fold increase in MT number seen in single sections of fully stimulated cells one would expect a total MT number of between 160-200 MTs in the serial sections. Because there are so many MTs in the centrosome region of the stimulated cells it is difficult to accurately determine total counts and to clearly identify all MTs ending on SFs. My best estimate is that a minimum of 8 MTs end on SFs in stimulated cell centrosomes. For the centrosome shown in figure (4) which has 12 SFs, therefore, at least 96 MTs end on SFs. A significant number of MTs, 53 in this series, could not be traced back to the SFs and appeared to end in PCM. This change in initiation activity from resting cell centrosomes to stimulated cell centrosomes is shown in the stereo reconstructions (figure 5) and suggests a growing importance of the MT-PCM association in the stimulated cell. The MT initiation capacity of the PCM may continue to increase as the cells enter mitosis since mitotic lymphocytes do not have any SFs (Brown, personal communication).

Increases in the MT initiation capacity of the stimulated cell centrosomes may be due to increases in the number of initiation sites, utilization of preexisting sites or a com-

bination of both of these. The increase in the number and size of the SBs and increases in the PCM may reflect increases in the numbers of MT initiation sites. An increase in the size of the pericentriolar cloud in mitotic HeLa cells was noted by Robbins and Gonatas (1964). Kuriyama and Borisy (1981b) also suggest that increases in "the amount of pericentriolar cloud or in its specific nucleating activity" could account for increased numbers of MTs in mitotic cells. These long term changes may be dependent on the synthesis of new MT initiating sites.

The activation of a second set of preexisting MT initiation sites on (or in) the centrosome that may become activated is suggested by the work of Hoffstein and coworkers (1976). They noted a large increase in MT content arising from the centrosomes of human polymorphonuclear leukocytes as soon as 15 minutes after con A addition, suggesting that the addition of lectin might activate the additional sites. This change is too rapid to be accounted solely by the synthesis of new MT initiating sites. During lymphocyte stimulation it appears that there is both an increase in the number of initiation sites and a utilization of preexisting sites.

In vitro MT assembly assays have been used to determine what structures in animal cells initiate MT assembly (Telzer et al., 1975; Gould and Borisy, 1977; Pepper and Brinkley,

1979; Brinkley et al., 1980), to investigate the composition of MTOCs (Snyder, 1980; Pepper and Brinkley, 1980) and to investigate changes in initiation capacity of MTOCs in mitotic and/or interphase cells (Weisenberg and Rosenfeld, 1975; Snyder and McIntosh, 1975; Telzer and Rosenbaum, 1979; Schliwa et al., 1979). Two of these studies (Snyder and McIntosh, 1975; Telzer and Rosenbaum, 1979) had the common observation that interphase MTOCs had little or no activity and that an increase in MT initiation was seen if mitotic MTOCs were assayed. Recently Kuriyama and Borisy (1981b), using centrosomes from interphase and mitotic CHO cells in in vitro assembly assays, showed that interphase centrosomes could assemble MTs, and confirmed that only centrosomes from mitotic cells showed any significant increases in MT initiating capacity.

The results of the in vitro assays using permeabilized lymphocytes clearly show that increases in MT assembly activity can be detected during interphase and correlated with SB number and size increases and an increase in the activity of the PCM. Several studies have shown increases in the MT initiation capacity of centrosomes of cells entering mitosis. These studies used cells that were continuously cycling whereas lymphocytes had to be activated to enter the cell cycle from the Go stage.

In this study the centrosomes of permeabilized cells incubated with 1mg/ml of phosphocellulose-purified tubulin initiated significantly more MTs when compared against the intact, untreated control. When the concentration was increased to 2mg/ml the MT count also increased. This result is unlike any other reported for *in vitro* assembly assays, where it has been possible to saturate (Brinkley *et al.*, 1981; Stearns and Brown, 1981) or nearly saturate (Gould and Borisy, 1977) the MT initiating sites in the MTOCs, ie. assemble the same or nearly the same number of MTs seen in intact cells. My results suggest that the SBs, and probably the PCM, have more initiation sites that are actually occupied in intact cells. These additional sites may have been activated or uncovered as a consequence of SB disaggregation due to the cold treatment, and reformation during the *in vitro* MT assembly incubation. Colchicine treatment does not appear to affect the structure of the SBs in the same way as cold since fewer additional MT initiating sites were exposed or activated. This could explain why more MTs were assembled *in vitro* using cold-treated cells than if colchicine-treated cells were used.

The possible existence of additional MT initiation sites in the centrosome that are not fully occupied *in vivo* is supported by the taxol experiments of Paatero and Brown (1982) and Brown and Little (1982) using resting and stimulated lymphocytes. Taxol has been shown to decrease the

critical concentration of tubulin *in vitro* (Schiff, Fant and Horwitz, 1979) and to promote random MT assembly *in vivo* at sites other than the centrosome in several types of cultured cells (Schiff and Horwitz, 1980). In contrast, lymphocytes treated with taxol have increased numbers of MTs and form one to few large bundles extending from the centrosome.

The *in vitro* results strongly suggest that the SBs are major MT initiating structures. It is possible, however, that SBs don't initiate MTs but serve as attachment points for MTs initiated elsewhere in the centrosome. De Brabander (1982) hypothesized the existence of a "domain" wherein MT initiation occurs due to a lowering of the tubulin critical concentration. The MTs would then attach directly to the SBs or become attached in the PCM. It might be possible to test the hypothesis by sampling at very short times during *in vitro* assembly. De Brabander also suggests that MTs which are attached to SBs are more stable than other centrosome-associated MTs. If this is true the resting cell MTs should be more stable and stimulated cells should have two populations of MTs differing in stability. This could be tested by examining the time course of MT disassembly using various antimicrotubule treatments in intact cells.

The increase in the number of MTs assembled during stimulation is not due solely to increases in the MT initiating

capacity of the centrosome. It has been clearly established that there is a 2.5 fold increase in the tubulin content of con A stimulated mouse splenic T lymphocyte populations at 48 hours, the same cells populations used in this study. The amount of tubulin rises from 0.20pg/cell to 0.51pg/cell (Waterhouse et al., 1983). Based on the known molecular weight of the tubulin dimer, the number of dimers per microneter, and the average length of MTs they showed that the tubulin content of resting lymphocytes was not enough to form all the MTs counted in fully stimulated cells, demonstrating that tubulin availability is a limiting factor in the development of the MT network in blast cells. The results of Waterhouse and coworkers (1983) showing a differential increase in tubulin content correlate well with the results of Kecskemethey and Schafer (1982) who showed that tubulin mRNA in resting bovine lymph node lymphocytes is at an undetectable level and rises tremendously as a result of con A stimulation.

It has been shown, for several types of tissue culture cells that an increase in the soluble tubulin pool, caused by MT depolymerizing drugs, results in decreased tubulin sythesis (Ben-Ze'ev, Farmer and Penman, 1979; Cleveland et al., 1981), and this has suggested an autoregulatory mechanism. Lymphocyte stimulation is a potentially useful model system to examine the relationship between increases in MT assembly and increases in tubulin synthesis. In this thesis

I have shown that there is an increase in the *in vitro* MT initiation capacity of the centrosome during stimulation. It would be of interest, now, using the stimulated lymphocyte as a model system to establish the temporal relationships between the increases in tubulin message, tubulin synthesis, MT initiation capacity of the centrosome, and the increased assembly of microtubules that accompanies the activation of lymphocytes.

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