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**LA THÈSE A ÉTÉ
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Role of Microtubules in Patching and
Capping of Lymphocyte Surface Receptors

A Thesis Submitted
to the School of Graduate Studies
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Kem A. Rogers

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ABSTRACT

The cytoskeleton has been implicated in the process of lymphocyte surface receptor capping induced by bivalent ligands. Experiments were performed to determine the role of one of the cytoskeletal elements, microtubules, in the patching and capping of surface IgG.

Using an improved method for the immunofluorescent detection of microtubules, I have examined their distribution in resting and concanavalin A (Con A) activated lymphocytes. The method involves extraction of the soluble cytoplasm of the cell with Triton X-100 in a microtubule stabilizing buffer. In some experiments a brief prefixation with paraformaldehyde is used to prevent the detergent extraction of labelled cell surface receptors in resting cells, and to stabilize the detergent sensitive microtubules of activated cells.

Resting cells appear to have between 5 and 15 stained fibers converging on the centriolar region. Electron microscopic examination of Con A activated lymphocytes shows a large increase in the number of cytoskeletal microtubules during blast transformation. With the increase in number of microtubules, there is a concomitant increase in the number of satellite bodies in the centrosomal microtubule organizing centre.

I examined the time course of disassembly of microtubules in resting lymphocytes exposed to low temperature or to the

anti-mitotic drug colchicine. The microtubule network is disassembled by a 45 min exposure to 4°C , and upon re-warming to 37°C reassembles in 3 to 5 min. The protocol of colchicine treatment reported by other laboratories to completely disassemble microtubule networks in all cells (10^{-4} M, 30 min) was found to be less than 50% effective; maximal disassembly requires a 4-8 h exposure to 10^{-5} M colchicine. Higher concentrations of the drug do not increase the rate of disassembly.

Using double labelling immunofluorescence, the microtubule network of patched and capped cells was examined. In lymphocytes patching and capping surface IgG, the microtubule network remains intact and apparently unaltered. Cap formation occurs exclusively over the centriolar region containing the centriole pair, Golgi and associated organelles. In cells treated with colchicine, the site of cap formation appears to be random with respect to the location of the centriole pair and other cytoplasmic organelles.

These results implicate the microtubule network in controlling the directional flow of receptor-ligand complexes as they are drawn towards one pole of the cell by the microfilament system. Two models for the role of microtubules in cap formation are discussed. The first involves the anchoring of integral proteins by microtubules to produce parallel tracks of frozen membrane which orient the lateral flow of receptor-ligand complexes. The second requires a direct association of the contracting microfilament system with the

microtubule network. Whatever the mechanism, it is clear that the microtubule network exerts spatial control over the site of cap formation.

Résumé

Il semblait que le cytosquelette ait un rôle à jouer dans la formation de capuchons par les récepteurs superficiels des lymphocytes, réaction provoquée par des ligands bivalents. On a effectué une série d'expériences pour déterminer le rôle que joue l'un des éléments du cytosquelette, les microtubules, dans la formation de plaques par des IgG superficiels.

Au moyen d'une technique perfectionnée d'immunofluorescence, j'ai étudié la distribution de microtubules dans des lymphocytes au repos et dans des lymphocytes traités à la concanavine A (Con A). Cette méthode comprend l'extraction au Friton X-100 du cytoplasme soluble de la cellule dans une solution tampon. Dans certains cas, on a eu recours à une brève fixation préalable au paraformaldehyde, afin d'éviter l'extraction accidentelle de récepteurs colorés à la surface des cellules au repos, et pour stabiliser les microtubules, sensibles au détergent, dans les cellules stimulées.

Les cellules au repos portent de 5 à 15 fibres colorées qui convergent sur la région centrolaire de la cellule. Un examen au microscope électronique des lymphocytes traitées au Con A permet de déceler une augmentation considérable du nombre de microtubules au cours de la blastogénèse. Cette augmentation correspond à une augmentation des autres corps dans le centre d'organisation des microtubules centrosomaux.

On a aussi étudié le délai de désassemblage des microtubules de lymphocytes au repos qui ont été exposées à de faibles températures ou à la drogue anti-mitotique colchicine. Le réseau de microtubules se désassemble après avoir été exposé pendant 45 min. à 4°C de

température, après que l'on a ramené la température à 37°C, il se reconstruit au bout de 3 à 5 min. Le protocole de traitement à la colchicine qui, selon les résultats publiés par d'autres laboratoires, désassemblerait complètement les réseaux de microtubules dans toutes les cellules (10^{-4} M), 30 min), s'est révélé-efficace à moins de 50%; un désassemblage maximal exige entre 4 et 8 h d'exposition à 10^{-5} M de colchicine. Une concentration plus élevée de colchine n'a pas accéléré le désassemblage.

Au moyen d'immunofluorescence double, on a pu étudier le réseau microtubulaire des cellules sur lesquelles apparaissaient plaques et capuchons. Dans les lymphocytes où les IgG superficiels formaient des plaques et des capuchons, le réseau de microtubules demeurait intact. La formation de capuchons ne se produisait qu'au-dessus de la région centriolaire où se trouvent le pair centriol, le golgi, et leurs organelles associées. Après le traitement à la colchicine, cependant, cette formation se produisait fortuitement par rapport à l'emplacement de le pair centriol et à d'autres organelles cytoplasmiques.

Les résultats indiqueraient donc que le réseau microtubulaire orienterait le mouvement des complexes récepteurs-ligands alors qu'ils sont tendus vers l'un des pôles de la cellule par le système de microfilaments. On discute deux modèles pour le rôle que jouent les microtubules dans la formation de capuchons. Le premier consiste à ce que les microtubules fixent des protéines intégrales pour produire des parallèles de membrane figée qui orienteraient le mouvement latéral de complexes récepteurs-ligands. Le second exige une association directe entre le système de micro-filaments en contraction et le réseau microtubulaire. Sans pouvoir cerner le processus qui est en cours lors de la réaction, on peut affirmer que le réseau microtubulaire

exerce une influence spatials sur l'endroit où à lieu la formation des
capuchons.

INTRODUCTION

The lymphocyte is found in virtually all higher animals which have developed an immune system. This cell exists in a resting state with little metabolic activity, and under certain conditions it can be induced by specific agents to undergo a variety of morphological and metabolic changes (termed blastogenesis) until it is a fully activated immune cell.

The structure and function of the cytoskeleton has been under investigation for a number of years. The components of the cytoskeleton are directly or indirectly involved in a wide variety of cell processes, acting as structural elements and/or agents of movement. Much of the evidence published to date has shown that the cytoskeletal network of lymphocytes may be directly or indirectly involved in the patching and capping of receptor-ligand complexes, some of which stimulate the lymphocyte to undergo blastogenesis.

In this thesis I report on an improved immunofluorescent technique for the accurate detection of microtubule networks in resting lymphocytes. With this technique I have examined the relationship of the microtubule network to the capping of receptor-ligand complexes and critically compare my results with those of others who have utilized less accurate methods.

LYMPHOCYTES IN CULTURE

The immune system, designed to recognize and destroy foreign or unwanted antigens, consists of two main families of lymphoid cells; namely T-cells or thymus dependent lymphocytes, and B-cells or bone marrow dependent (bursa equivalent) lymphocytes. The former are responsible for cell mediated immunity, while the latter are considered to be responsible for humoral immunity (Le Douarin, 1977).

When lymphocytes are isolated and cultured in vitro, they can be stimulated by a variety of agents to undergo blast transformation (blastogenesis, activation, stimulation, mitogenesis). These agents include the plant lectins (concanavalin A, phytohemagglutinin, wheat germ agglutinin, pokeweed mitogen), lipopolysaccharide, specific antigens, and chemical reagents such as periodate. All of these agents are polyclonal mitogens (except specific antigens); polyclonal because cells within the population having different antigenic specificities respond in common to the agent, and mitogens because they induce cells in a G_0 or resting state to enter G_1 , pass through S and G_2 , and eventually undergo mitosis. When lymphocytes are induced to transform they undergo a variety of morphological and biochemical changes, several of which have been well characterized.

Resting lymphocytes are normally 5-8 μm in diameter, have a large condensed nucleus, scant cytoplasm with a small number of organelles, and few microtubules. During

blastogenesis they undergo an increase in total cell size (9-18 um in diameter), the chromatin decondenses, the ratio of cytoplasmic to nuclear area increases, and the number and/or activity of most cellular organelles increases (Biberfeld, 1971; Shands, Leavy, Smith, 1973). Finally the blast cell enters mitosis and divides. Biochemically a large number of parameters have been monitored during lymphocyte transformation. There is an early increase in the level of sugar, amino acid and nucleoside transport into the cell, changes in the permeability of the plasma membrane to cations, increased RNA and protein synthesis, replication of DNA, and finally mitosis (Mendelsohn, Skinner and Kornfield, 1971; Parker, 1974; Wedner and Parker, 1976; Whitney and Sutherland, 1972). The incorporation of ³H-thymidine into the DNA of cells in S-phase has been used as a general measure of blast transformation.

THE COMPONENTS OF THE CYTOSKELETON

The cytoskeleton of lymphocytes consists primarily of the microtubule and microfilament networks, the first functioning as a structural element while the second is involved in contractile processes (reviewed by Loor, 1977).

1: Microtubules:

Microtubules are structural organelles found in all eukaryotic cells during some stage of their life cycle. They form a part of a large number of structures including

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mitotic spindles, eukaryotic flagella, cilia, basal bodies, centrioles, and cytoplasmic cytoskeletal elements (Porter, 1966; Snyder and McIntosh, 1975).

In cross-section, microtubules appear to be hollow cylinders 240 Å in diameter, made up of 13 individual protofilaments (Tilney et al., 1973). They are composed of two major polypeptides, termed alpha tubulin and beta tubulin. There exists a close similarity among the alpha tubulins (and also among the beta tubulins) isolated from various species, with respect to their molecular weight, amino acid composition and immunological cross reactivity. These monomers normally associate as heterodimers with a molecular weight of 110,000 and a sedimentation coefficient of 6S. The heterodimer is the subunit which assembles into microtubules (for a recent review of the biochemistry of microtubules see Kirschner, 1978).

Microtubules have been implicated in a variety of cell functions. They are arranged in highly structured patterns in cilia, flagella and axostyles which function in cell motility in a variety of organisms (Satir, 1965; Brokaw, 1972; Bloodgood, 1975). Microtubules function in intracellular transport, providing a structural framework for the movement of chromosomes (Harris, 1962), the transport of proteins in nerve axons (Fernandez et al., 1971), the movement of pigment granules during contraction and dispersion in erythrophores (Byers and Porter, 1977), and the movement of endocytotic vesicles to the region of

the Golgi in ovarian granulosa cells (Albertini and Anderson, 1977). They are also involved in the determination and maintenance of cell shape (Gibbins, Tilney, and Porter, 1966; Brown and Bouck, 1973).

In vivo, microtubules are in constant dynamic equilibrium with their subunits (Inoue and Sato, 1967). It is for this reason that many of the studies undertaken to determine the role of microtubules in specific cell processes have involved the use of treatments which reversibly disassemble the microtubule system and disrupt these processes. These treatments include the use of the alkaloids colchicine and vinblastine (Borisy and Taylor, 1967; Bensch and Malawista, 1968), low temperature (Inoue and Sato, 1967), and high hydrostatic pressure (Zimmerman and Marshland, 1964; Inoue and Sato, 1967). In these experiments, reassembly of the microtubules is correlated with return of function.

2: Microfilaments:

In all non-muscle cells actin can be found in the form of microfilaments, 40 to 60 Å in diameter. Both biochemical and electron microscopic observations have demonstrated that actin is a major constituent of muscle cells (for a review see Pollard and Weihing, 1974), and that actin-like proteins from non-muscle cells are very similar to skeletal muscle actin (Bray, 1972).

Actin is a double helical filament (Hataro, Totsuka

and Oosawa, 1967) assembled from a 42,000 molecular weight monomer. In non-muscle cells actin is found to exist in two forms, namely F-actin or filamentous actin and as G-actin or monomeric actin (Bray, 1976).

Actin filaments have been identified within cells using a variety of light and electron microscopic techniques. Heavy meromyosin (HMM) binding can be used to label actin filaments at the electron microscope level giving the characteristic arrowhead pattern in muscle cells (Huxley, 1963) and non-muscle cells (Ishikawa, Bischoff, and Holtzer, 1969). Bundles of actin filaments known as stress fibers have been seen in living non-muscle cells using Nomarski optics (Goldman *et al.*, 1975), and in fixed non-muscle cells examined in the transmission electron microscope (Goldman, 1975). The presence of actin in these bundles has been confirmed using fluorescently labelled HMM (Aranson, 1965) and indirect immunofluorescence (Lazarides and Weber, 1974).

Other proteins, common to the contractile apparatus of muscle cells, have been identified and localized in non-muscle cells in association with microfilaments. These include the structural proteins myosin (Weber and Groeschel-Stuart, 1974), tropomyosin (Lazarides, 1975) and alpha-actinin (Lazarides, 1976).

Microfilaments are found in a variety of contractile organelles in non-muscle cells. These include structures involved in cytokinesis, patching and capping, endocytosis

and exocytosis, cell adhesion to a substratum, cell locomotion, membrane ruffling and maintenance of cell shape (Buckley and Porter, 1967; Schroeder, 1970; Taylor et al., 1971; Orr, Hall, and Alison, 1972; Goldman and Knipe, 1973; Wessels et al., 1973; and Sanger, 1975).

A variety of treatments which disrupt microfilament function have been used to determine the rôle of microfilaments in different cell processes. Examples of such treatments include the use of azide to prevent ATP production necessary for contraction, disruption of the microfilaments by cytochalasins, and induction of contraction by Ca^{++} (Wessels et al., 1971; Schreiner and Unanue, 1976a).

THE ROLE OF THE CYTOSKELETON IN PATCHING, CAPPING AND LYMPHOCYTE ACTIVATION

It has been demonstrated by a large number of researchers that components of the cytoskeleton are involved in the patching and capping of surface receptors by specific ligands, and in the stimulation of lymphocytes by specific mitogens.

1: Patching and capping of cell surface receptors:

Upon exposure of lymphocytes to a variety of multivalent cell surface ligands at 37°C, the specific receptor crosslinked by the ligand will redistribute to form

patches on the cell and, in an energy dependent process, will migrate to one pole of the cell to form a cap. These ligands include anti-Ig antibody, lectins, anti-H-2 antibody, anti-Thy-1 antibody, anti-lymphocytic antibody (ALS), Fc receptors, viruses and viral antigens. Only anti-Ig, lectins and viral antigens will form caps spontaneously at 37°C. Anti-H-2, anti-Thy-1, ALS and Fc receptors require the presence of another antibody, or crosslinking agent. An example would be the incubation of a T-lymphocyte with goat anti-Thy-1 followed by rabbit anti-goat to induce cap formation (Schreiner and Unanue, 1976b).

The best characterized ligands are concanavalin A and anti-Ig antibody. Both cap spontaneously at 37°C, but with striking differences that may indicate a variation in mechanism (Table 1). As with all ligands, they must be multivalent to be capped. They require that the cell be metabolically active, thus their capping can be inhibited by exposure of the cell to the metabolic inhibitor sodium azide (Taylor et al., 1971). Concanavalin A at high concentrations (50 ug/mL) will effectively inhibit the capping of other surface receptors, as well as its own (Yahara and Edelman, 1972). Anti-Ig shows no such "freezing" of other receptors, and will only inhibit migration of Ig at very high concentrations (500 ug/mL) (Taylor et al., 1971).

Colchicine, a disruptor of microtubules, has little

TABLE 1
Comparison of Anti-Ig and Con A Induced Capping

	Anti-Ig	Con A
Multivalency	Required	Required
Energy metabolism	Required	Required
Dose dependency	Some inhibition at high doses	Inhibition at all doses
Effects on capping of other molecules	No inhibition	Dose dependent inhibition
Cytochalasin B	Some inhibition at high doses (10-30 ug/mL)	Marked inhibition at low doses (10 ug/mL)
Colchicine	No inhibition: some enhancement	Marked enhancement: permits capping of Con A
Association with motility	Capping precedes motility	Capping follows motility

or no effect on the percentage of cells capping anti-Ig (Taylor et al., 1971; Loor, Forni and Pernis, 1972), but it will enhance anti-Ig capping on cells inhibited by Concanavalin A (Edelman, Yahara and Wang, 1973). Cytochalasin B, an inhibitor of microfilament function, will inhibit anti-Ig capping (Taylor et al., 1971), but is more effective in inhibiting Concanavalin A capping (Edelman, Yahara and Wang, 1973). Morphological studies utilizing immunofluorescence and electron microscopy have been carried out to show the relationship between the patching and capping of surface receptors and the cytoskeletal elements of the cell. It has been shown using double label immunofluorescence that "sub-caps" of the contractile proteins actin, myosin and alpha-actinin are found below the capped ligand (Gabbiani et al., 1977; Schreiner et al., 1977; Bourguignon, Tokuyasu and Singer, 1978; Geiger and Singer, 1979).

It has also been demonstrated that patching of surface receptors is sufficient to redistribute actin, myosin and alpha-actinin into "sub-patches" (Geiger and Singer, 1979). It has been reported that tubulin in capped cells also forms "sub-caps" (Gabbiani et al., 1977; Yahara and Kakimoto-Sameshima, 1978), however there is evidence to the contrary (Bourguignon, Tokuyasu and Singer, 1978).

Several mechanisms for capping have been proposed, each taking into account some or all of the observations

stated above.

In 1972, de Petris and Raff proposed a counter-current mechanism in which patches of receptors are formed by the crosslinking ligand, and are then collected into a trailing cap when the rest of the membrane is displaced forward by the activity of the cytoskeleton which is moving the cell forward. It is this movement of the cell that makes the capping process energy dependent.

Bretscher in 1976 suggested that capping involved a rapid continuous oriented flow of lipid molecules which sweep slowly diffusing patches of ligand crosslinked proteins to the site of lipid resorption forming a cap, while not affecting uncrosslinked proteins capable of rapid lateral diffusion out of the lipid flow.

Schreiner and Unanue (1976c) have proposed two mechanisms for capping. One form is a movement dependent capping, similar to the mechanism of de Petris and Raff, in which large crosslinked complexes formed on the surface of the cell would accumulate to the trailing part of the cell. This mechanism does not require interaction between the surface receptors and the cytoskeletal system, and would be utilized in capping of plant lectins such as Con A and ligands requiring a secondary crosslinking agent. The other mechanism depends on selectively induced interactions between the cell surface receptor-ligand complex and the cytoskeleton. This mechanism would be utilized in the capping of anti-Ig and does not require

that the cell be in motion, however in lymphocytes this capping will induce translatory movement.

The latest theory proposed by Bourguignon and Singer (1977) suggests that all capping phenomena are mediated by a similar active mechanism. They propose that membrane associated actin is directly or indirectly bound to an integral membrane protein X which is ubiquitous in the membranes of eukaryotic cells. When any receptor in the membrane is aggregated by its specific external multi-valent ligand, the aggregate binds effectively to X, whereas unaggregated receptors do not bind to X. As a result of the linkage to X, aggregates of that single receptor thus become bound to actin (and myosin) on the cytoplasmic surface of the membrane. The bound actin and myosin then serve to collect the receptor aggregates into a cap presumably by a sliding filament mechanism.

Once the ligand-receptor complex has been capped, it may: 1) be endocytosed into the cell interior and fused with lysosomes; 2) be shed from the cell surface into the surrounding environment; 3) remain on the cell surface for various periods of time; 4) dissociate, leaving the receptor free on the cell surface (Schreiner and Unanue, 1976b).

Little information has been obtained concerning the role of microtubules in patch and cap formation in lymphocytes. This lack of information is due primarily to the difficulty in visualizing microtubules in resting

lymphocytes. The few microtubules in these cells are difficult to quantitate by thin section electron microscopy (see for example Yahara and Edelman, 1975).

It is known that colchicine does not inhibit the capping process, and may indeed enhance it (Edelman, Yahara and Wang, 1973). For example, colchicine will release the inhibition of anti-Ig capping caused by high concentrations of Con A, presumably by depolymerizing microtubules. The effect of colchicine led Edelman, Yahara and Wang (1973) to believe that this freezing of receptors by Con A is due to a direct association of the Con A receptors with the microtubules and that the microtubule network modulates the mobility and distribution of cell surface receptors.

Recently, Yahara and Kakimoto-Sameshima (1978) utilized an immunofluorescent technique to examine the microtubule system in resting lymphocytes prior to and after capping of cell surface receptors. Prior to exposure of the cells to a ligand, they found that a microtubule network could be visualized in over 90% of the cells stained. Each cell contained between 5 and 20 "tubular structures" which seemed to originate from an organizing centre. After capping the surface Ig in lymphocytes with rhodamine-goat-anti-mouse IgG (R-GaMIgG) they found that normal microtubule organization was absent from capped cells. The region beneath the cap stained very brightly with anti-tubulin antibody, indicating that

a "sub-cap" of tubulin had formed (see also Gabbiani et al., 1977). Patching of Ig, induced by incubating the cells with R-GaMIgG in the presence of the metabolic inhibitor sodium azide, was sufficient to cause a disorganization of the microtubule network. From these results, Yahara and Kakimoto-Sameshima (1978) concluded that microtubules are redistributed during patch and cap formation, and that the microtubule organization is actually modulated by patch and cap formation, rather than the microtubules modulating the formation of patches and caps as was suggested earlier (Edelman, Yahara and Wang, 1973).

2: Lymphocyte activation:

The mechanism by which the binding of a mitogen to the lymphocyte surface is able to trigger that cell to undergo blastogenesis is not well understood. The binding of the mitogen to the cell surface and some of the biochemical changes which ensue are well characterized, but the signal which links these two events is as yet unknown.

Using drugs to disrupt microfilaments and microtubules, it has been demonstrated that these two cytoskeletal elements may be directly involved in the stimulation of lymphocytes by lectins and other mitogens, and that they may be the signal transducer triggering blastogenesis. Cells exposed to various mitogens, such as specific antigens or the lectins PHA and Con A, can be inhibited from undergoing blastogenesis by exposing them

to the drug cytochalasin B. This drug does not interfere with the binding of the activating ligand to the cell surface, but appears to mediate its effects by binding to the microfilament system or some related cytochalasin sensitive structure, thus disturbing normal microfilament function. These observations suggest that microfilaments play an important role in the stimulation of lymphocytes (Roberts and La Via, 1976; Greene, Parker and Parker, 1976).

Disruption of microtubules with the anti-microtubule agents colchicine and vinblastine has been shown to interfere with the activation of lymphocytes by plant mitogens. Edelman and his coworkers have determined the time course of inhibition of blastogenesis produced when these agents are added at varying times of culture (Wang, Gunther, and Edelman, 1975; Gunther, Wang and Edelman, 1976). From their results they have developed a model in which microtubules are necessary for the initiation of DNA synthesis brought on by the binding of the mitogen to cell surface receptors.

Despite the large number of reports describing loss of function (ie: response to mitogen) with disruption of microtubules in lymphocytes by anti-mitotic drugs, few of these reports have been accompanied by a direct study of the effect of these drugs on the microtubules themselves. Probably the most complete electron microscopic study of the structural changes occurring during the mitogenic stimulation of human lymphocytes was that of Biberfeld

(1971), who showed that there was a large increase in the number of microtubules in the centriolar region during blastogenesis. The microtubule network in blast cells has been shown by electron microscopy to be completely disassembled by a 4 h exposure to 10^{-5} M colchicine (Thyberg, Maskalewski and Friberg, 1977). Lower concentrations of the drug, commonly used to inhibit lymphocyte activation, were not tested. Yahara and Edelman (1975) had reported that most microtubules disappear from cells examined by electron microscopy after treatment with 10^{-4} M colchicine for 30 min; however they encountered great difficulties in observing microtubules in the majority of resting lymphocytes, even in those that had not been treated by the drug. In their recent report Yahara and Kakimoto-Sameshima (1978) showed that the microtubule networks in resting lymphocytes were completely disassembled by a 30 min exposure to 10^{-4} M colchicine at 37°C . They found however that 10^{-5} M colchicine was not as effective during the same time period, and that microtubules were not significantly affected by lower concentrations (10^{-6} M). It should be noted that 10^{-6} M colchicine is the concentration normally utilized to inhibit lymphocyte activation.

STATEMENT OF THE PROBLEM

It is clear from a survey of the literature that there is confusion concerning the relationship of the microtubule network to the redistribution of lymphocyte surface receptors crosslinked by multivalent ligands. Colchicine treatment has been used in capping studies to determine the role of microtubules in this process, but these studies have not been accompanied by a direct examination of the effect of this drug on the microtubules in these cells. Further confusion has been created by the use of ligands with multiple receptor classes. One such ligand is Con A, which requires that the microtubule network be disassembled before it will induce capping.

I have carefully examined the sensitivity of lymphocyte microtubules to low temperature and to colchicine. I have used the ligand anti-IgG in my studies because it is specific for one class of receptor, and can induce the redistribution of its receptors on cells with an intact microtubule network. Using double labelling immunofluorescence, I have shown that lymphocytes patching and capping surface IgG have intact microtubule networks. The results clearly show that the microtubule network exerts spatial control over the site of cap formation in lymphocytes.

SUPPLIES

MATERIAL

Rhodamine goat anti-mouse IgG
Rhodamine goat anti-human IgG
Poly-L-lysine
Fluorescein goat anti-rabbit IgG
Fluorescein rabbit anti-goat IgG
Pipes

EGTA

Polyethylene glycol 6000
Triton X-100
Glutaraldehyde (EM Grade)
Sodium borohydride
Imidazol
Paraformaldehyde

SUPPLIER

Cappel
Cappel
Sigma
Hyland
Cappel
Sigma
Eastman
Baker
Fisher
Polyscience
Baker
Sigma
Polyscience

MATERIALS AND METHODS

CELL CULTURES

Mouse splenic lymphocytes were aseptically isolated from 10-14 weeks old Balb/c mice obtained from Bio-Breeding Laboratories. Human peripheral lymphocytes were obtained from healthy volunteers by venous puncture using a heparinized needle.

1: Purification of murine splenic lymphocytes:

Mice were sacrificed by cervical dislocation, their spleens aseptically removed and washed in isotonic saline (0.9%) to remove fatty tissue. The spleens were teased with forceps into RPMI 1640 made 10% with fetal calf serum (FCS). RPMI 1640 in all experiments was buffered to pH 7.2 with either 0.2% NaHCO_3 for use in a CO_2 purged incubator or 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) for use in sealed culture flasks. Clumps of cells were broken by passing the suspension through a 20 gauge needle several times. The suspension was layered on a 100% FCS cushion cooled to 4°C , and the large debris allowed to settle through. The suspension was then removed and centrifuged in an IEC Clinical Centrifuge at a setting of 6 (1000g) for 3 min. The pellet was resuspended in cold 0.83% NH_4Cl (aqueous) for 7 min. to lyse red blood cells and the suspension layered on a 100% FCS cushion by adding the FCS to the bottom of the

suspension using a pasteur pipette. The lymphocytes were spun through this cushion at 700g (setting 5) for 4 min., washed once in RPMI 1640, and resuspended at a concentration of 2×10^6 /mL in RPMI 1640 made 10% with FCS.

Cells were stimulated with concanavalin A (Con A) at a concentration of 2 ug/mL.

2: Purification of human peripheral lymphocytes:

Human venous blood was mixed 2:1 with RPMI 1640 and layered on a 15 mL cushion of cold Ficoll-Hypaque in a 50 mL conical centrifuge tube and spun at 1500 RPM in a Beckman model J6 centrifuge at 20°C. The red blood cells pellet through the Ficoll-Hypaque leaving the lymphocytes at the serum/ Ficoll-Hypaque interface. The lymphocytes were removed, washed twice with RPMI 1640, and cultured at a concentration of 1 to 1.5×10^6 cells/mL in RPMI 1640 made 10% with FCS or autologous serum.

For both mouse and human lymphocytes, number and viability were determined by trypan blue exclusion using a hemocytometer. To prevent bacterial contamination 100 units penicillin G and 100 ug streptomycin sulfate per mL culture were added.

Cultures were treated with freshly prepared solutions of colchicine at the indicated concentrations in RPMI 1640 supplemented with 10% FCS.

ASSAY OF STIMULATION

Stimulation of lymphocytes by Con A was measured by the standard technique of incubating cells with the DNA precursor ^3H -thymidine, which is incorporated into the DNA of cells stimulated to enter S-phase by Con A (Wang, Gunther and Edelman, 1975).

Cells were incubated in 250 uL Limbro wells: 200 uL of cells at $2 \times 10^6/\text{mL}$ were added to each well followed by 50 uL of ^3H -thymidine to give a final concentration of 2 uCi/mL (specific activity 42 Ci/mM). Cells were exposed to ^3H -thymidine for 4 h and harvested onto glass fiber filters using a Flow Titertek Cell Harvester. The filters were air dried, placed in 3 mL of Scintilene and counted in a Beckman LS-233 liquid scintillation counter.

IMMUNOFLUORESCENT LABELLING

1: Cell surface labelling

Rhodamine isothiocyanate (RITC) coupled to goat anti-mouse IgG (immunoglobulin-G) (R-GaMIgG) or goat anti-human IgG (R-GaHIgG) was exhaustively dialyzed against phosphate buffered saline pH 7.0 (PBS: 7.65 gm NaCl, 0.725 gm NaHPO_4 , 0.212 gm KH_2PO_4 per litre) to remove azide. Cells suspended in RPMI 1640 supplemented with FCS were exposed to the indicated concentration of either label and plated onto poly-L-lysine coated coverslips. These coverslips were prepared by flooding them with

0.1% poly-L-lysine (aqueous) for 60 sec, washing with distilled water, and blotting dry. Cells adhere firmly to the coverslips, greatly facilitating the processing of the cells as the coverslips are passed from solution to solution.

Following incubation with RITC-coupled label, cells were washed in PBS for 15 sec and fixed for 60 sec in 3% PBS buffered paraformaldehyde pH 7.0. This fixative was prepared by dissolving 3 gm of paraformaldehyde in 80 mL of boiling water made basic with 100 μ L of 1N NaOH. 10 mL of 10x stock PBS was then added, the pH adjusted to 7.0, and the volume brought to 100 mL. After fixation the cells were washed in PBS and mounted on a slide in 50% (V/V) glycerol in PBS at pH 7.6 for examination, or they were further processed for immunofluorescent staining of the microtubule networks.

2: Immunofluorescent staining of microtubules:

a) Antibodies: The immunofluorescent staining was carried out using antisera produced in either rabbit or goat directed against purified pig brain tubulin. The antisera were a generous gift from Dr. V. I. Kalnins and DR. J. A. Connolly (Department of Anatomy, University of Toronto). The secondary labels used were FITC-conjugated goat anti-rabbit IgG (F-GARs) or FITC-conjugated rabbit anti-goat IgG (F-RAGs). These were prepared by incubating with 80 mg/mL of rat liver acetone powder and 40 mg/mL mouse spleen acetone powder for 1 h at 37°C or overnight at 4°C which

removes non-specific, and perhaps specific, binding of the secondary antibody directly to cellular components. The acetone powders were prepared by homogenizing the liver or spleen in 100% acetone, and washing the precipitate with excess acetone. The powder was air dried and stored at -20°C . When required, the acetone powder was washed with PBS until the high speed supernatant of PBS was clear.

b) Fixation and staining of cells: Cells for immunofluorescent staining were plated onto poly-L-lysine coated coverslips and processed in one of two ways.

The first procedure was a modification of the method of Osborn, Webster and Weber (1978) in which cells were washed twice for 30 sec at room temperature (RT) in the stabilization buffer SB-1 (0.1M piperazine-N,N'-bis(2-ethane sulfonic acid), 1mM ethylene glycol-bis-(beta-aminoethyl ether)-N,N'-tetraacetic acid(EGTA), 4% polyethylene glycol 6000, pH 6.7) and extracted for 4 min at RT in SB-1 containing 1% Triton X-100. The cells, now cytoskeletons, were washed twice for 30 sec in SB-1, fixed for 10 min at RT in SB-1 containing 1% glutaraldehyde and washed twice for 4 min in 1-2 mg/mL sodium borohydride in PBS to reduce free aldehyde groups. The cells were washed twice in PBS for 2 min each, and stained with antibodies to tubulin.

The second procedure involved a prefixation step in which the cells were lightly fixed with a 60 sec exposure to 3% paraformaldehyde in PBS, pH 7.0. This prefixation step was used in all experiments where a cell

surface component was labelled with an RITC-conjugate, and the microtubule system was subsequently stained with an FITC-conjugate. The prefixed cells were extracted with 1% Triton X-100 in the stabilization buffer SB-2 (50 mM Imidazol, 50 mM KCl, 0.5 mM MgCl₂, 1 mM EGTA, 0.1 mM disodium ethylenediaminetetraacetate (EDTA), 1 mM 2-mercaptoethanol, 4 M glycerol, pH 6.7) for 30 to 60 min. The cells (cytoskeletons) were washed twice for 30 sec in SB-2, fixed in 1% glutaraldehyde in SB-2 for 10 min, and reduced in NaBH₄ as described above.

Following either fixation procedure the cytoskeletons were incubated with antiserum to tubulin (diluted 1:30 with PBS) for 45 min at RT in a humid chamber, thrice washed in PBS for 4 min each and incubated for 45 min with the appropriate fluorescein conjugated secondary antibody (dilution with PBS dependent on supplier and lot number). The cytoskeletons were thrice washed in PBS for 4 min each and mounted on a slide in 50% (V/V) glycerol in PBS, pH 7.8, for examination.

Fluorescently labelled cells were examined with a Zeiss Universal microscope equipped with epifluorescence optics, and photographed on Kodak Tri-X Pan film developed in Microdol-X, or on Ilford FP4 developed in Microphen.

FERRITIN ANTIBODY LABELLING OF MOUSE IgG

Ferritin conjugated goat anti-mouse IgG was used at 200 ug antibody/mL to label IgG on the surface of mouse

splenocytes. Cells at $1 \times 10^7/\text{mL}$ were exposed to the label at 37°C for the indicated period of time and processed for electron microscopic examination.

TRANSMISSION ELECTRON MICROSCOPY

Cells were pelleted by gentle centrifugation and fixed at 20°C for 1.5 h in 4% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.0. They were washed three times in 0.05 M sodium phosphate and postfixed at 4°C in 1% osmium tetroxide in 0.05 M sodium phosphate buffer pH 7.0. Dehydration by a graded acetone series was followed by infiltration with Spurr's hard resin mixture (Spurr, 1969) which was polymerized at 60°C for 18 h. Sections cut with a Dupont diamond knife on a Sorval Porter-Blum MT-2B ultramicrotome were stained for 7 min with uranyl acetate (2% in 50% ethanol) and 5 min with lead citrate (Reynolds, 1963).

RESULTS

LYMPHOCYTE ACTIVATION: GENERAL OBSERVATIONS1: ^3H -thymidine incorporation:

Resting lymphocytes, purified from Balb/c mouse spleen or human peripheral blood are in a G_0 stage of the cell cycle. They can be stimulated to enter G_1 , and subsequently S, by exposing them to the plant mitogen Concanavalin A (Con A). The level of activation by Con A can be measured by adding ^3H -thymidine to the culture medium for a period of four hours at 37°C , and harvesting the cultures on glass fiber filters which retain the macromolecules synthesized by the cell. Using this technique it is possible to measure the entry of cells into the S-phase of the cell cycle, since ^3H -thymidine is incorporated into newly synthesized DNA. The activation of lymphocytes by Con A can be inhibited by the concomitant exposure of the cells to the alkaloid colchicine, the effect of which is presumably a consequence of microtubule disassembly (Wang, Gunther and Edelman, 1975; Gunther, Wang and Edelman, 1976).

To ensure that the tissue culture techniques used permitted mouse splenocytes to respond to Con A in the usual manner, and to determine if the colchicine used in subsequent experiments was effective in inhibiting blastogenesis, ^3H -thymidine measurements were carried out 48 h

after the time of addition of these drugs (Table 2). Colchicine is 80% effective in inhibiting lymphocyte activation by Con A.

2: Light and electron microscopic observations of lymphocyte activation:

The resting mouse splenocyte is 5-7 μm in diameter, has scant cytoplasm, and a large nucleus (fig. 1A). Upon addition of Con A at 2 $\mu\text{g}/\text{mL}$ resting T-cells that are stimulated grow in size (Fig. 1B-D), until they reach the blast stage (Fig. 1E). During this growth period, the chromatin of the activated lymphocyte decondenses, nucleoli appear, the cytoplasm to nucleus ratio increases, and the cell enters mitosis (Fig. 1F).

Figures 2 and 3 illustrate the major morphological differences between resting mouse lymphocytes (Fig. 2A-D) and lymphocytes activated by a 48 h exposure to Con A (Fig. 3A-D). Due to the limited number of microtubules present in resting mouse splenocytes, they are not easily detected by electron microscopy. When detected, they are most frequently seen near the centrioles, an observation consistent with the immunofluorescent staining (Fig. 2D), showing the microtubules converging on the centriolar region. Figure 2C is a typical view of the centriolar region of a resting cell; one microtubule is seen here in cross section.

Resting cells activated by Con A show a progressive

TABLE 2

Effect of Colchicine on ^3H -Thymidine Incorporation into Con A Stimulated Lymphocytes. Colchicine and Con A were added simultaneously.

Cell Category	^3H -Thymidine (CPM)
Resting control	2,838 \pm 830
Con A stimulated	223,379 \pm 14,367
Con A stimulated + 10^{-6}M colchicine	36,423 \pm 946

Fig. 1A-F: Phase contrast photomicrographs of living mouse splenocytes at various stages of blast transformation after stimulation with 2 ug/mL Con A. A: 0 h B: 12 h C: 24 h D: 36 h E,F: 48 h. Note the increase in volume of cytoplasm and nucleus, the appearance of a nucleolus (D,E) and the eventual mitosis (F). X 2,500

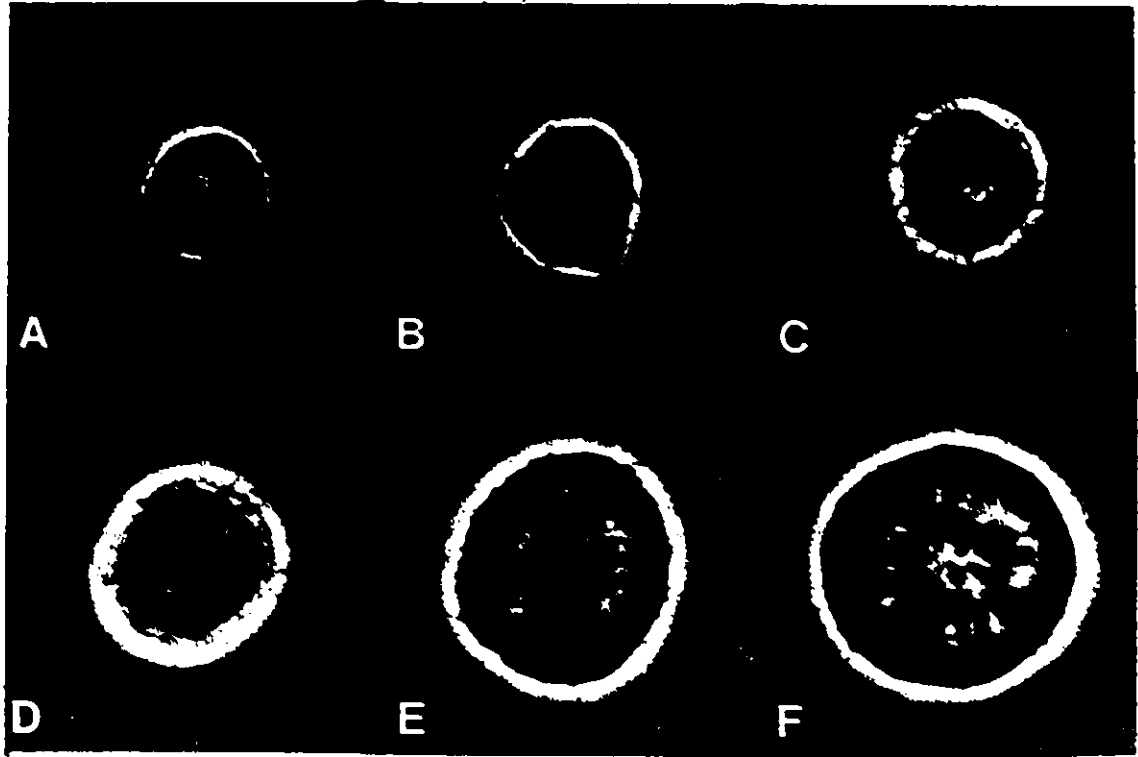


Fig. 2A-D: Microscopy of untreated resting mouse lymphocytes.

A: Phase contrast of four resting cells. The nucleus occupies most of the cell volume. X 2,000.

B: Electron micrograph showing the condensed nucleus and the few organelles in the centriolar region of a resting cell. X 28,000.

C: High magnification view of the centriolar region showing a single microtubule in cross-section. X 53,000.

D: Immunofluorescent staining showing the overall view of the microtubule network radiating from the centriolar region. X 2,200.

The distribution of microtubules in large populations of resting lymphocytes would be difficult to determine by electron microscopy (C). Such is not the case with immunolabelling of microtubules in these cells (D).

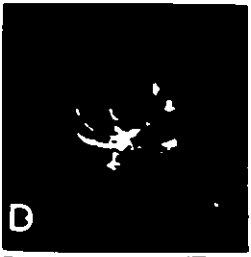
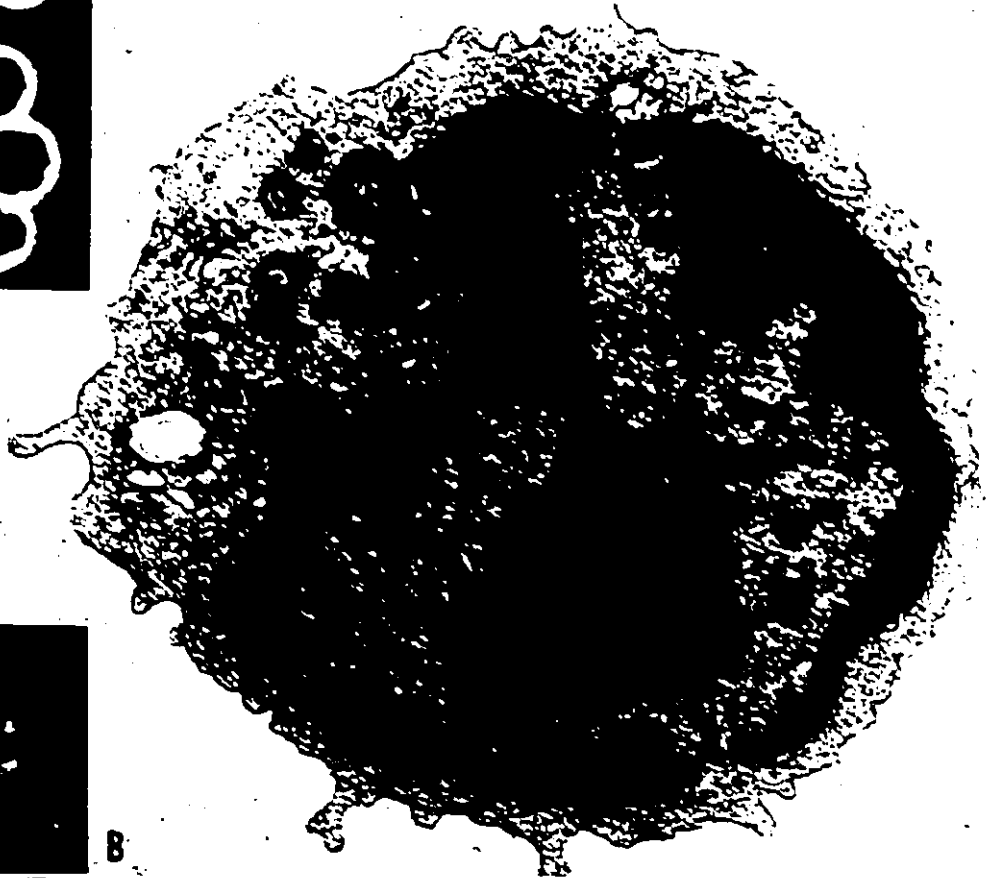
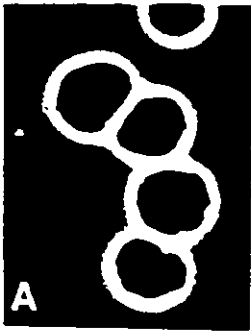


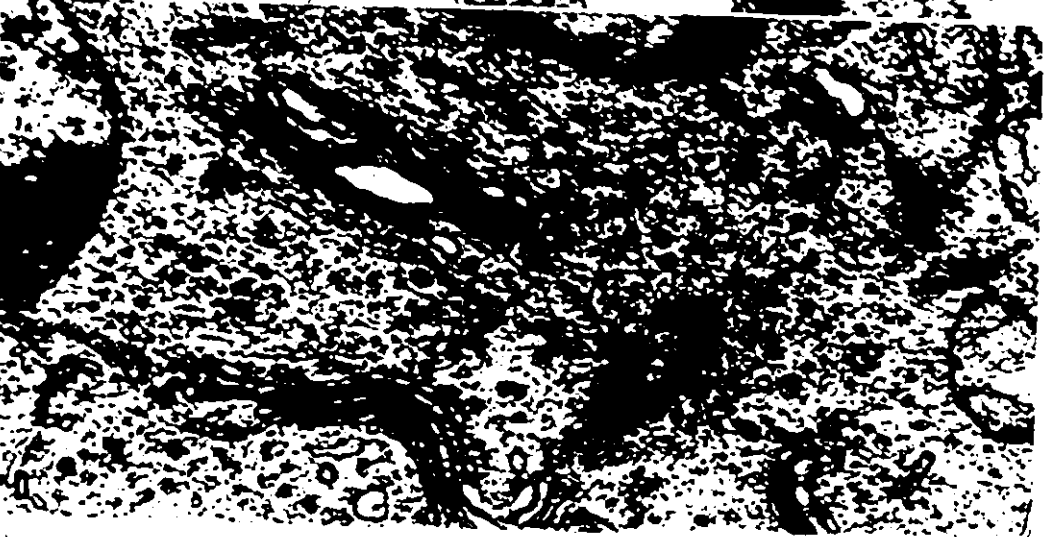
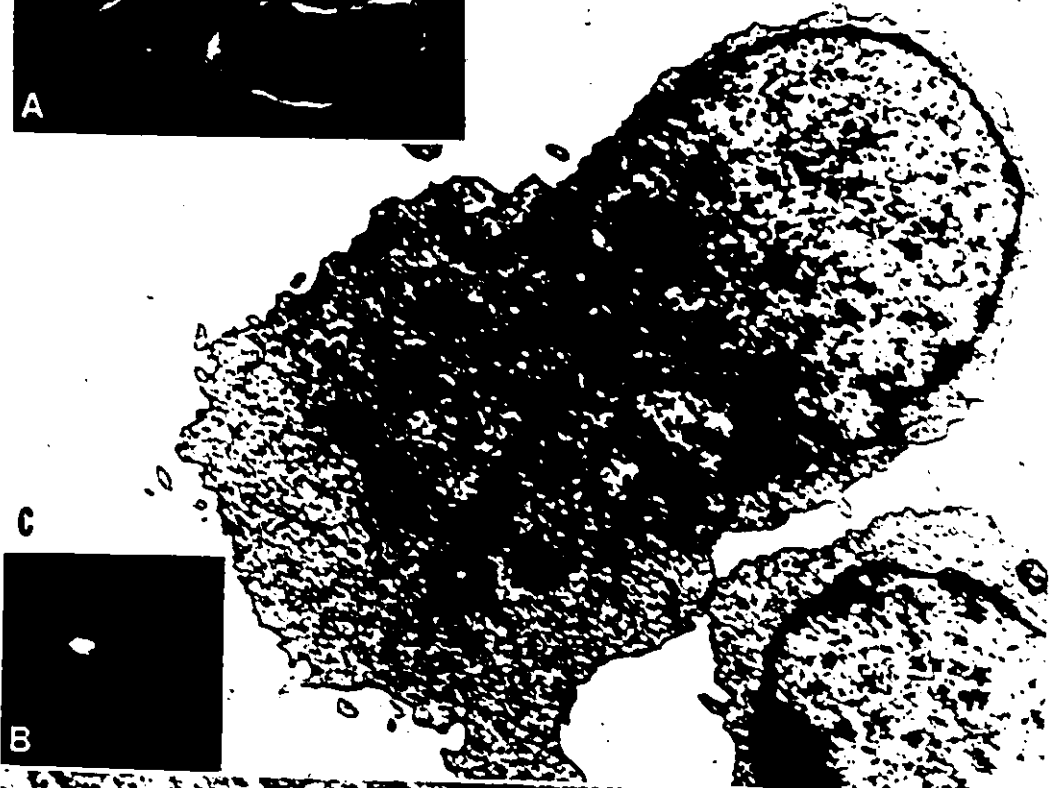
Fig. 3A-D: Microscopy of mouse lymphocytes stimulated by a 48 h exposure to 2 ug/mL Con A.

A: Phase contrast micrograph of stimulated cells at the same mag. as Fig. 1A. The cell volume has increased several fold. X 2,200.

B: Immunofluorescent staining showing the overall view of microtubules radiating from the centriolar region. X 2,200.

C: Electron micrograph showing the decondensed nucleus and the increased numbers of organelles in the centriolar region of a stimulated cell. X 9,800.

D: High magnification view of the centriolar region showing the large increase in numbers of microtubules in the stimulated cell. X 40,000.



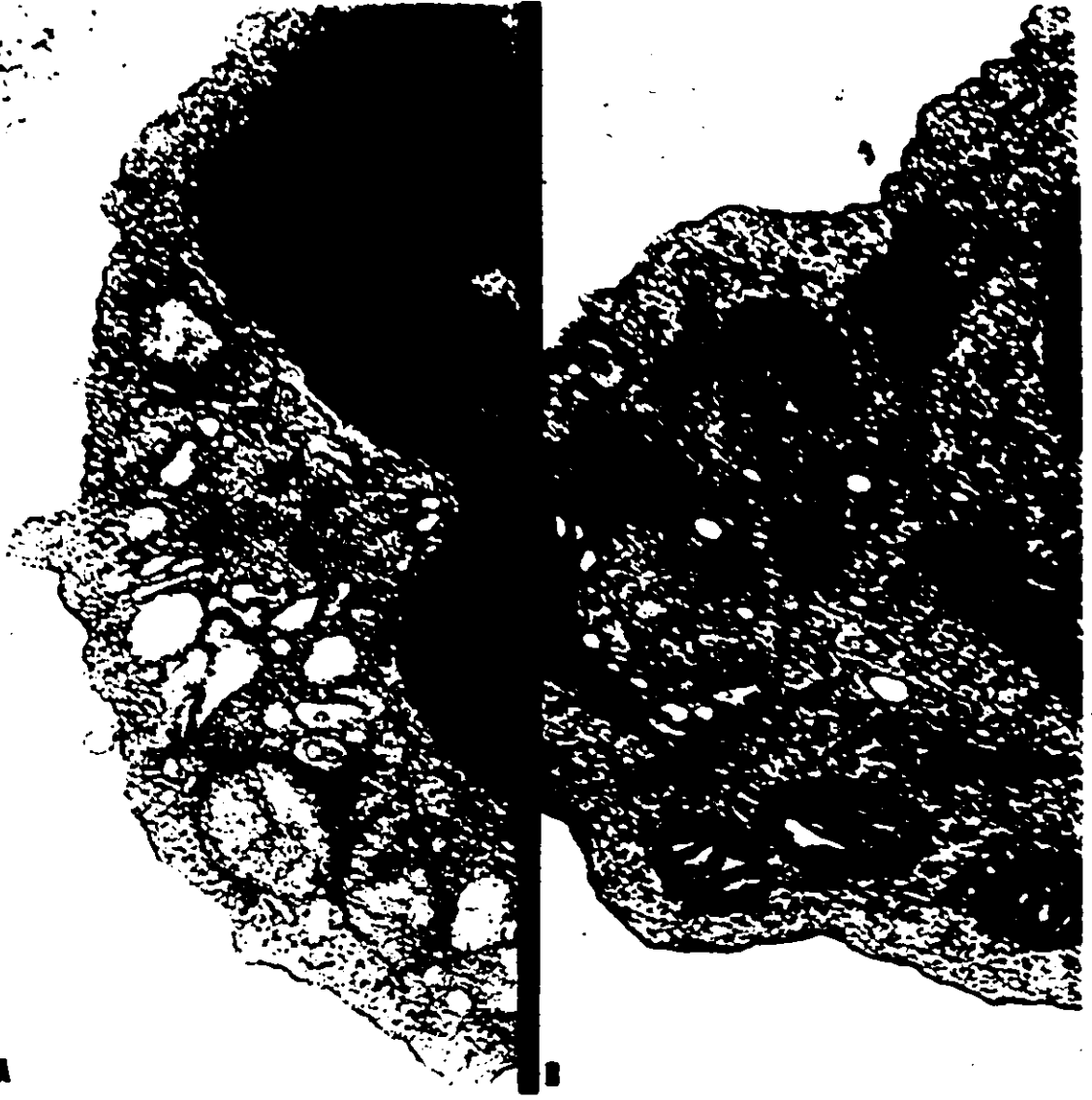
increase in the number of microtubules during blast transformation. Figure 4A-E shows comparable sections through the centriolar region of stimulated cells fixed at various times of culture. The increase can be detected within the first 12 h (Fig. 4B), and the numbers of microtubules continues to grow (Fig. 4C,D) until the blast stage is reached (Figs. 3D, 4E). At later times (Fig. 4D,E) of culture, the numbers of amorphous electron dense structures called satellite bodies also increases. The microtubules appear to originate from these structures (Fig. 4E).

IMMUNOFLUORESCENT DETECTION OF MICROTUBULE NETWORKS IN RESTING AND STIMULATED LYMPHOCYTES

The difficulty in detecting microtubule networks in large populations of resting lymphocytes by electron microscopy was circumvented by developing an immunofluorescent technique to specifically label tubulin containing structures in these cells.

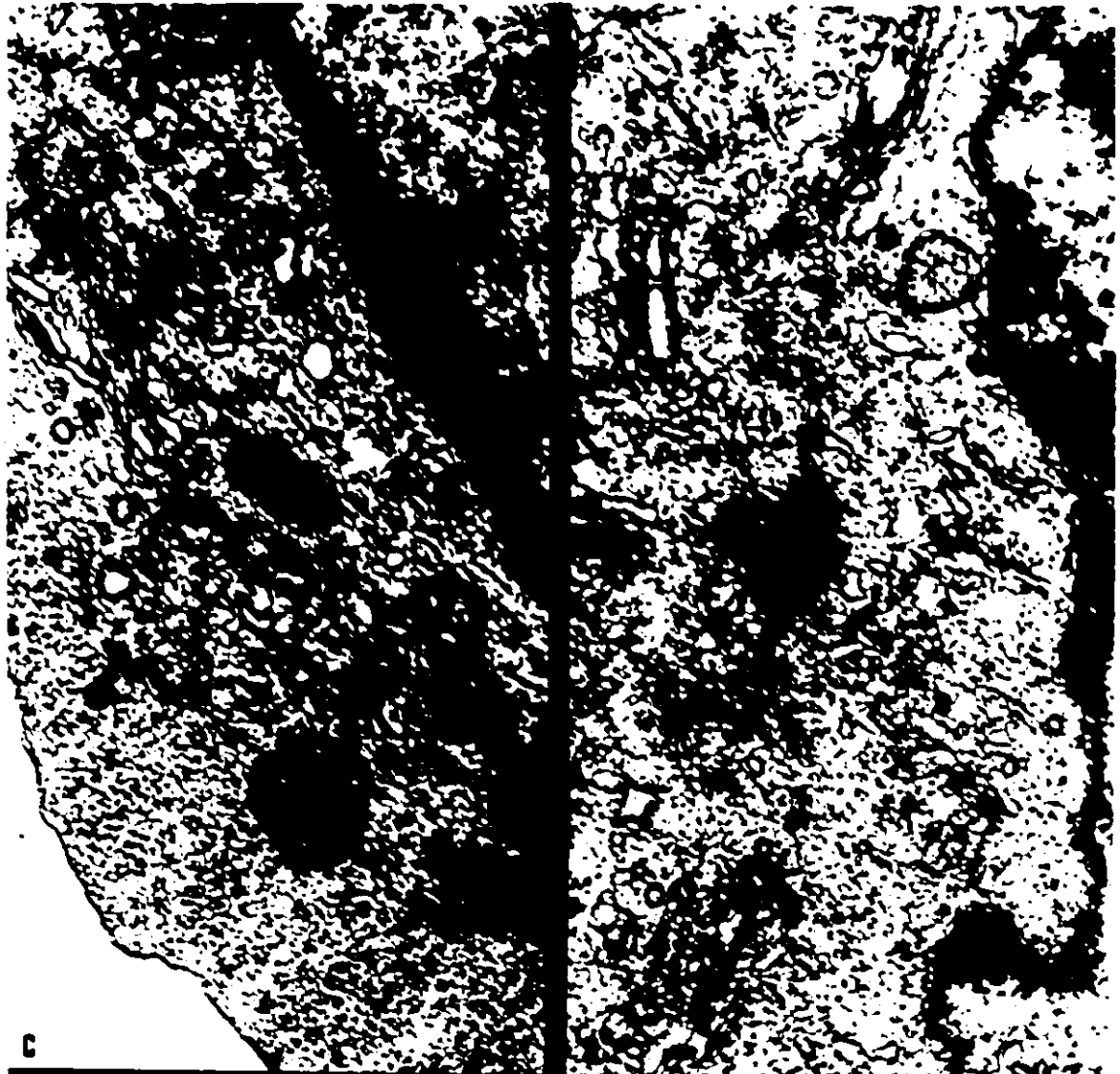
Classical immunofluorescent techniques of fixation and labelling, utilizing the fixatives methanol, acetone and paraformaldehyde in a variety of combinations, proved inadequate in maintaining intact microtubule networks. Of the techniques attempted, only two were satisfactory. The first is a modified version of the recently developed fixation method of Osborn, Webster and Weber (1978). The cells are plated onto poly-L-lysine coverslips and ex-

Fig. 4A-E: Electron micrographs of sections taken through the centriolar region of mouse splenocytes exposed to 2 ug/mL Con A, and fixed at various times of culture.
A: 0 h B: 12 h C: 24 h D: 36 h E: 48 h
Note the increased number of microtubules (B-E) and concomitant increase in the number of satellite bodies (D,E). X 4,500.



A

B



tracted with Triton X-100 in stabilization buffer-1 (SB-1). The buffer stabilizes the microtubule network during the detergent extraction of the soluble cytoplasm. The resultant "cytoskeletons" are fixed with glutaraldehyde and the free aldehyde groups reduced with sodium borohydride to prevent non-specific binding of the antibodies.

This procedure allows us to visualize the microtubules in over 85% of a population of resting lymphocytes. The cells appear to have between 5 and 15 stained fibers converging on the centriolar region of the cell (Fig. 2D; Fig. 5A,B,C). Photographic detection of the microtubules is difficult because of the cell size and shape. These small (5-7 μm) round cells must be examined using oil immersion, which limits the depth of field to less than $1/3$ the diameter of the cell, making it necessary to focus up and down through the cell to see the complete microtubule network.

The fixation method described above cannot be used to stain microtubules in 48 h Con A blasts because the general morphology of the cells is poorly maintained by the SB-1 of Osborn, Webster and Weber (1978). A second fixation procedure was developed which involves the use of paraformaldehyde in a short prefixation step to maintain intact microtubules during the time the cell cytoplasm is extracted with Triton X-100 in the microtubule stabilization buffer, SB-2 (Bershadsky *et al.*, 1979). The cytoskeletons are fixed a second time with glutaraldehyde, reduced with

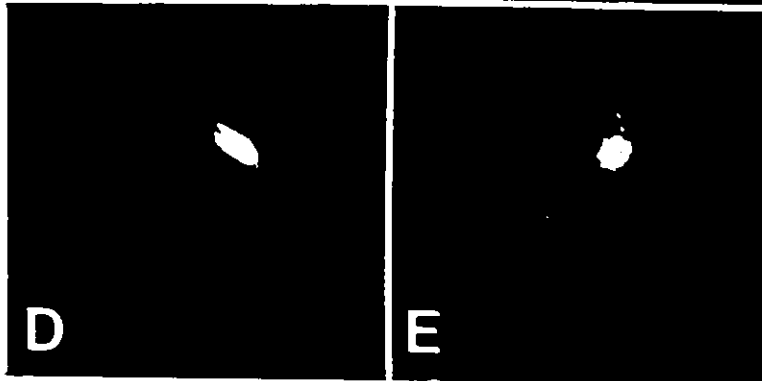
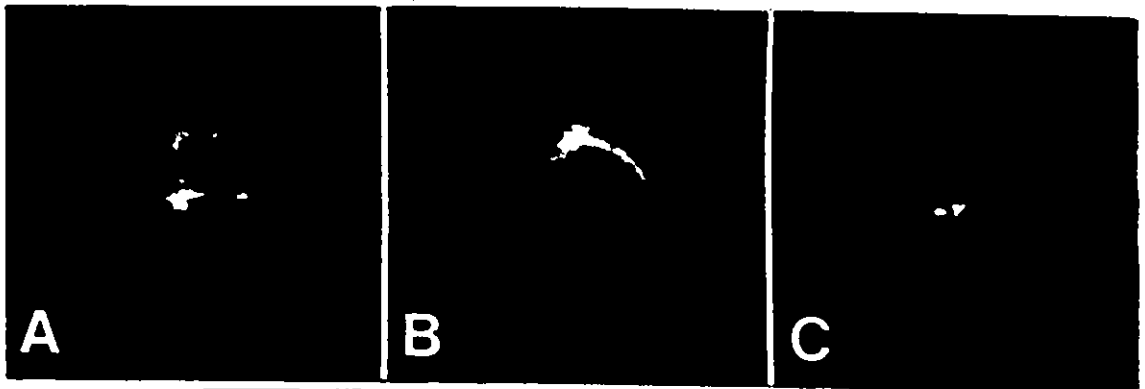
Fig. 5A-H: Immunofluorescent staining of microtubule networks of control and treated resting mouse lymphocytes. X 4,500.

A-C: Representative views of stained microtubule networks in control cells.

D: Cells treated for 45 min at 4°C. The microtubules of most cells have disassembled, leaving a brightly staining centriolar region.

E: Cells treated for 8 h with 10^{-6} M colchicine, which disassembles the microtubules in most cells.

F-H: Examples of cells treated for 30 min (F) and 60 min (G) with 10^{-6} M colchicine or (H) 30 min with 10^{-4} M colchicine. All show staining of microtubule networks.



sodium borohydride, and stained in the usual manner. Initial results gave very high background fluorescence. This was reduced by incubating the fluorescein-conjugated goat anti-rabbit with mouse spleen powder, which effectively removes any direct binding of the conjugate to non-tubulin containing structures.

This procedure permits the visualization of the large number of microtubules originating at the centriolar region of a blast cell as a bright diffuse fluorescence, with some fibers being distinguished (Fig. 3D; Fig. 6A). The microtubule networks of blasts in various stages of mitosis can also be detected (Fig. 6B-I). In Fig. 6C, fibers are clearly seen running from centriole pair to centriole pair. In Fig. 6H and I the nuclear envelope has reappeared, and a cytoplasmic bridge with unstained mid-body is all that remains of the cleavage furrow. Cytoplasmic microtubules are again visible in Fig. 6I. Although individual microtubules are not clearly seen in these preparations, they can be easily and accurately detected in blasts by electron microscopy.

EFFECT OF LOW TEMPERATURE AND COLCHICINE ON MICROTUBULES

In order to determine the relationship between microtubule networks and the patching and capping of surface IgG, it was important to establish the sensitivity of lymphocyte microtubules to low temperature and the microtubule disrupting drug colchicine.

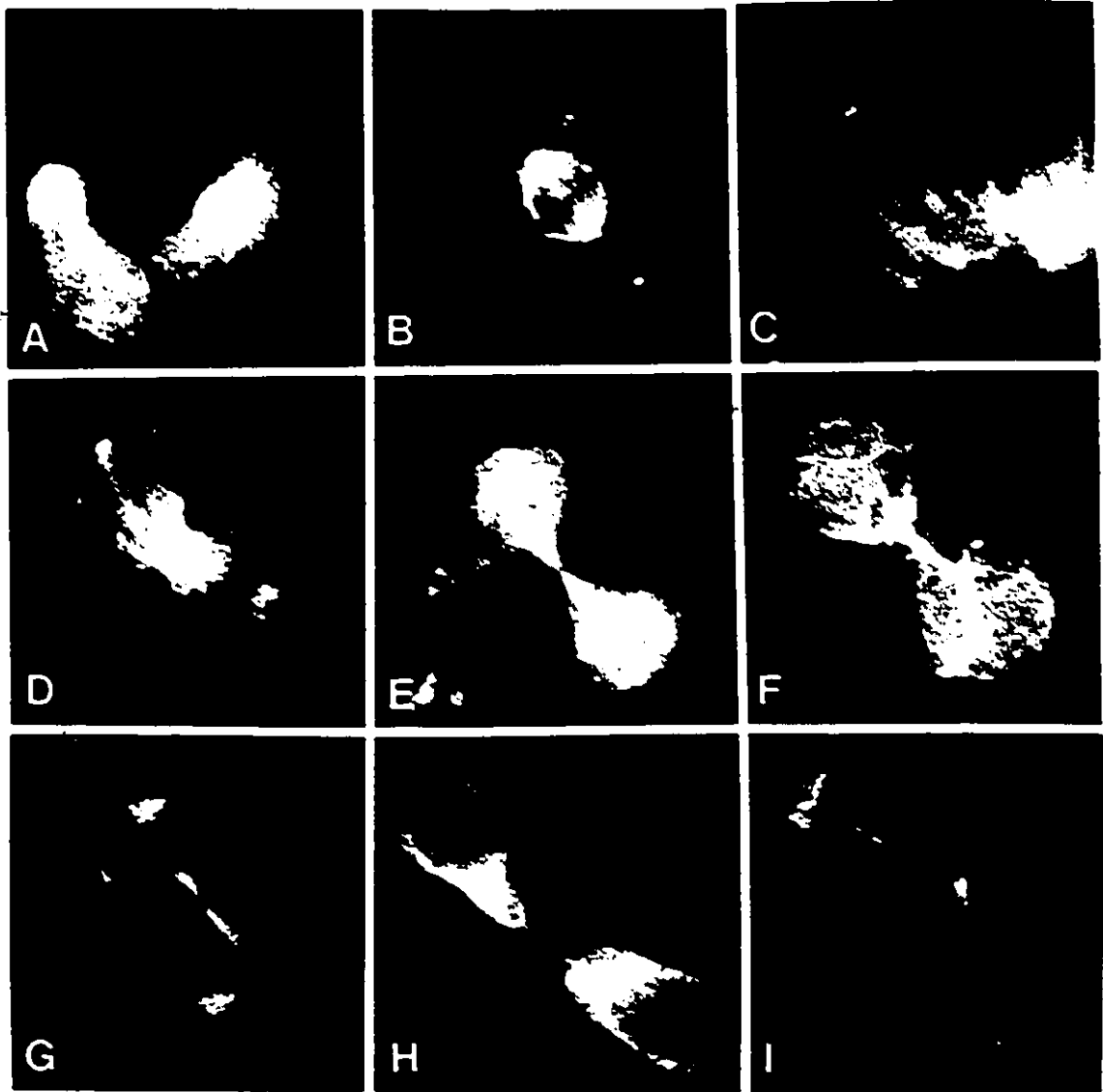
Fig. 6A-I: Immunofluorescent staining of microtubule networks of 48 h Con A stimulated mouse lymphocytes. X 2,500.

A: Blast cell showing intense fluorescence in the region of the centrioles.

B-G: Blast cells in various stages of mitosis.

H,I: Blast cells have completed mitosis, leaving a strongly staining cytoplasmic bridge and unstained mid-body.

In several of these micrographs, microtubules or bundles of microtubules are clearly seen.



1: Low temperature effects:

Experiments designed to study the relationship between capping and the cytoskeleton often utilize a protocol which involves incubating the cells with a specific ligand at 4°C for 30 min, and then raising the temperature to 20°C or 37°C to allow the surface receptors to migrate (Unanue, Perkins and Karnovsky, 1972; Schreiner and Unanue, 1976a; Albertini and Anderson, 1977; Bourgeois, Tokuyosu and Singer, 1978). It is well established that exposure of the microtubules to low temperature causes them to disassemble.

Cells were incubated for various periods of time at 4°C, and processed for immunofluorescent staining of microtubules (Fig. 7). Results are expressed as a percentage of cells containing detectable microtubule networks. The disassembly of the microtubules is rapid, with over 50% of the cells lacking detectable microtubules after a 15 min exposure. Over 70% of the population of cells lose their microtubules during a 30 min cold incubation and maximal disassembly requires 45 min (Fig. 5D). Even after long exposures, up to 20% of the population contains stained structures resembling microtubules.

To determine the rate at which cells recover their microtubules after cold depolymerization, cells exposed to 4°C for 45 min were incubated at 37°C for various periods of time (Fig. 8). Within the first 2 min, 50% of the cells recover their microtubule networks, and only

Fig. 7: Rate of microtubule disassembly at low temperatures. Resting mouse lymphocytes were exposed to 4°C for various periods of time, plated onto coverslips at 4°C for the last 5 min of the incubation period, and processed for immunofluorescent staining of microtubules. Results are expressed as a percentage of control cells which were incubated for 90 min at 37°C before staining.

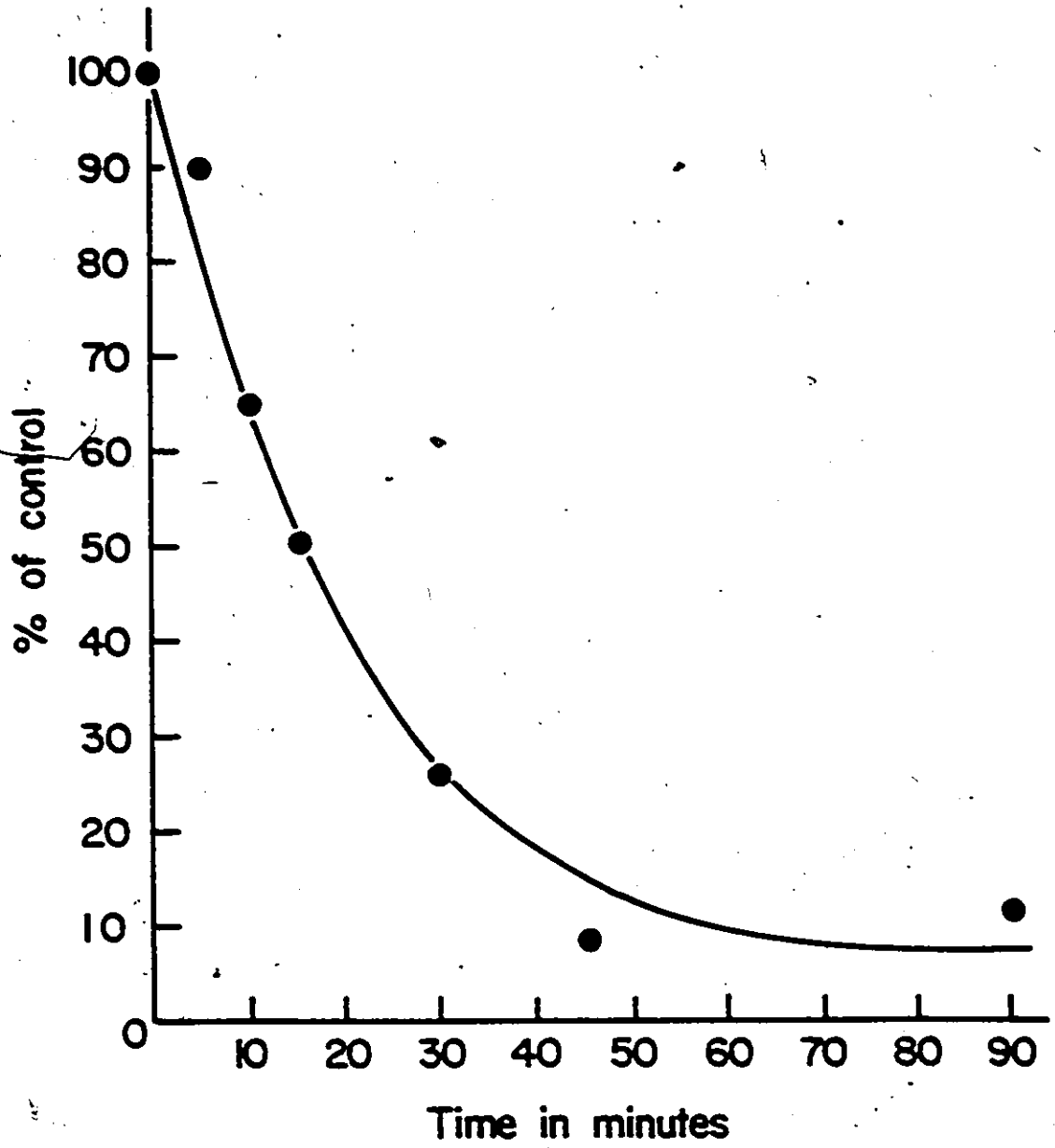
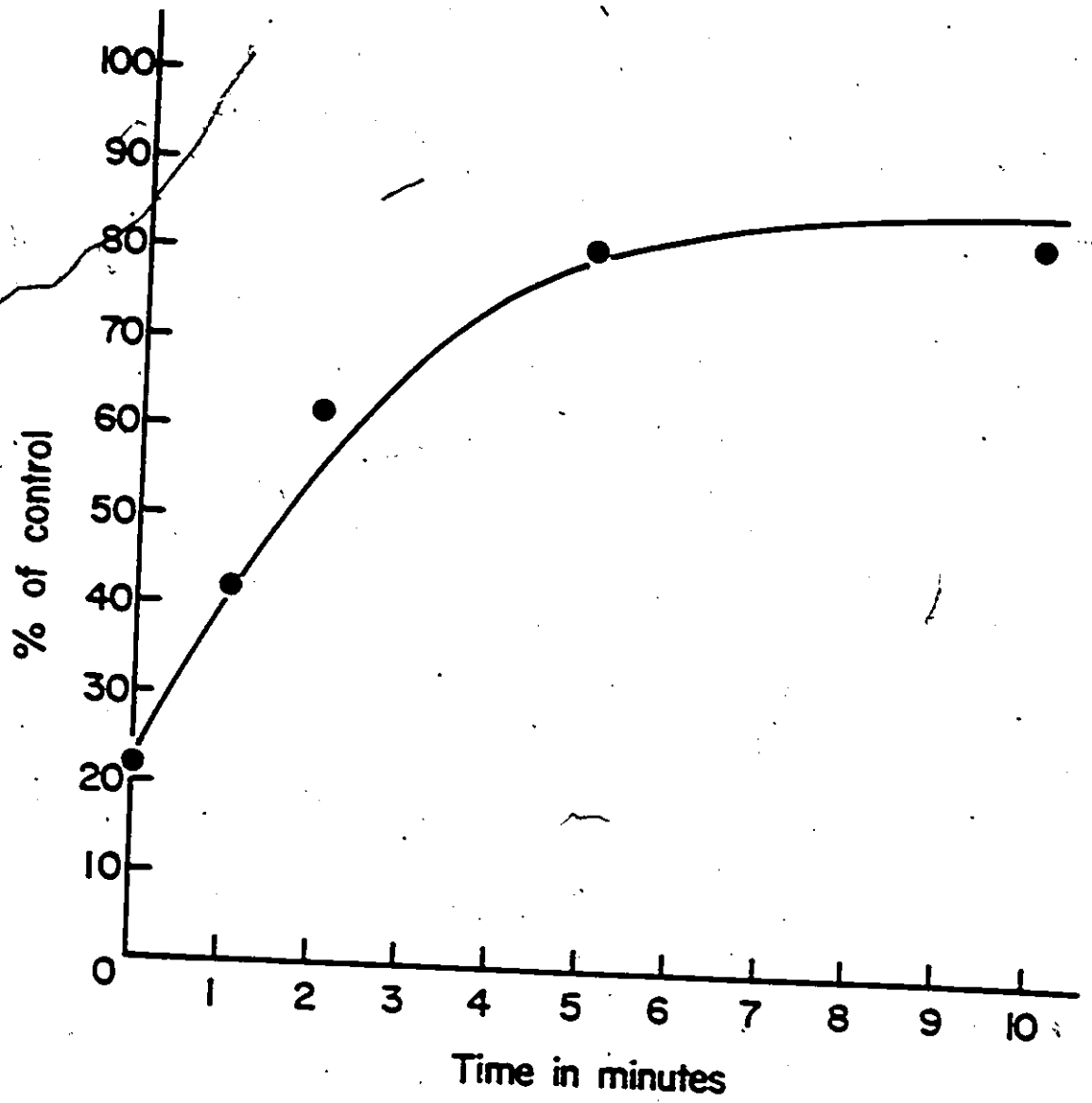


Fig. 8: Rate of reassembly of microtubule networks of cold treated cells. Resting mouse splenocytes were incubated at 4°C for 45 min, and plated onto coverslips for 5 min at 4°C . The cells were then incubated at 37°C for varying times, and processed for immunofluorescent staining of microtubules. Results are expressed as percent of control cells which were not exposed to cold treatment.



5 min is required for maximal recovery.

2: Colchicine effects:

Experiments were carried out to determine the concentration of colchicine which would optimally disassemble microtubules, and to determine the time course of disassembly by the drug.

The disassembly of microtubules by colchicine is a process which is both time- and concentration-dependant, with cells showing a marked variation in their sensitivity to the drug. 50% of mouse splenocytes exposed to colchicine for 60 min still have detectable microtubule networks (Fig. 9). Concentrations of colchicine below 10^{-6} M do not have any effect during this time period, and higher concentrations do not increase the rate of depolymerization (Fig. 10). The microtubule networks of human peripheral blood lymphocytes show a similar sensitivity to the drug (Fig. 11).

It is clear that the exposure of resting lymphocytes to high concentrations of colchicine for short periods of time is not sufficient to disassemble the microtubules in all cells (Fig. 5F,G,H). In fact, exposures of 6-8 h are required to disassemble microtubules in over 90% of the cells. Even after this time period, microtubules are detected in up to 10% of the population (Fig. 12). During these long exposures, concentrations as low as 10^{-8} M colchicine have significant effects on microtubule

Fig. 9: Percentage of mouse splenocytes with microtubule networks after a 60 min exposure to increasing concentrations of colchicine. After the treatment cells were plated onto coverslips and processed for immunofluorescent staining of microtubules. Over 85% of control cells show microtubule networks. At concentrations of 10^{-7} M or less, colchicine has no effect during this incubation period.

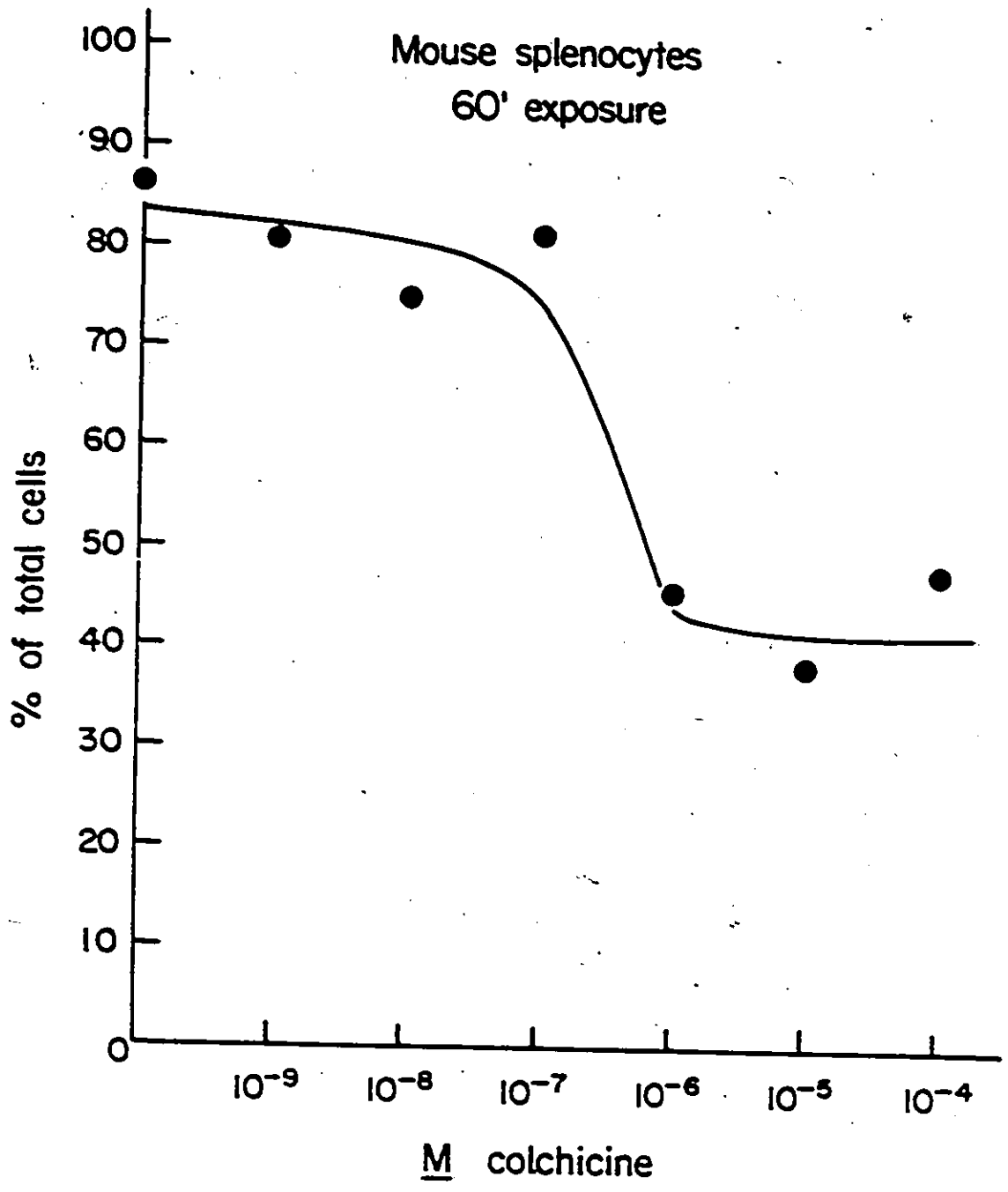


Fig. 10: Rate of disassembly of microtubule networks in resting mouse splenocytes exposed to one of three concentrations of colchicine (10^{-6} M Δ , 10^{-5} M \circ , 10^{-4} M \square) for varying periods of time at 37°C . The cells were then plated onto coverslips and processed for immunofluorescent staining of microtubules. After a 60 min exposure to colchicine, 40% of the population still have intact microtubule networks.

Mouse splenocytes

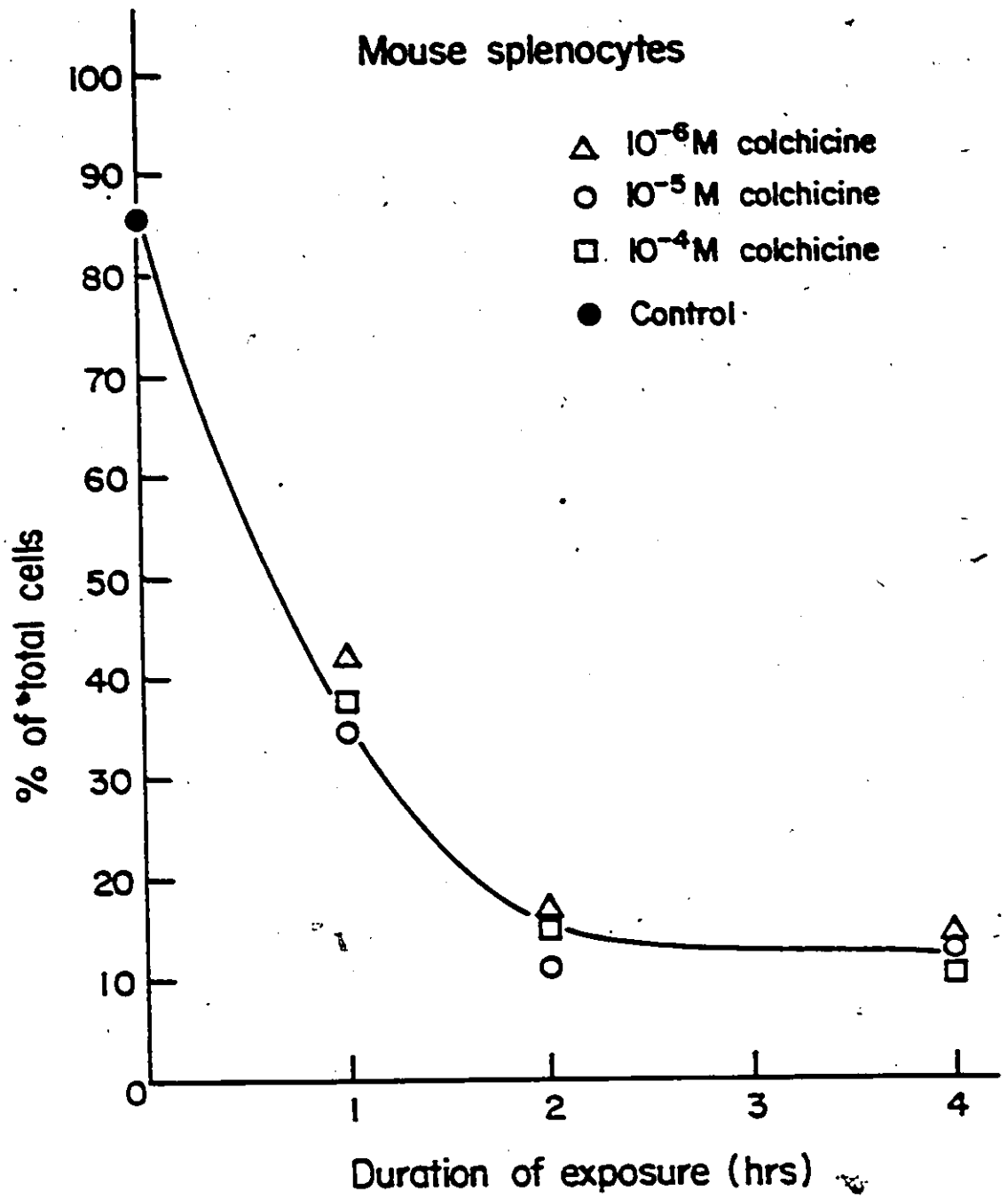


Fig. 11: Rate of disassembly of microtubule networks in resting human peripheral blood lymphocytes exposed to one of three concentrations of colchicine (10^{-6} M Δ , 10^{-5} M \circ , 10^{-4} M \square) for varying periods of time at 37°C . The cells were then plated onto coverslips, fixed once in paraformaldehyde, and processed for immunofluorescent staining of microtubules. After 60 min exposure to colchicine, over 50% of the population still have intact microtubule networks.

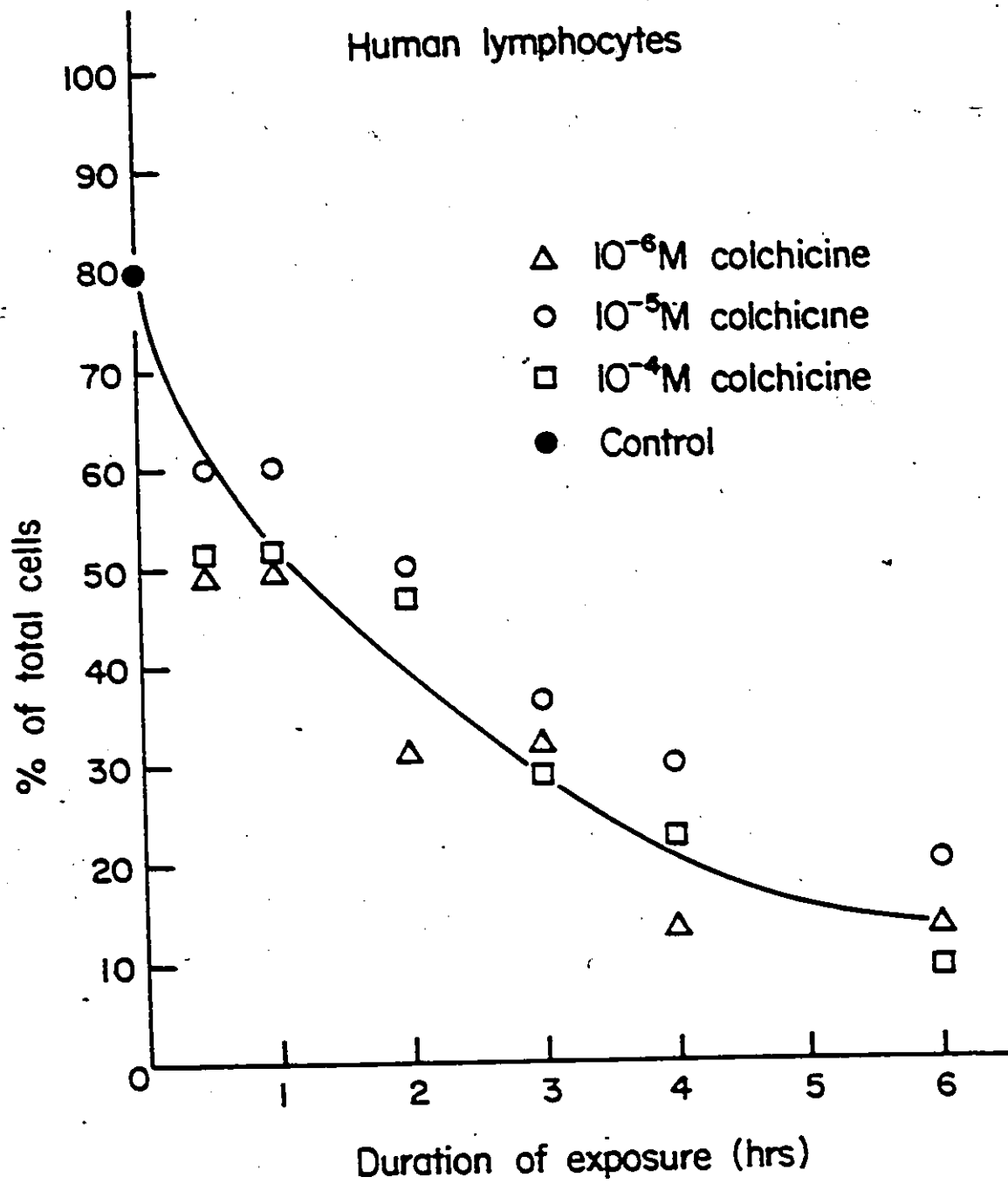
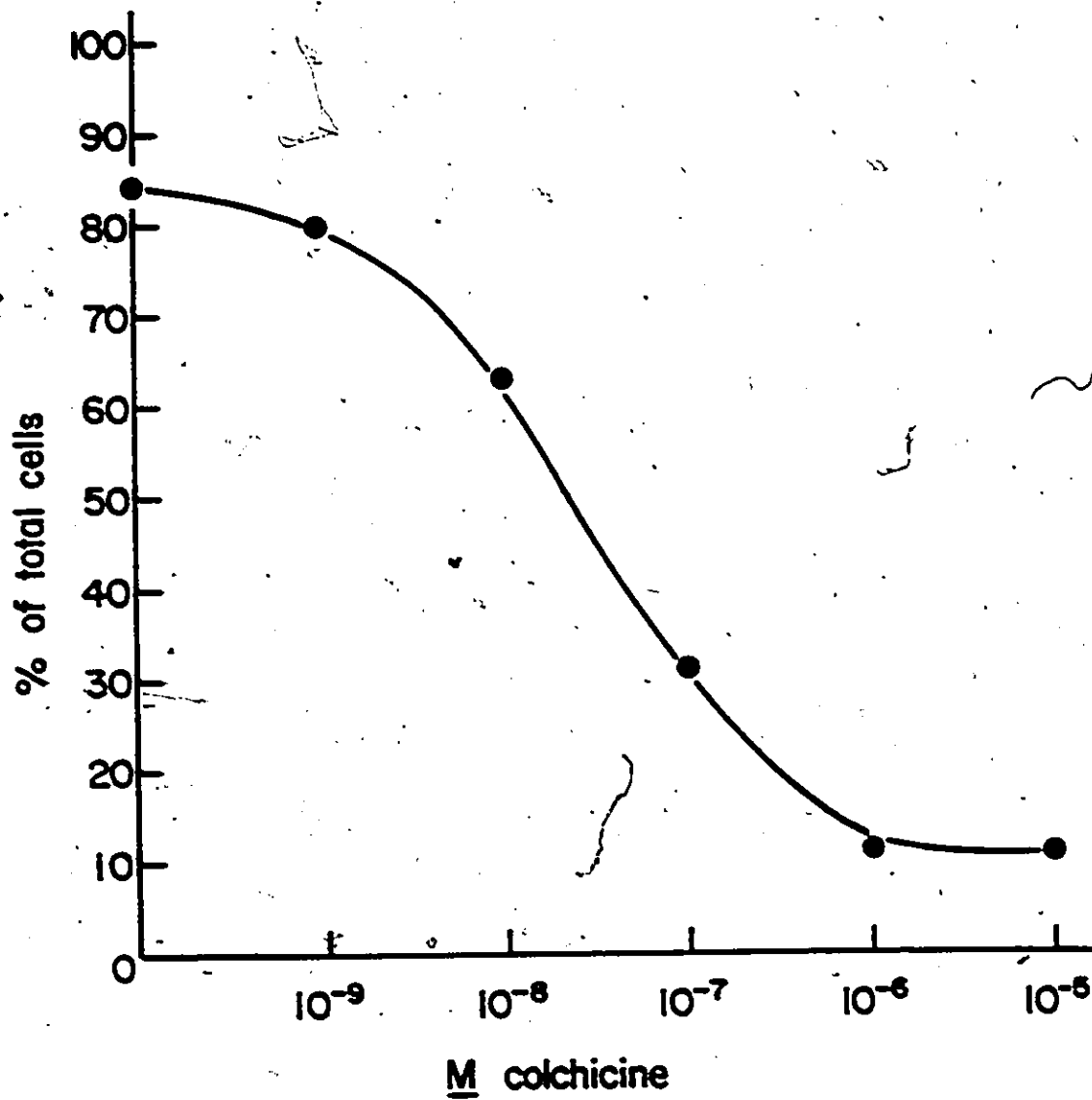


Fig. 12: Percentage of mouse splenocytes with microtubule networks after an 8 h exposure to increasing concentrations of colchicine. After the treatment cells were plated onto coverslips and processed for immunofluorescent staining of microtubules.



networks (Fig. 12).

48 h Con A blasts were exposed to 10^{-6} M colchicine, harvested at various times of culture, and prepared for examination by electron microscopy. The microtubules in these cells have a similar sensitivity to colchicine as do the resting cells. Microtubules are still detected in sections through the centriolar regions of blast cells treated with the drug for 30 to 60 min (Fig. 13A,B). Longer exposures leads to disassembly of all the microtubules.

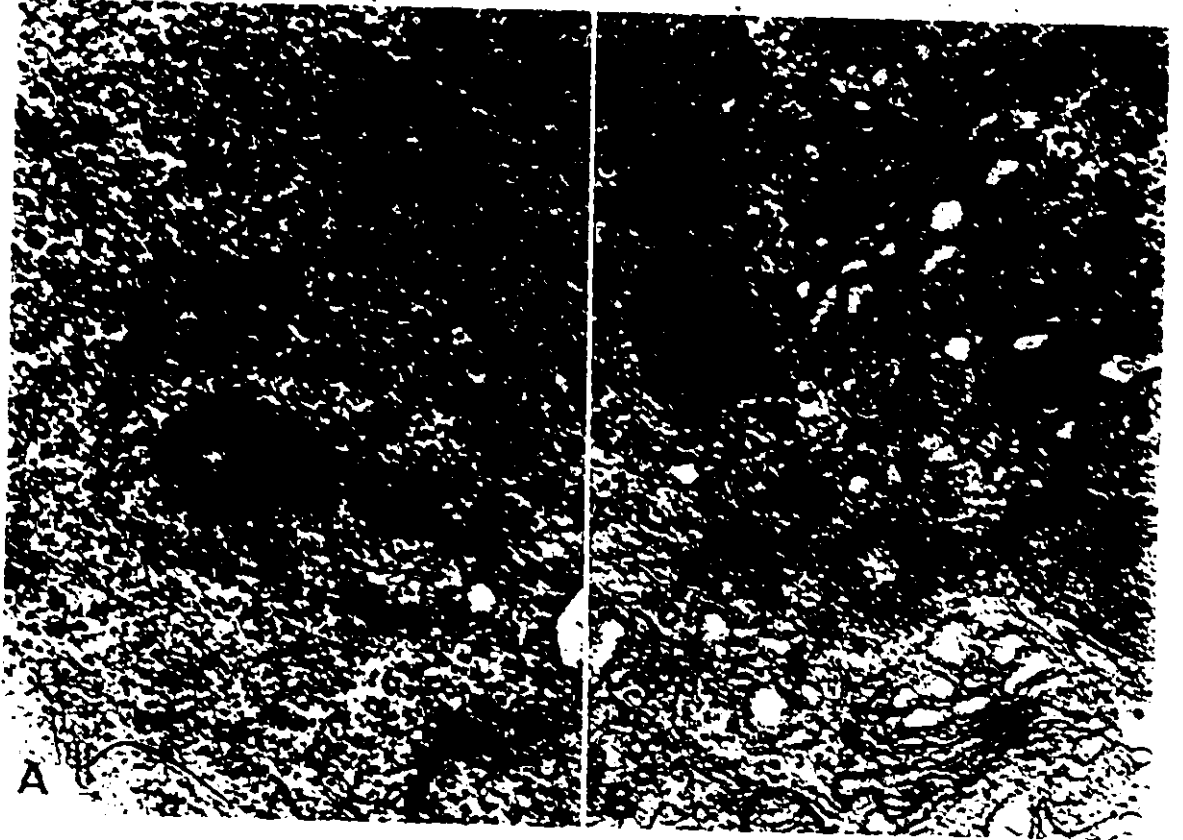
IMMUNOFLUORESCENT DETECTION OF SURFACE IgG AND MICROTUBULES IN THE SAME CELL

When resting lymphocytes are exposed to anti-immunoglobulin (anti-Ig) the anti-Ig antibody binds to surface receptors, and this antibody-receptor complex clusters to form patches which subsequently migrate to one pole of the cell to form a cap. The capped complexes are then quickly internalized.

To determine the effect of IgG capping on lymphocyte microtubule networks, a double labelling technique was employed which involved labelling the surface receptors with rhodamine goat-anti-mouse (or human) IgG (R-GaMIgG), prefixation in paraformaldehyde and processing for fluorescein immunolabelling of microtubules in the same cells. Comparison of surface labelled cells before and after extraction showed no difference in intensity or

Fig. 13A,B: Electron micrographs of 48 h Con A stimulated lymphocytes treated with 10^{-6} M colchicine for 30 min (A) or 60 min (B). Note that the microtubules are still evident. X 52,000.

2



4

location of fluorescence.

The time course of patching, capping and internalization of R-GaMIgG by mouse splenocytes is shown in figure 14. Patching is rapidly followed by capping and internalization of the label. A cell has capped its receptor-ligand complexes when it has cleared over half its surface of the rhodamine label. From figure 14 we see that 7 min is the time point at which maximal capping has occurred before the label becomes completely internalized. Cells which have capped R-GaMIgG show typical uropod formation in the region of the cap. They have intact microtubule networks (Fig. 15A-C) converging on the brightly fluorescent centriolar region which is always found on the same half of the cell as the capped R-GaMIgG. Similar experiments with human peripheral lymphocytes gave the same result (Fig. 16B,C).

Two of the sequential stages in the capping of surface receptors, "ring formation" and patching, can be artificially induced. When high concentrations of Con A are added to cells shortly before the addition of R-GaMIgG, IgG receptors are frozen in place, and the R-GaMIgG exhibits a diffuse fluorescence over the cell surface, giving the appearance of a ring in the microscope. As the concentration of Con A is increased, the percentage of cells exhibiting rings increases, until at 200 ug/mL, 80% of the cells are affected (Fig. 17). When cells have been treated in this manner, their microtubule networks remain

Fig. 14: Rate at which resting mouse splenocytes patch, cap and internalize R-GaMIG. Cells were exposed to 200 ug/mL R-GaMIG and processed at various times of culture. They were plated onto coverslips for 60 sec, washed once in PBS, and fixed in paraformaldehyde. Abscissa represents time elapsed between addition of label and fixation in paraformaldehyde. Patched cells ○. Capped internalized cells ●. Capped cells only □. At 7 min, maximal capping has occurred with the least amount of label internalized.

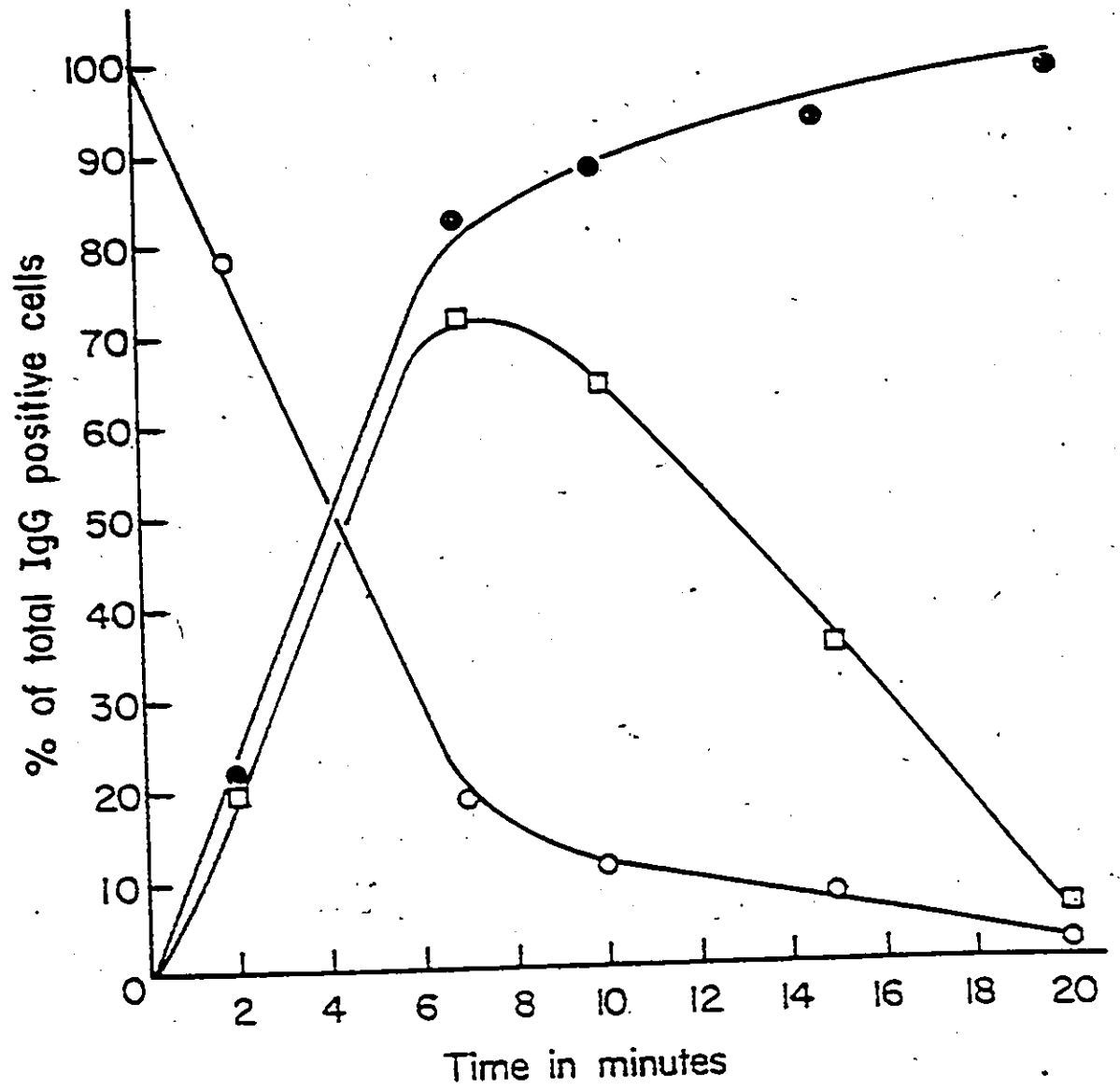


Fig. 15A-C: Immunofluorescent detection of surface IgG and microtubules in the same cell. Resting mouse splenocytes were exposed to 200 ug/mL R-GaMIgG and plated onto coverslips for 7 min at 37°C. They were washed once in PBS, fixed in paraformaldehyde, and processed for immunofluorescent staining of microtubules.

A-C: Phase contrast, R-GaMIgG, and FITC-immunolabelling of microtubules in the same cell. The cap is always located over that half of the cell containing the centriolar region.
X 31,000.

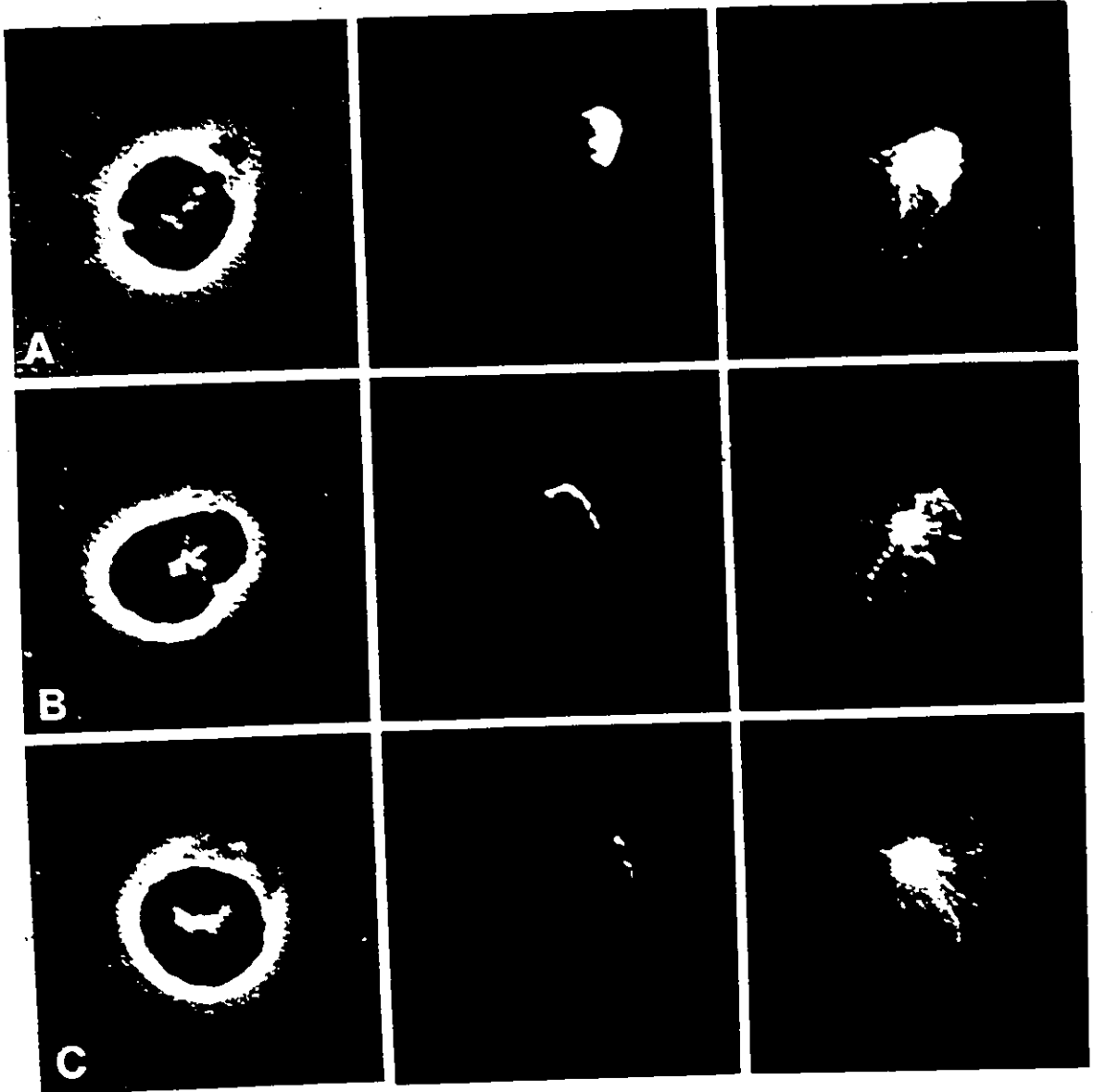


Fig. 16A-C: Immunofluorescent detection of surface IgG and microtubules in the same cell. Resting human lymphocytes were exposed to 200 ug/mL R-GaHIgG for 60 min and then plated onto coverslips for 15 min at 37°C. They were washed once in PBS, fixed in paraformaldehyde, and processed for immunofluorescent staining of microtubules.

A: Phase contrast, R-GaHIgG patches, and Fitc-immunolabelling of microtubules in the same cell.

B: R-GaHIgG cap and Fitc-immunolabelling of microtubules in the same cell.

C: Phase contrast, R-GaHIgG cap and Fitc-immunolabelling of microtubules in the same cell.

In all cases the formation of patches (A) and their aggregation to one pole of the cell to form a cap (B,C) does not appear to dis-aggregate the microtubules. The microtubules in C appear to converge under the cap region. X 3,200.

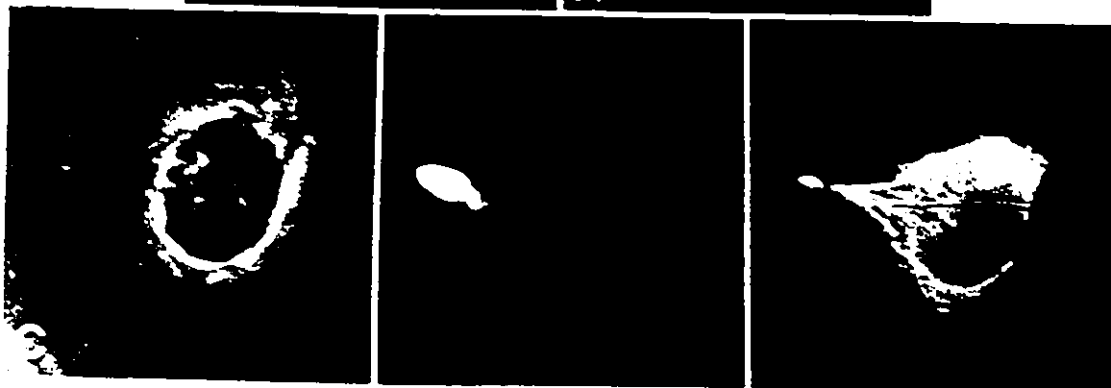
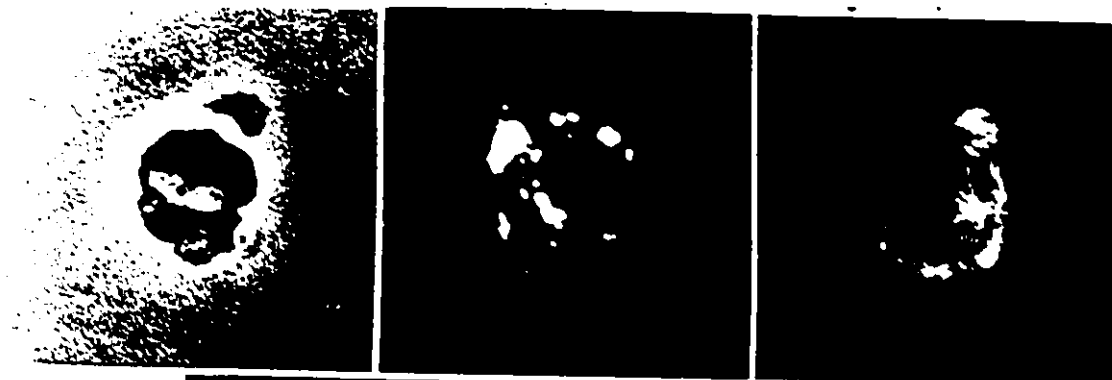
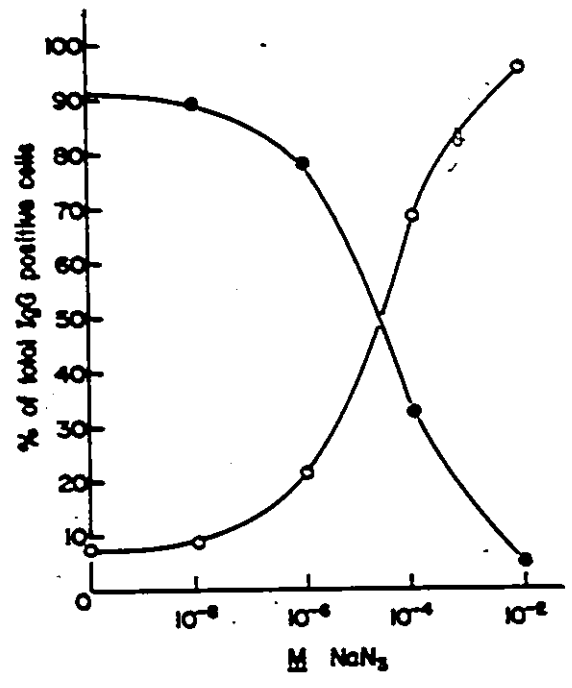
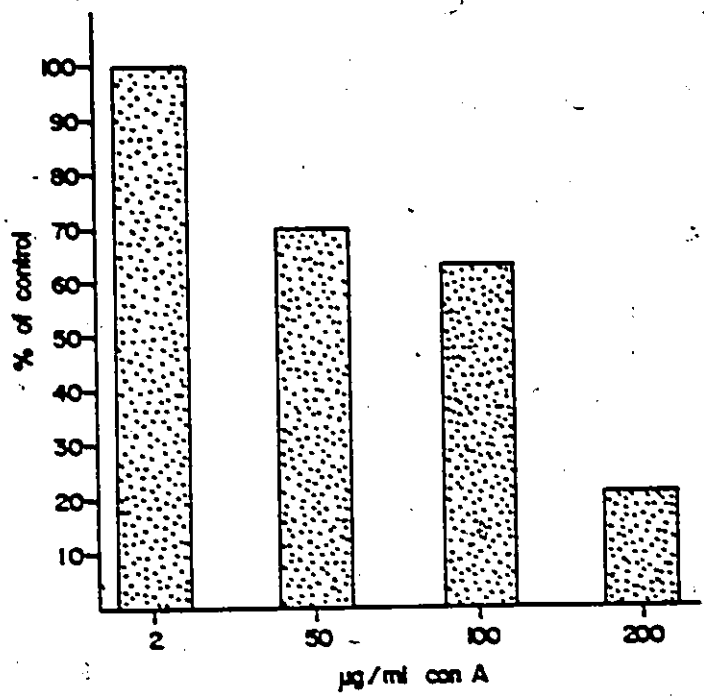


Fig. 17: Effect of increasing concentrations of Con A on the capping of R-GaMIgG. Resting mouse splenocytes were exposed to Con A for 5 min prior to (and during) incubation with 200 ug/mL R-GaMIgG for 10 min at 37°C. IgG positive cells were scored for caps. All other cells showed diffuse fluorescence (rings). Results are expressed as a percentage of control cells not treated with Con A.

Fig. 18: Effect of increasing concentrations of azide on the patching and capping of R-GaMIgG. Resting mouse splenocytes were exposed to the drug for 5 min prior to incubation with 200 ug/mL R-GaMIgG for 10 min at 37°C in the continued presence of the drug. IgG positive cells were scored for patches ○ or caps ●. Maximal inhibition of capping occurred at 10^{-2} M sodium azide. Over 90% of IgG positive cells display patched receptors.



unchanged (Fig. 19B).

Con A inhibition of IgG capping can be released by incubating the cells with colchicine to disassemble microtubules. The cell is then able to patch and cap the R-GaMIgG (Fig. 19C), but the cap is no longer located directly over the centriolar region in all cells.

Sodium azide at a concentration of $10^{-2}M$ is 98% effective in inhibiting the capping of surface IgG (Fig. 18). In the presence of this drug, cells can only patch the receptors. The microtubule networks in patched cells appear to be unchanged (Fig. 16A, 19A).

EFFECT OF COLCHICINE ON R-GaMIgG CAPPING OF SURFACE RECEPTORS

When Con A frozen IgG receptors were released by colchicine, the caps no longer exclusively formed over the region of the centriole. It was important to determine if this result was related to the presence of Con A, or if indeed a relationship exists between the location of the cap and the integrity of the microtubule network.

Cells were exposed to increasing concentrations of colchicine for 8 h and incubated with R-GaMIgG (Table 3). The drug did not significantly affect the percentage of cells capping surface IgG. When the concentration of the drug was sufficient to depolymerize microtubules (see Fig. 12), there was no longer an association of the cap with the centriolar region. This result is expressed as

Fig. 19A-C: Immunofluorescent detection of surface IgG and microtubules in the same cell. Resting mouse lymphocytes were treated with: A) 10^{-2} M sodium azide for 5 min; B) 200 ug/mL Con A for 5 min; C) 10^{-4} M colchicine for 30 min prior to exposure to 200 ug/mL Con A for 5 min. They were then incubated with 200 ug/mL R-GaMIgG and plated onto coverslips for 7 min at 37°C. The cells were washed once in PBS, fixed in paraformaldehyde, and processed for immunofluorescent staining of microtubules.

A: Phase contrast, R-GaMIgG patches, and FITC immunolabelling of microtubules. The microtubule network is still intact.

B: Phase contrast, R-GaMIgG ring, and FITC immunolabelling of microtubules. The microtubule network is still intact.

C: Phase contrast, R-GaMIgG cap, and FITC immunolabelling of microtubules. The microtubules have been disassembled. Caps formed after colchicine treatment are randomly located on the cell surface with respect to the centriolar region. X 3,000.

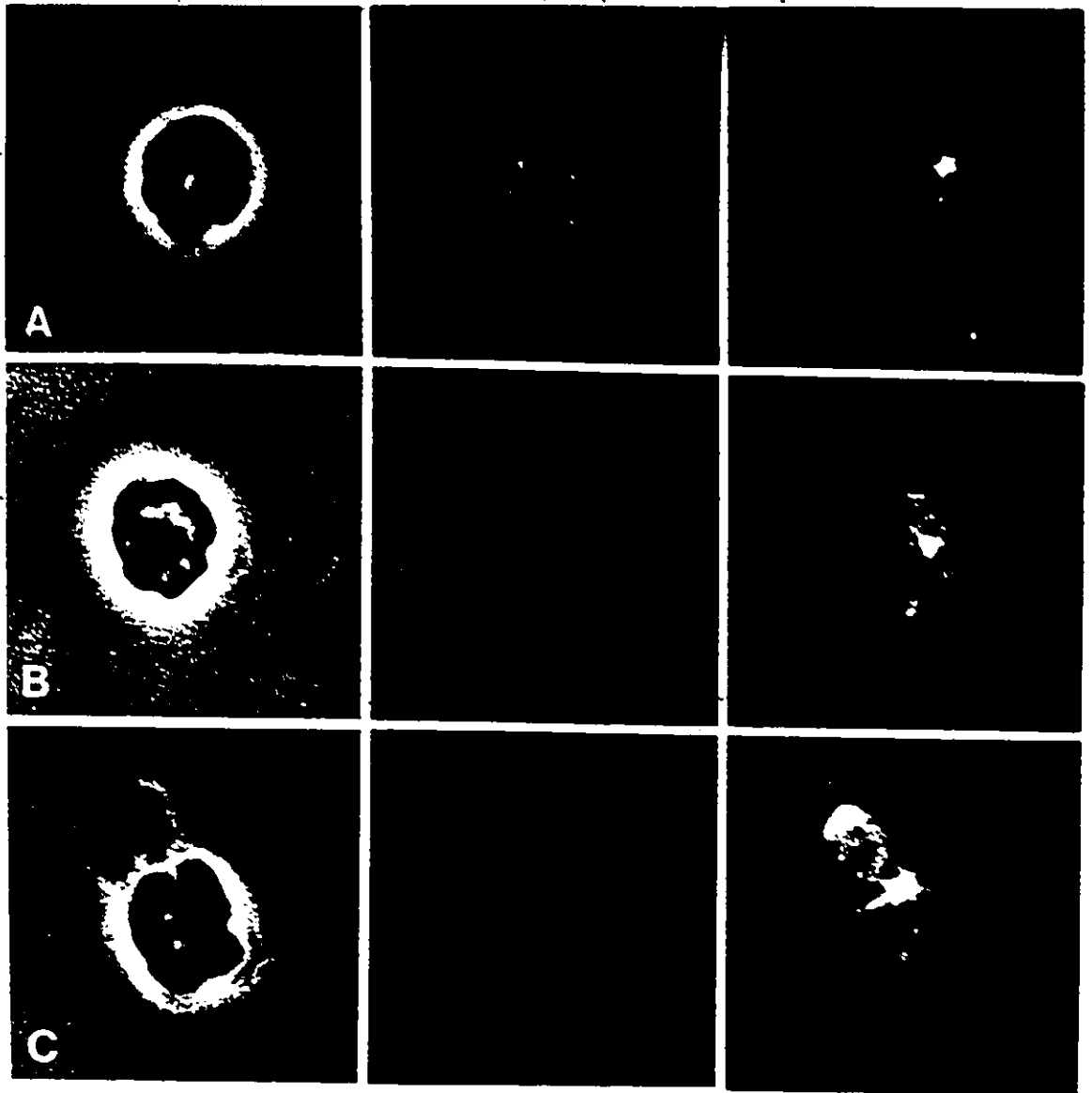


Table 3: Effect of colchicine on capping of R-GaMIgG. Resting mouse splenocytes were treated with increasing concentrations of colchicine for 8 h at 37°C, followed by a 7 min incubation with 200 ug/mL R-GaMIgG at 37°C. The cells were then processed for immunofluorescent staining of microtubules. Cells were scored for number of IgG positive cells with caps, and % of capped cells with the centriole located on the same half of the cell as the cap.

Colchicine <u>M</u>	% IgG positive cells capped	% cells with cen- triolo under cap
0	80	95
10 ⁻⁹	72	98
10 ⁻⁸	80	92
10 ⁻⁷	75	63
10 ⁻⁶	94	56
10 ⁻⁵	84	68
10 ⁻⁴	81	70

the percentage of capped cells with the centrioles located in the same half of the cell as the rhodamine label. Cells in colchicine treated preparations often cap the surface IgG at a point opposite to the centrioles (Fig. 20A-F). They also stain brightly with anti-tubulin in the region of the cap (Fig. 20F). Preabsorption of anti-tubulin with purified tubulin did not reduce the intensity of this fluorescence, indicating that it is not a tubulin containing structure, although it did prevent detection of the centrioles in these cells.

Observation of the position of the centrioles relative to the cap was extended to the electron microscope level using ferritin conjugated GaMIgG as a surface label. The location of the receptor-ligand complex can be more accurately detected using electron microscopy. Control cells, and cells treated with $10^{-5}M$ colchicine for 8 h were incubated with the ferritin conjugated GaMIgG for 7 min at $37^{\circ}C$. The ferritin is most clearly seen in unstained sections (Fig. 21); after only 7 min some of the label has been internalized. The centrioles are difficult to detect in these sections, and for photographic purposes they were stained. Figure 22A clearly shows the cap has formed over the centriolar region of the control cell, but the colchicine treated cell has capped the label at a position opposite the centriole pair (Fig. 22B).

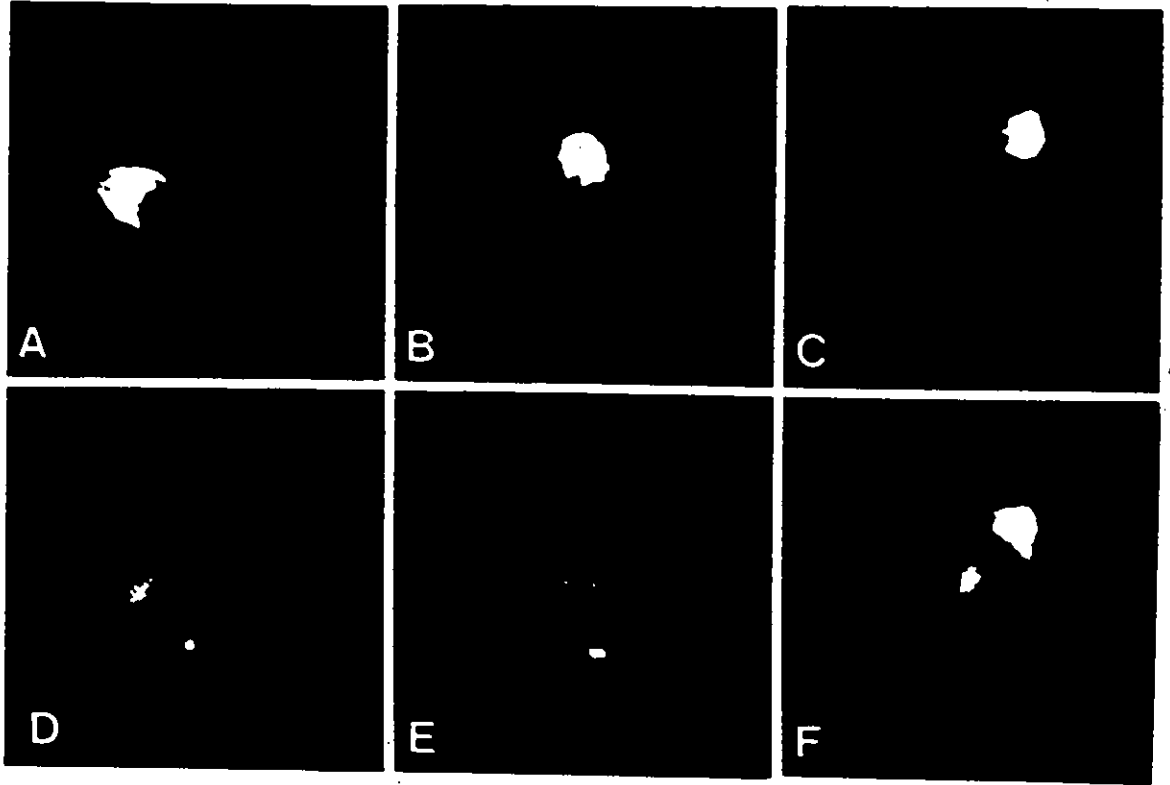
Fig. 20A-F: Resting mouse splenocytes were treated for 8 h with 10^{-5} M colchicine at 37°C . They were then exposed to 200 ug/mL R-GaMIgG and plated onto coverslips for 7 min at 37°C . They were washed once in PBS, fixed in paraformaldehyde, and processed for immunofluorescent staining of microtubules.

A,B,C: R-GaMIgG caps.

D,E,F: Fitc-immunolabelling with anti-tubulin antibody.

A&D, B&E, and C&F are three individual cells labelled with each fluorochrome. X 2,500.

The microtubules have been disassembled, and caps formed after the colchicine treatment are randomly located on the cell surface with respect to the centriolar region.



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Fig. 21: Electron micrograph of a resting mouse splenocyte incubated with 200 ug/mL ferritin-GaMIgG for 7 min at 37°C. The section was not stained so that the label contained in endocytotic vessicles could be more clearly seen. Note that the "capped" ferritin-GaMIgG is localized on that half of the cell containing the centriolar region (arrow). X 35,000.

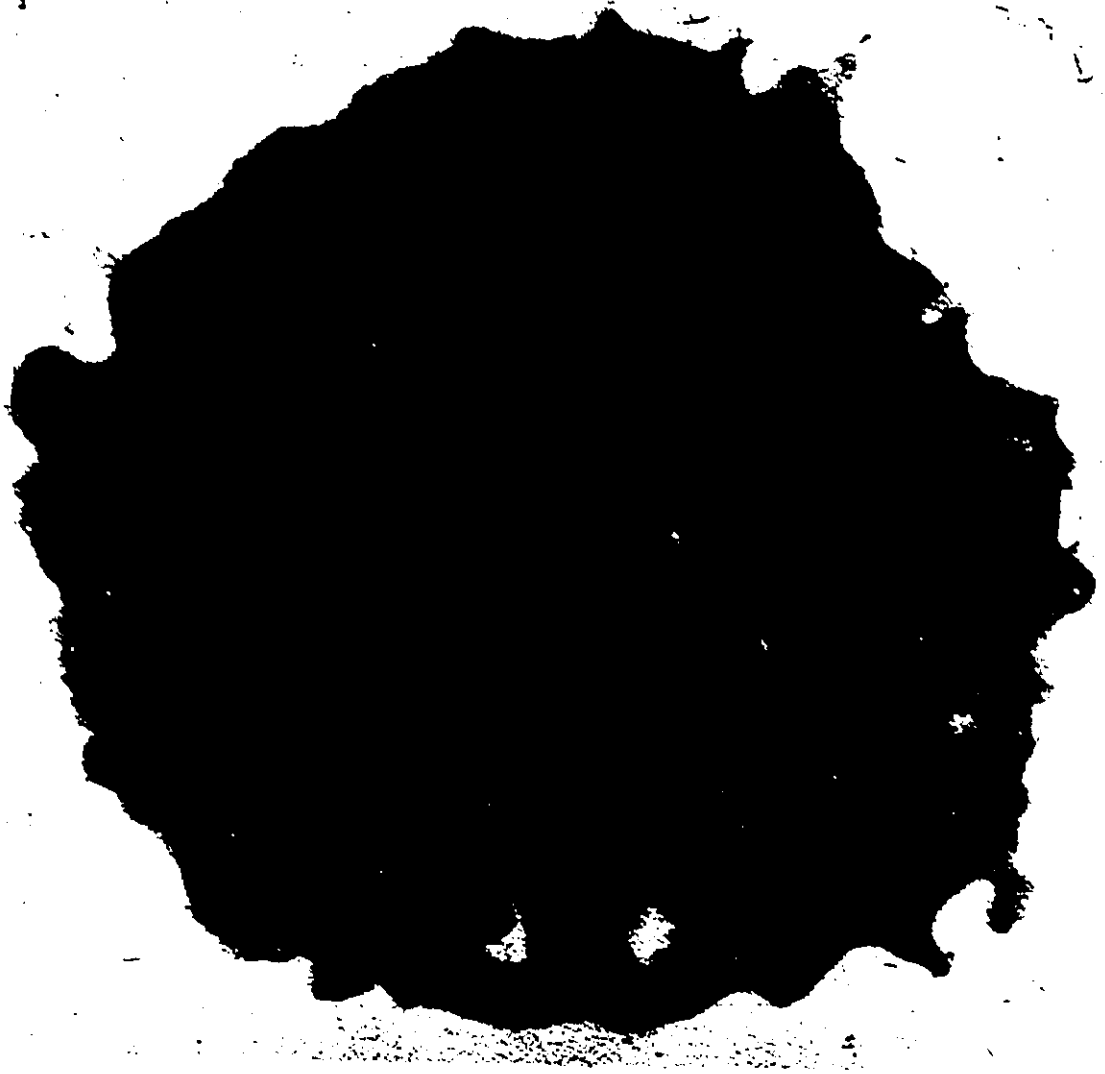
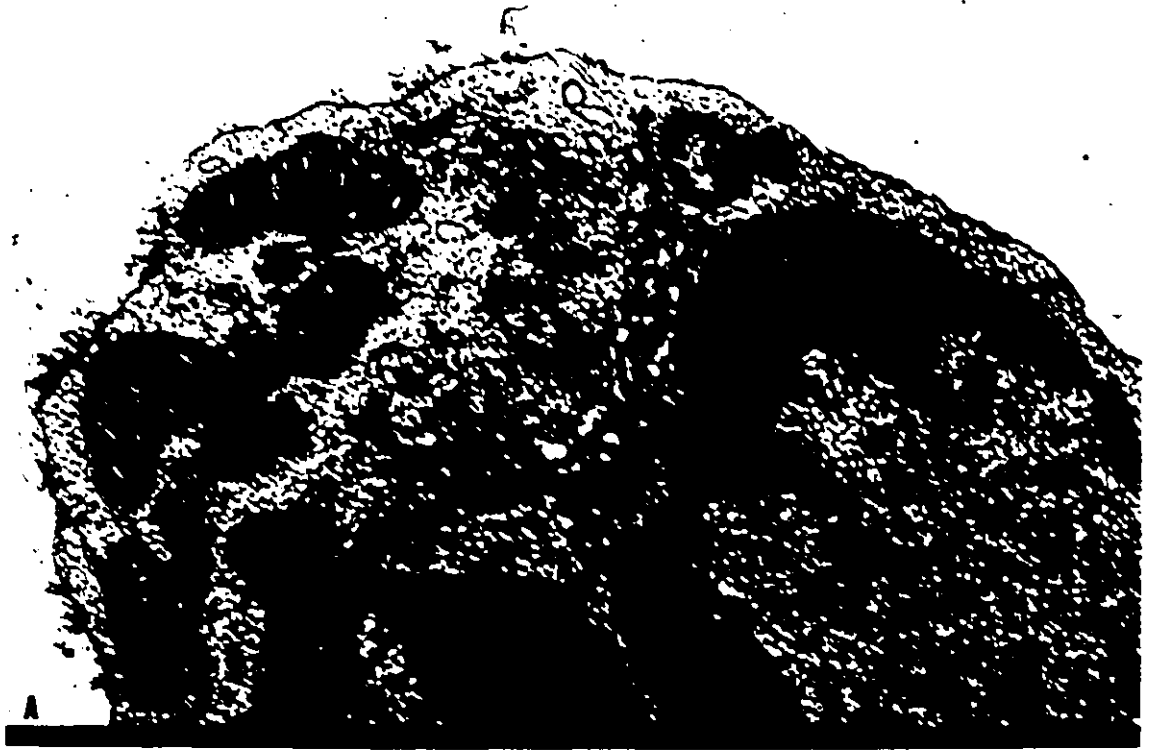


Fig. 22A,B: Electron micrographs of control and treated cells incubated with 200 ug/mL ferritin-GaMIgG for 7 min at 37°C.

A: Control cell showing that the cap is located directly over the centriolar region. X 45,000.

B: Cell treated with 10^{-5} M colchicine for 8 h prior to incubation with ferritin-GaMIgG. The cap (between arrows) is located over a region of the cell that does not contain the centriole pair. X 45,000.



DISCUSSION

It was first demonstrated by Edelman's group that intact microtubules or related structures are required for the Con A activation of resting lymphocytes. They suggested that microtubules, in association with other elements of the cytoskeleton, have a regulatory function in the transmission of signals, generated by Con A binding to the cell surface, to the nucleus resulting in activation. Their results indicated that cells committed to enter S-phase are insensitive to colchicine inhibition. They defined commitment as that time point at which the presence of Con A is no longer required for activation. Prior to commitment, colchicine is a potent inhibitor of mitogenesis, presumably because of its effect on microtubules. Once the cells are committed, microtubules would no longer be required for the cells to undergo blast transformation (McClain and Edelman, 1976; Gunther, Wang and Edelman, 1976).

Although this signal transmission hypothesis of Edelman's group cannot be excluded, alternative interpretations of their results are possible. Microtubules are associated with a large number of cytoplasmic processes, either directly or indirectly, and in their absence these processes are inhibited to various degrees (for reviews see Roberts, 1974; Stephens and Edds, 1976). The disassembly of microtubules leads to the disorganization of certain cytoplasmic organelles, and thus interferes with the function

of these organelles (Thyberg et al., 1977a). In lymphocytes, the phytohemagglutinin induced development of the Golgi complex and other cellular organelles is inhibited by colchicine (Thyberg, Maskalewski and Friberg, 1977b). It is conceivable that the inhibition of lymphocyte activation by the drug is a consequence of cytoplasmic organelle disorganization leading to a general depression of cellular metabolism, and not due to direct interference with the transmission of a signal from the cell membrane to the nucleus.

One would expect that if microtubule disassembly affected general cellular metabolism, colchicine would inhibit blast transformation both before and after commitment. Such a result was recently published by Rudd et al. (1979) in which they demonstrated that colchicine can inhibit transformation at any time of culture, before or after commitment, provided that sufficient time was allowed to elapse between addition of the drug and assay of DNA synthesis.

In association with Yahara, Edelman has also suggested that microtubules are involved in the patching and capping of lymphocyte surface receptors. Again their studies are based on the use of the drug colchicine. They demonstrated that Con A will not induce the patching and capping of its receptors, and will inhibit the mobility of other surface receptors, unless the cells have been exposed to conditions which disrupt or disassemble the microtubule network. They

constructed a model in which the binding of ligands to cell surface receptors would result in the anchoring of the receptor-ligand complexes to the microtubule network of the cytoplasm. Thus the binding of Con A to its receptors would lead to the anchoring of other surface receptors by the microtubule network, either directly or indirectly, by other elements of the cytoskeleton. Destruction of the microtubule networks by cold or colchicine frees anchored receptors, which are then capped by the cell following crosslinking by the appropriate ligand. They concluded that the microtubule networks of lymphocytes play a primary role in modulating cell surface receptor mobility (Yahara and Edelman, 1972; Edelman, Yahara and Wang, 1973; Yahara and Edelman, 1973; Yahara and Edelman, 1975; Yahara and Edelman, 1976; Edelman, 1976).

Recently, Yahara and Kakimoto-Sameshima (1978) concluded that it is the microtubule organization that is modulated by patch and cap formation. Using an immunofluorescent technique for microtubule detection they examined the distribution of microtubules in resting lymphocytes which had patched or capped surface IgG. They found that the microtubules in these cells appeared to be disaggregated (disassembled), and that a subcap of tubulin forms under the IgG cap. I infer from their studies that they are suggesting that anti-IgG can induce the capping of IgG in the absence of microtubule disrupting conditions because the formation of this receptor-ligand complex can itself

cause the disassembly of microtubules.

This is obviously incorrect because my results show that neither patching nor capping of surface IgG has any detectable effect on the integrity of the microtubule network.

IMMUNOLABELLING OF MICROTUBULES

The lack of detailed studies of microtubule distribution in resting lymphocytes has been due in past to the difficulty in detecting these microtubule networks by electron microscopy (Yahara and Edelman, 1975). Immunofluorescence is an excellent technique for the detection of the microtubule network in the entire cell at one time, and can be used to examine a large number of cells in the same population. Until recently, the use of immunofluorescence to detect microtubules in lymphocytes has yielded unsatisfactory results; Gabbiani *et al.* (1977) and Bourguignon, Tokuyasu and Singer (1978) were unable to detect microtubule networks in resting cells. Using immunoperoxidase labelling, Dighiero *et al.* (1978) could only detect microtubule networks in 40% of the cells examined. Yahara and Kakimoto-Sameshima (1978) were successful in detecting microtubule networks in a high percentage of cells (90%), but they were unable to detect intact microtubules in resting lymphocytes exposed to anti-Ig. One of the main reasons for the wide variability in the results was the type of fixation used. Fixation is usually the key to successful (and accurate)

detection of any structural protein by immunofluorescence (Webster, Osborn and Weber, 1978).

Some of the technical problems encountered in using immunofluorescence to detect microtubules in lymphocytes are directly related to the morphology of these cells. They are round, relatively thick (5-8 μm) and grow in suspension. An "ideal" cell for this type of assay is flat (less than 2 μm thick), well spread and grows attached to a substrate. As a cell increases in thickness, there is a corresponding increase in the level of background staining due to the trapping (specific and non-specific) of fluorescently conjugated antibodies. In round cells, a high level of background fluorescence comes from those stained structures which are above or below the plane of focus.

Microtubule networks have been detected by immunofluorescence in a variety of "ideal" cell types (Brinkley, Fuller and Highfield, 1975; Weber, Bibring and Osborn, 1975; Osborn and Weber, 1976). Microtubules detected by immunofluorescence in cells fixed with glutaraldehyde were shown to have a one to one correspondance with microtubules as seen by electron microscopy in the same cell (Osborn, Webster and Weber, 1978). Other fixatives, such as formaldehyde and methanol-acetone do not adequately preserve microtubules, although they appear to maintain the antigens in a pattern resembling that seen in glutaraldehyde fixed cells examined by electron microscopy (Sato, Ohnuki and Fujiwara, 1976). The use of formaldehyde and methanol-

acetone can lead to artifacts which are dependent on the degree to which microtubule networks are maintained during the staining procedures. The sensitivity of the microtubules may depend not only on the cell type in question, but also on the treatments the cell has been subjected to prior to fixation. This may account for the inability of Yahara and Kakimoto-Sameshima (1978) to detect intact microtubules in patched and capped cells.

All the studies in this thesis use a glutaraldehyde fixation similar to that published by Osborn, Webster and Weber (1978). To reduce background staining, the soluble cytoplasm is extracted with Triton X-100 in a stabilizing buffer which prevents disassembly of the microtubule network. The network remains in contact with this buffer until the glutaraldehyde fixation is complete. The use of paraformaldehyde in a brief prefixation step was necessary in double labelling experiments to prevent the detergent extraction of IgG from the plasma membrane. Prefixation was also necessary for the detection of microtubule networks in blast cells; these networks are more sensitive to detergent treatment than those of resting cells.

This is the first report of an immunofluorescent technique which can detect microtubule networks in stimulated as well as resting lymphocytes. Yahara's inability to do so (Yahara and Kakimoto-Sameshima, 1978) may be a reflection of his use of the fixative formaldehyde. His statement that stimulated lymphocytes do not possess well organized

microtubule networks is certainly incorrect. It is obvious from the electron micrographs in figures 3D and 4B-E that Con A stimulated mouse lymphocytes possess a highly organized microtubule network in which the number of microtubules increases during blast transformation. A similar organization of microtubules in phytohemagglutinin stimulated human lymphocytes was reported by Biberfeld (1971).

THE STUDY OF PATCH AND CAP FORMATION

When attempting to correlate the results of the many published studies of cap formation, care must be taken to ensure that the studies are indeed comparable. Capping studies have been performed under a variety of conditions, which can lead to very different results, and with a variety of cell types, making it difficult to construct a unifying model for patch and cap formation.

Capping experiments have been carried out at a variety of temperatures between 20°C and 37°C (Yahara and Edelman, 1975; Schreiner and Unanue, 1976b). This can affect the rate at which capping occurs (Taylor et al., 1971) and at lower temperatures the endocytosis of the capped receptor-ligand complex is inhibited (Schreiner and Unanue, 1976b). Often the cells are exposed to the ligand at 4°C for periods of 30 min or more (Unanue, Perkins and Karnovsky, 1972; Schreiner and Unanue, 1976a; Albertini and Anderson, 1977; Bourguignon, Tokuyasu and Singer, 1978) which allows receptor-ligand binding, but inhibits patch and cap formation.

Over 50% of the lymphocytes labelled at 4°C with anti-Ig show capping within 90 sec after the temperature is raised to 37°C (Taylor et al., 1971). Considering the sensitivity of microtubules to low temperature, and the rate at which they repolymerize at 37°C (see Fig. 8), it is obvious that receptors capping under these conditions will do so in the absence of a complete microtubule network.

The receptor on the cell surface and the specificity of the ligand used to crosslink it must also be considered. Con A and anti-Ig cap their respective lymphocyte surface receptors by different mechanisms (see the Introduction). In addition, although anti-Ig is specific for a single type of surface receptor (immunoglobulin), Con A binds to all glycoproteins containing alpha-D-glycosyl and sterically related residues (Goldstein, Hollerman and Smith, 1965). One of these Con A receptors is Ig (Andersson, Lafleur and Melchers, 1974). De Petris (1975) demonstrated that low concentrations of Con A could cap all surface Ig, whereas the capping of surface Ig by anti-Ig leaves the majority of Con A receptors spread diffusely over the cell surface. His experiments also indicated that at low concentrations of Con A, the glycoprotein receptor Ig preferentially bound Con A, either because of its higher affinity for the lectin, or its valency.

Con A capping has been studied using a variety of other cell types, including neutrophils, SV 40 transformed fibroblasts, mastocytoma cells, macrophages and ovarian

granulosa cells. With the exception of ovarian granulosa cells, all of these cell types (and lymphocytes) require a prior treatment with colchicine to disassemble their microtubules before Con A will induce the capping of its surface receptors. This suggests that the Con A receptors in these cell types are anchored directly or indirectly by a microtubule network. In contrast, the capping of Con A receptors of ovarian granulosa cells is not inhibited by the presence of an intact microtubule network. This latter capping system closely resembles the capping of lymphocyte Ig; cap formation is rapid at 37°C (10 min), and is followed by endocytosis of the receptor-ligand complex. However, the microtubule networks in these cells are not disaggregated by patch and cap formation, indeed the network is required for normal cap formation (Albertini and Anderson, 1977).

To determine the role of microtubules in the capping of lymphocyte surface receptors, it is of advantage to specifically disassemble the microtubule network, and to examine the effect this has on the capping process. It was first demonstrated by Taylor's group that the binding of colchicine to microtubular proteins leads to the disassembly of microtubules in vivo. (Borisy and Taylor, 1967). Other studies have shown that the sensitivity of microtubules to the drug varies from cell type to cell type, and between different microtubule assemblies within the same cell (Behnke and Forer, 1967; Tilney and Gibbins, 1968). In a variety of cell types, the colchicine disassembly of micro-

tubules can require several hours of treatment (Tilney and Gibbins, 1969; Brown and Bouck, 1973).

My results show that the disassembly of lymphocyte microtubules is both a time- and concentration-dependent process, with over 45-50% of the cells having intact microtubules after a one hour exposure to high concentrations of the drug. Once the critical concentration for disassembly has been reached (10^{-7} - 10^{-6} M) the rate of disassembly can not be accelerated by increasing the concentration of the drug (10^{-5} - 10^{-4} M). At any one concentration of colchicine, different cells in the culture show a marked difference in the rate at which their microtubules disassemble. While a small number of cells have no detectable microtubules after a 30 min exposure, the remainder require much longer treatments. This difference in sensitivity may be a reflection of the mixture of cell types found in a given population of splenic lymphocytes.

Having established the sensitivity of lymphocyte microtubules to colchicine, it was possible to compare the capping of surface receptors in the presence and absence of a microtubule network. The ligand used was anti-IgG, because it is specific for one cell surface receptor, and because it can induce capping without prior disruption of the microtubule network. All experiments were carried out at 37°C, without prior incubation at 4°C.

Using double labelling, immunofluorescence to detect surface IgG and microtubules in the same glutaraldehyde

fixed cell, I have clearly shown that the microtubule networks of resting lymphocytes, which have bound, patched or capped anti-IgG, are intact. The suggestion that a "sub-cap" of tubulin forms under the anti-IgG-IgG cap (Gabbiani et al., 1977; Yahara and Kakimoto-Sameshima, 1978) may be an artifact of immunofluorescent staining. The microfilament network which forms a "sub-cap" during capping may act non-specifically to trap both the primary antibody to tubulin, and the fluorescently-conjugated secondary antibody.

In the presence of an intact microtubule network, cap formation occurs exclusively over the region of the centriole pair, which is found associated with the Golgi complex of the cell. When resting lymphocytes cap surface IgG in the absence of a microtubule network, cap formation appears to occur randomly over the cell surface, and the organelles normally associated with the centriole pair are found distributed throughout the cytoplasm.

SPECULATIONS ON THE ROLE OF MICROTUBULES IN CAP FORMATION

It is clear that the microfilament system is directly involved in capping, but the relationship of the microtubule network to this process is not well documented in the literature. Microtubules do not provide a motive force, otherwise colchicine would inhibit the capping process. It has been speculated that the microtubule network in lymphocytes may act to guide the general movement of the

microfilament system (Loor, 1978). This thesis is the first direct demonstration that such a relationship exists. An intact microtubule network is required to control the site of cap formation relative to the position of certain cytoplasmic organelles. It is not known whether it is the region of cap formation on the membrane, or the spatial arrangement of cytoplasmic organelles, or both, that is controlled by the microtubule network in these cells.

Microtubules may control, directly or indirectly, the directional flow of receptor-ligand complexes as they are drawn towards one pole of the cell by the microfilament system. Microtubules may exercise direct control over the site of cap formation by freezing parallel tracks of membrane via association with certain membrane proteins (for example the class of anchored Con A receptors). These parallel tracks would run along the microtubules themselves and converge over the centriolar region. The lateral movement of the receptor-ligand complexes in the membrane would be guided by these tracks to collect over the centriolar region. Disassembly of the microtubule network would release the tracks of frozen membrane, and cap formation would occur randomly over the cell surface.

It is equally possible that the microtubule network is associated with the microfilament system, and acts to direct the lateral forces exerted by the microfilaments on the surface receptors. This latter model was suggested for the capping of Con A receptors on ovarian granulosa cells.

It was demonstrated by electron microscopy that patches form over individual microtubules in the cell in association with the microfilament system (Albertini and Anderson, 1977). An association between these two filament systems in lymphocytes remains to be established.

Although the evidence indicates that the microtubules are associated in some way with the control of cap formation, it is possible that their role involves organization of the cytoplasmic organelles in the centriolar region, and that the formation of the cap over this region is controlled by an unidentified system unrelated to the microtubule network.

The requirement for spatially controlled cap formation may be related to the final fate of the receptor-ligand complexes. Cap formation in lymphocytes is often followed by a rapid internalization of the receptor-ligand complexes, a process termed receptor-mediated endocytosis. The endocytic vesicles containing the receptor-ligand complexes then fuse with lysosomes produced by the Golgi, and the contents are degraded by the lysosomal enzymes (Schreiner and Unanue, 1976b). I suggest that spatial control may be required to ensure that endocytic vesicles are transported to the Golgi region so that they can be processed by the lysosomes. Random cap formation in the absence of an intact microtubule network may interfere with this process.

Techniques must now be developed to test the above models, and to determine the association of microtubules with the capping process. The problem is a biochemical one; we must

demonstrate an association, either direct or indirect, between microtubules and the anchored surface receptors, and we must determine if a direct relationship exists between the microfilament system and the microtubule network.

It is now recognized that receptor-mediated endocytosis has a fundamental role in the growth, nutrition and differentiation of animal cells. This process is used by a variety of cell types to internalize proteins and peptides (ligands) bound to specific receptors. In all cases studied thus far, the receptor-bound proteins enter the cell through coated pits. The fate of internalized receptor-ligand complexes varies from cell type to cell type; in some both the receptor and the ligand are degraded by lysosomes, while in others the ligand is degraded and the receptor returned to the cell surface (for a review, see Goldstein, Anderson and Brown, 1979).

The role of microtubules in the transport of endocytic vesicles in lymphocytes has not yet been determined. In ovarian granulosa cells, a clear association is seen between endocytic vesicles moving to the Golgi region, and the microtubule network in these cells (Albertini and Anderson, 1977). It remains to be seen whether colchicine will interfere with the degradation of receptor-ligand complexes in lymphocytes, and whether this interference is directly due to microtubule disassembly, or due to a general disorganization of the cytoplasmic organelles.

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