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CYTOSKELETAL REORGANIZATIONS IN CYTOTOXIC T LYMPHOCYTES FOLLOWING CONJUGATE FORMATION AND HYPERTHERMIA

by

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in the
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John David Knox

"It's a rat race out there,
so you might as well be a big rat."

The Valley Perfesser
(Lake Dorée, 1988)

To my family

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CONTENTS

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ABBREVIATIONS

APC	:	antigen-presenting cell
anti-Ig	:	anti-immunoglubulin
ATP	:	adenosine triphosphate
Ca^{++}	:	calcium
CHO	:	Chinese hampster ovary
con A	:	concanavalin A
^{51}Cr	:	radioactive chromium, a τ-emitter
CTL	:	cytotoxic T lymphocyte
EGTA	:	ethylene glycol bis (ß-aminoethylether) N,N,N',N'-tetra-acetic acid
EM	:	electron microscopy
FCS	:	fetal calf serun
FITC	:	fluoroscein isothiocyanate
GA	:	Golgi apparatus
GDP	:	guanosine diphosphate
GLU	:	detyrosinated tubulin
GTP	:	guanosine triphosphate
MAP	:	microtubule associated protein
MEM	:	minimal essential medium
Mg^{++}	:	magnesium
MHC	:	major histocompatibility complex
MTOC	:	microtubule organizing centre
LFA	:	lymphocyte function associated protein
PBS	:	phosphate buffered saline
PCM	:	pericentriolar material
PIPES	:	piperazine N,N'-bis (2-ethane sulfonic acid)
SDS	:	sodium dodecyl sulfate
sIg	:	surface immunoglobulin
TC	:	target cell
TCR	:	T cell receptor
TRITC	:	tetramethylrhodamine isothiocyanate
TYR	:	tyrosinated tubulin

ABSTRACT

The function of cytotoxic T lymphocytes (CTLs) is to recognize and lyse the specific target cells (TCs) that elicit their generation. Upon binding of a CTL to an appropriate TC a rapid orientation of the Golgi apparatus (GA) and microtubule organizing centre (MTOC) occurs within the CTL so that the two organelles face the TC. This polarization of the CTL is dependent upon the presence of microtubules and is thought to be a prerequisite for the effective lysis of the TC. The rapid movement of the MTOC and the link to an easily assayable function, TC lysis, make CTLs an attractive system in which to study cytoskeletal dynamics and functions. This thesis presents the results of my investigation of the process of cell-mediated cytotoxicity and in particular the role of the microtubules and intermediate filaments in MTOC/GA orientation.

Immunofluorescence microscopy was used to determine if the orientation of the MTOC is accompanied by the posttranslational modification of tubulin. My results indicate that, if a more stable subset of microtubules is formed during the orientation of the MTOC towards the TC, it does not persist long enough for the posttranslational modification of tubulin to occur. This result does not support, but does not rule out the involvement of the selective stabilization of a subset of microtubules in the orientation of the MTOC.

Immunofluorescence microscopy also demonstrated that conjugation results in the formation of filamentous vimentin

aggregates in CTLs similar to those induced by microtubule disassembling drugs. However, intermediate filament aggregates are not formed in all conjugated CTLs and there is no evidence to suggest that their formation is required for lytic activity.

Immunofluorescence, electron microscopy (EM), and radioactive chromium (^{51}Cr) cytolytic release assays were used to investigate the effect of hyperthermia on the cytolytic activity and cytoskeleton of CTLs. A hyperthermia treatment of $42^{0}C$ for 30 min results in a marked inhibition, followed by a transient recovery of cytolytic activity that peaks within 6 h. The killing step, or steps, affected by the hyperthermia treatment were found to occur subsequent to TC recognition and binding. The hyperthermia treatment did not result in microtubule disassembly, but did cause a reversible loss of the characteristic radial MTOC-microtubule array in most CTLs which was accompanied by an aggregation of the pericentriolar material (PCM) about the centrioles. The close correlation observed between the loss and recovery of microtubule organization and the loss and recovery of cytolytic activity suggest that the initial inhibitory effect of hyperthermia on cytolytic activity is due to the disruption of MTOC-microtubule organization.

The microtubule stabilizing drug taxol was used to test the relationship between the hyperthermia-induced disorganization of the microtubule network and its effect on cytolytic activity. The microtubules of taxol-treated cells remain associated with the centrosome, but the characteristic radial array is replaced

by large bundles. This microtubule organization has no effect on centrosome organization, orientation of the MTOC, nor on cytolytic ability. The taxol stabilized microtubule network is unaffected by the hyperthermia treatment; however, taxol-treatment does not prevent the aggregation of the PCM, the loss of MTOC orientation, and an inhibition of cytolytic activity. These results show that the normal radial organization of microtubules is not required for the functional polarization of CTL, but suggest that this process is dependent upon centrosome organization.

It is proposed that 50% of the cytolytic activity of the effector cells used in this study is due to the synthesis and secretion of a lytic protein subsequent to TC binding and that the remaining activity is occurring via a protein synthesis-independent apoptotic pathway. Hyperthermia initially has at least a partial effect on both lytic pathways. To what extent the recovery of cytolytic activity is dependent upon the recovery of each of the pathways could not be determined. The final results presented suggest that the long-term loss in cytolytic activity may be due to hyperthermia-induced effector cell death.

RÉSUMÉ

La fonction des lymphocytes cytotoxiques T (CTLs) est de reconnaître et de lyser les cellules cibles spécifiques qui ont entraîné leur production. Lorsqu' un CTL s'attache à une cellule cible apppropriée, l'appareil de Golgi et le centre organisateur des microtubules (MTOC) du CTL se réorientent rapidement vers la cellule cible. Cette polarisation des CTLs requiert la présence de microtubules, et semble être nécessaire à la lyse de la cellule cible. Ce déplacement rapide du MTOC et le lien avec une fonction facile à tester, la lyse de cellules cibles, font des CTLs un système favorable pour l'étude de la fonction et de la dynamique du cytosquelette.

Dans la présente étude, j'ai d'abord utilisé la microscopie par immunofluorescence pour déterminer si la réorientation du MTOC était accompagnée de modifications post transductionnelles de la tubuline. Mes résultats indiquent que, si un sous-ensemble de microtubules plus stables est formé durant la réorientation du MTOC vers la cellule cible, ce sous-ensemble n'est pas présent assez longtemps pour subir des modifications post transductionnelles au niveau de la tubuline. Ceci ne supporte pas mais n'écarte pas non plus l'implication de la stabilisation sélective d'un sous-ensemble de microtubules lors de la réorientation du MTOC.

La microscopie par immunofluorescence a aussi été utilisée

pour démontrer que l'association du CTL avec une cellule cible entraîne la formation d'agrégats de vimentine. Ces agrégats sont similaires à ceux observés lors de traitements avec des drogues qui entraînent la désorganisation des microtubules. Il est donc possible que l'agrégation des filaments intermédiaires soit due au détachement de la vimentine des microtubules, ceci afin de permettre une rapide réorientation du MTOC.

L'immunofluorescence, la microscopie électronique, ainsi que le test de libération de ^{51}CR par les cellules lysées, ont été utilisés pour étudier les effets de l'hyperthermie sur l'activité cytolytique et sur l'organisation du cytosquelette des CTLs. Un traitement des CTLs pendant 30 min à $42^{0}C$ entraîne une forte inhibition de leur activité cytolytique, suivie d'une récupération transitoire avec un maximum en moins de 6 heures. L'(les) étape(s) du processus lytique ayant été affectée(s) par le traitement d'hyperthermie semble(nt) avoir lieu après l'attachement du CTL à la cellule cible. Le traitement hyperthermique n'entraîne pas le désassemblage des microtubules. Toutefois, il se produit un changement réversible dans l'organisation radiale caractéristique des microtubules autour du MTOC dans la plupart des CTLs, accompagné d'une agrégation du matériel péricentriolaire (PCM) autour des centrioles. L'étroite corrélation observée entre la perte et la réorganisation des microtubules et la perte et la récupération de l'activité cytolytique suggère que l'effet inhibiteur initial de l'hyperthermie sur la fonction cytolytique est une conséquence de

la désorganisation du complèxe microtubules-MTOC.

Le taxol, une drogue stabilisatrice des microtubules, a été utilisée afin de vérifier la relation entre la désorganisation du réseau de microtubules par l'hyperthermie et l'effet de celle-ci sur la capacité cytolytique de ces cellules. Les microtubules des cellules traitées avec le taxol demeurent associés avec le centrosome, mais leur organisation radiale caractéristique est remplacée par d'épais faisceaux. Cette nouvelle organisation des microtubules n'a aucun effet sur l'organisation du centrosome, l'orientation du MTOC, ainsi que sur l'activité cytolytique. Le réseau de microtubules, lorsque stabilisé par le taxol, n'est pas affecté par le traitement hyperthermique. Cependant, le traitement au taxol n'empêche ni l'agrégation du PCM, ni la perte de réorientation du MTOC, ni l'inhibition de l'activite cytolytique dues à l'hyperthermie. Ces résultats indiquent que l'organisation radiale des microtubules n'est pas requise pour la polarisation des CTLs, mais suggèrent que ce procédé est dépendant de l'organisation du centrosome.

Il est suggéré que 50% de l'activité cytolytique des CTLs utilisés ici résulte de la synthèse et de la sécrétion de protéines lytiques après l'attachement à la cellule cible, tandis que l'autre 50% entraîne l'apoptose des cellules cibles sans nécessiter de synthèse protéique. Initialement, l'hyperthermie a un effet au moins partiel sur ces deux modes lytiques. Or, il n'a pas été possible de déterminer à quel point la récupération de l'activité cytolytique dépend de chacun de ces deux modes.

Enfin, les derniers résultats présentés suggèrent que la perte à long-terme de l'activité cytolytique pourrait être due à la mort des CTLs à cause de l'hyperthermie.

FOREWORD

A polarization of the Golgi apparatus (GA) and microtubule organizing centre (MTOC) is required for the migration of many eucaryotic cells and thus is involved in a wide range of physiological processes such as the development of the embryo, wound healing, and tumour metastasis (Singer and Kupfer, 1986). A similar polarization of the MTOC/GA is observed during certain cell-cell interactions such as the binding of a T helper or cytotoxic T lymphocyte (CTL) to an appropriate target cell (TC). This polarization of the MTOC/GA was first reported by Geiger et al. (1982) and has since been shown to occur subsequent to conjugate formation and to be a prerequisite for the effective lysis of the TC (Kupfer et al., 1983; 1985). Understanding how cells develop polarity is therefore of fundamental interest and since cell mediated cytotoxicity is the principal mechanism of tumour, viral, and transplant immunity determining the function of the polarization of the MTOC/GA in TC lysis may have important medical implications.

To present my research in the proper context, short surveys of several areas of literature are provided in the introduction. The first two sections contain brief reviews of the structure and dynamics of the microtubules and intermediate filaments respectively. In the third section the organization of the intermediate filaments and microtubules in unactivated lymphocytes is described and earlier work investigating the reorganization of

these two cytoskeletal components that takes place during the patching and capping of lymphocyte cell surface receptors is discussed. TC recognition and binding by the CTL is covered in the fourth section and the directed secretion model for cell-mediated cytolysis is introduced. Finally the rationale of the experimental strategy of the present study is presented.

INTRODUCTION

MICROTUBULES

The tubulin heterodimer, consisting of one α-tubulin and one ß-tubulin subunit, is the basic building block from which microtubules are assembled. When warmed to 37^0C in the absence of Ca^{++} and in the presence of guanosine triphosphate (GTP) the tubulin dimers polymerize. In the polymerized form the tubulin dimers are aligned 'head to tail' in parallel rows called protofilaments. Each microtubule consists of the staggered arrangement of 13 such protofilaments side by side around a hollow core.

The head to tail arrangement of the tubulin dimers results in the two ends of the microtubule being structurally distinct. In vitro polymerization studies demonstrated that the two ends of microtubules have different assembly rates (Summers and Kirschner, 1979). Addition of tubulin occurs preferentially at the '+' end of the microtubule and the opposite end, defined as the '-' end, may be either growing or shrinking depending on the tubulin concentration. At a particular tubulin concentration known as the critical concentration (C_c) the addition of dimers at the + end will be balanced by the loss of dimers at the - end.

Each tubulin dimer has two guanosine nucleotide binding sites, one of which is non-exchangeable and invariably contains guanosine triphosphate (GTP). The other exchangeable site can bind either GTP or guanosine diphosphate (GDP), but the dimer will only

polymerize readily if liganded to GTP (Weisenberg, 1972). The hydrolysis of the GTP in the exchangeable site to GDP occurs shortly after the incorporation of the dimer into a microtubule. Hydrolysis is not required for assembly as microtubules form when non-hydrolysable analogues are substituted for GTP (Penningroth et al., 1976). During periods of rapid assembly the rate of GTP hydrolysis is slower than the rate of assembly (Hill and Carlier, 1983) suggesting that a GTP 'cap' is formed at the + end of a microtubule.

In vivo, most microtubules arise from the centrosome which acts as a cap for the - end. When Mitchison and Kirschner (1984a,b) examined individual microtubules growing from purified centrosomes they found that when the concentration of tubulin was reduced to below C_c some microtubules continued to grow while others depolymerized quickly from the free end. The presence of growing and shrinking microtubules within the same population of microtubules has been termed 'dynamic instability'.

The key feature of the model that best explains the dynamic instability of microtubules is the segregation of the assembly and disassembly events by the essentially irreversible step of GTP hydrolysis. According to the model, in a growing microtubule the hydrolysis of GTP lags behind dimer addition, resulting in the formation of a GTP cap at the + end of a microtubule. GTP is thought to somehow stabilize the interaction between the tubulin subunits and thereby confer stability to the microtubule. When the microtubule stops growing, the subsequent elimination of the GTP

cap leads to the destabilization of the intersubunit bonds and results in the rapid depolymerization of the microtubule (Kirschner and Mitchison, 1986). In live cells, 50-80% of the shrinking microtubules convert back to the growth phase (Sammak and Borisy, 1988), indicating that the disassembly events can be either complete or incomplete.

The dynamic instability of microtubules suggests a means by which reorganizations of the microtubules can be effected. Kirschner and Mitchison (1986) have hypothesized that since the whole array is very dynamic the selective stabilization a particular subset of microtubules would rapidly transform the entire array. They suggested that microtubules might first be stabilized by an end interaction and then stabilized along their entire length by a time dependent process.

There are three ways in which this time dependent stabilization could occur; 1) association of microtubules with accessory proteins localized in different parts of the cell, 2) posttranslational modification of the α and ß-tubulin subunits, or 3) by a combination of these mechanisms (Piperno and Fuller, 1985). Five posttranslational modifications of tubulin have been described in the literature; removal of the carboxy-terminal tyrosine (Gundersen et al., 1984), acetylation (Piperno and Fuller, 1985; Maruta et al., 1986), and polyglutamylation (Eddé et al., 1990) of α-tubulin, and the phosphorylation (Gard and Kirschner, 1985), and polyglutamylation (Lee et al., 1990) of ß-tubulin. The posttranslational modifications that have been tested,

detyrosination and acetylation, have not been shown to dramatically alter the behaviour of the tubulins in vitro (Kumar and Flavin, 1982; Maruta et al., 1986); however, these modifications all take place in regions of the α and ß-tubulin subunits that are exposed on the outer surface of the microtubule lattice and participate in the binding of several microtubule associated proteins (MAPs) (Eddé et al., 1990; Lee et al., 1990). MAPs promote the assembly and stability of microtubules (for reviews see Vallee, 1990; Cleveland, 1990), thus by affecting the interaction of MAPs the posttranslational modifications may regulate microtubule dynamics and function.

INTERMEDIATE FILAMENTS

The intermediate filament proteins are a large multigene family of proteins that form at least five distinct types of intermediate filaments: type I and type II keratins in epithelial tissue; type III intermediate filaments consisting of vimentin, desmin, glial fibrillary acidic protein, and peripherin; type IV neuronal intermediate filaments; and type V nuclear lamins (for reviews see Steinhert and Roop, 1988; and Klymkowsky et al., 1989). The intermediate filament subunits all have limited, but pronounced, amino acid sequence homologies. The most characteristic feature is the presence of a central rod domain of ≈310 amino acids that contains a conserved pattern of heptad and nonheptad repeat domains. The structural uniformity of intermediate filaments is apparently due to the tendency of these

central rod domains to form α-helical coiled-coil dimers. The central rod domain is flanked by non-α-helical N-terminal 'head' and C-terminal 'tail' domains of variable size and chemical characteristics.

Once dimers have formed two dimers combine to form a tetramer. Antibody decoration experiments of assembled tetramers have suggested that the dimers in a tetramer are arranged antiparallel to one another (Stewart et al., 1989). Tetramers then assemble in an unknown manner to form protofilaments and a typical intermediate filament contains 6 to 8 protofilaments. This number is variable from filament to filament within a cell and may vary along the length of an individual filament (Aebi et al.,1988).

The cytoplasmic intermediate filaments (types I-IV above) can be divided into two groups on the basis of their ability or inability to form homopolymeric intermediate filaments. Types III and IV can form homopolymeric intermediate filaments, but when co-expressed within a cell they also can co-polymerize with one another (Monteiro and Cleveland, 1989). The cytokeratins are obligatorily heteropolymeric, and in order to form an intermediate filament the type I acidic cytokeratins and the type II neutral/basic cytokeratins must be present in a 1:1 ratio. It is unclear whether this constraint is imposed at the level of the dimer or the tetramer (Hatzfeld and Franke, 1985).

The non-polar organization suggests that the C_c for polymerization is the same for both ends of an intermediate filament. Therefore, it is not surprising that the dynamics of

intermediate filaments are distinctly different from the microtubules and microfilaments which are clearly polar structures. Very little intermediate filament protein is found in a soluble form and the intermediate filaments themselves are insoluble under physiological conditions; however, newly synthesized (Blikstad and Lazarides, 1983; Albers and Fuchs, 1987; 1989) or injected (Vikstrom et al., 1989) subunits are rapidly incorporated into the existing intermediate filament network. In addition to the vectorial assembly or incorporation/exchange of subunits occurring along individual filaments, the whole intermediate filament network can undergo dramatic reorganizations. For example, during surface immunoglobulin cap formation the vimentin intermediate filaments are also capped (Paulin-Levasseur and Brown, 1987b; and see below).

Despite the fact that intermediate filaments are now known to be highly dynamic components of the cytoskeleton (Steinert and Liem, 1990), the function of the intermediate filaments remains unknown. Defining the functions of the intermediate filaments has in part been made difficult by the absence of drugs that specifically disrupt intermediate filament organization. Two methods that have been shown to disrupt the existing intermediate filament network are the intracellular injection of anti-intermediate filament antibodies (Gawlitta et al., 1981) and the expression of mutated intermediate filament genes within cultured cell lines (Albers and Fuchs, 1987). Studies using these two techniques (for review see Klymkowsky et al., 1989) have shown that the disruption of intermediate filament organization has no obvious

effect on cellular morphology, motility or the intracellular distribution of organelles. The identification of some cell types in gastropods (Bartnik et al., 1986) and of cell lines (Paulin-Levasseur et al., 1988) that do not contain intermediate filaments has further indicated that intermediate filaments are not critical to the viability of eukaryotic cells.

The evolutionary conservation of the intermediate filament proteins and the absence of effects following the disruption of intermediate filament organization has led to the suggestion that the intermediate filaments must function at higher levels of biological organization than that of the single cell (Klymkowsky et al., 1989). Alternatively, the tissue-specific expression exhibited by the intermediate filament subunits has led to the suggestion that the functions of the intermediate filaments may only be readily observable in cells that are still performing their differentiated functions (Gawlitta et al., 1981; Klymkowsky et al., 1983)

THE UNACTIVATED LYMPHOCYTE

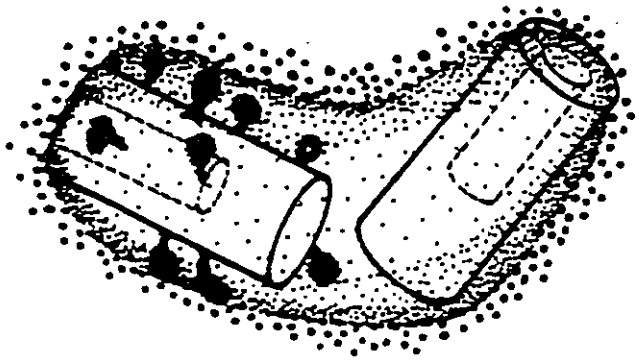
The small lymphocytes present in the peripheral blood and the lymphatic system continuously circulate throughout the body as quiescent cells. These cells, each committed to a single antigenic specificity, represent the millions of different lymphocyte clones that are capable of responding to an almost infinite number of foreign antigens. In mature unactivated B and T cells DNA is tightly packed into large heterochromatin masses, exhibits very

little transcriptional activity, and the nucleus is surrounded by only a thin layer of cytoplasm. When a foreign antigen is presented to an unactivated lymphocyte possessing a corresponding receptor a specific immune response, such as the generation of cytotoxic, helper, suppressor, or other effector cells, may be triggered. In all such cases the specific immune response is preceded by the proliferation and expansion of clones capable of recognizing and responding to that particular antigen. The proliferative response involves large increases in transcription and translation, in the volume of the nucleus and cytoplasm, and in the number of microtubules assembled from the centrosome.

The single centrosome of the unactivated lymphocyte is usually located adjacent to a nuclear cleft and, invariably, the Golgi apparatus is associated with it. The organization of the centrosome of unactiviated mouse splenic lymphocytes has been examined by electron microscopy of serial sections and found to consist of a pair of centrioles, satellite bodies, and pericentriolar material (Schweitzer and Brown, 1984). Immunofluorescence staining with anti-tubulin reveals a sparse microtubule system which forms a radial array extending from the centrosome and around the periphery of the cell near the plasma membrane. Frequently, the vimentin intermediate filaments parallel the distribution of microtubules and also appear to extend from the centrosome (Paulin-Levasseur and Brown, 1987a,b).

The initial interest in the microtubule system of lymphocytes was stimulated by the pioneering studies of Edelman and coworkers

Figure I.1. Diagramatic Representation of the Centrosome of an Unactiviated Lymphocyte. The two centrioles are oriented at right angles to one another. The dashed cylinder in the centrioles represents the dense core of material present at the distal ends of the centrioles. Nine satellite bodies are shown and are exclusively associated with one of the centrioles. The large stipple represents the ribosomes which appear excluded from the pericentriolar material, shown in fine stipple.



which implicated the submembranous array of microtubules as a likely candidate for a role in signal transmission (reviewed in Edelman, 1976). A part of the evidence supporting this role was derived from studies of the effects of anti-microtubule drugs, such as colcemid, on the process of cell surface receptor redistribution termed capping (for review see Bourguignon and Bourguignon, 1984). Capping involves the cross-linking of lymphocyte cell surface receptors by multivalent ligands, such as the plant lectin concanavalin A (con A) or anti-immunoglobulin (anti-Ig). This induces the temperature-dependent redistribution of receptor-ligand complexes to form patches which are subsequently transported in an energy-dependent manner to one pole of the cell. Ligand-mediated receptor patching and capping has been suggested to be important in a number of immune responses (Bourguignon and Bourguignon, 1984; Kupfer and Singer, 1989a).

Although it has been clear for some time that the microtubule system is involved in the redistribution of lymphocyte surface receptors (Edelman, 1976), the precise function of microtubules and intermediate filaments in this process is unclear. The surface immunoglobulin-anti-Ig (sIg-anti-Ig) cap is normally formed over the region of the cytoplasm containing the centrosome. A colchicine or colcemid treatment that results in the disassembly of the microtubules, does not inhibit cap formation, but the cap is then formed at any region on the cell surface with respect to the centrosome (Rogers et al., 1981). These results led the authors to suggest that the submembranous microtubule system provided the

spatial control to direct the microfilament-dependent translocation of the sIg-anti-Ig complexes to form a cap over a specified region of the cytoplasm. Subsequent studies by Paatero and Brown (1982) examining the effects of the microtubule assembly-promoting drug taxol on the organization of microtubules and on the capping of sIg, are consistent with this suggestion.

In lymphocytes, taxol causes an extensive reorganization of the microtubule system and the formation of one to a few centrosome-associated bundles of microtubules. Concomitantly, the centrosome with its associated Golgi apparatus is displaced from its normal position in the nuclear cleft to a position near the plasma membrane. The microtubules of the bundles in taxol-treated cells no longer form a submembranous array, but most frequently pass through the cytoplasm near the surface of the nucleus (Brown et al., 1985). This reorganization of microtubules by taxol has the same effect as the removal of the microtubules by colcemid, leading to random cap position with respect to the centrosome (Paatero and Brown, 1982). This indicates that it is not just the presence of microtubules, but it is their presence in a particular organization, that is required to direct the site of cap formation over the region of the cell containing the centrosome and Golgi apparatus where endocytosis occurs.

The radial pattern of microtubules appears to be maintained throughout patch and cap formation though some reports (e.g. Yahara and Kakimoto-Sameshima, 1978) to the contrary exist. In contrast, coincident with cap formation vimentin is relocalized as a diffuse

accumulation under the cap and discrete filament arrays are no longer detected by immunofluorescence (Bourguignon and Bourguignon, 1981; Dellagi and Brouet, 1982; Paulin-Levasseur and Brown, 1987b). This led to the suggestion that this intermediate filament reorganization might function in the capping process.

The disassembly of microtubules leads to the formation of a ring-like structure of vimentin filaments which shows no spatial relationship to the position of the centrosome (Paulin-Levasseur and Brown, 1987a,b). It is interesting that coincident with cap formation, which under these conditions also is not related to the position of the centrosome, this ring-like structure disappears and vimentin forms a diffuse aggregate under the cap indistinguishable from that seen in control cells (Paulin-Levasseur and Brown, 1987b). Clearly the formation of the diffuse aggregate and its localization to the cap can occur independent of the microtubule system.

In taxol-treated lymphocytes vimentin filaments co-localize with the microtubule bundles and remain co-localized with the stable microtubule bundles during cap formation (Paulin-Levasseur and Brown, 1987b). This result shows that neither the formation of the vimentin aggregate nor its relocalization to the cap is a requirement for cap formation. These results, however, do not rule out a role for the intermediate filament system in capping and it is possible (as discussed in Paulin-Levasseur and Brown, 1987b) that this system contributes to the directionality of sIg redistribution by transducing spatial information between

microtubules and microfilaments.

As it has been observed in a number of cell types including lymphocytes (Thyberg and Moskalewski, 1985 for review), the disassembly of microtubules by colcemid or colchicine produces a disorganization of the Golgi apparatus and a dissociation from the centrosome. Recent studies have shown that microtubule disassembly is required, but not sufficient for Golgi apparatus fragmentation and dispersal (Turner and Tartakoff, 1989); whereas, microtubules are absolutely required for the reassembly of the Golgi apparatus (Ho et al., 1989). Also as described above and observed in other cell types (Bloom et al., 1985), microtubule disassembly leads to a collapse of the intermediate filament system, suggesting that the microtubule system functions as a scaffold for its maintenance. Therefore, the minimal microtubule array in the unactivated lymphocyte appears to carry out some of the same functions in maintaining cell organization and polarity as the more elaborate microtubule arrays in cycling cells.

CTLs AND THE DIRECTED SECRETION MODEL

The function of mature lymphocytes is dependent upon their ability to synthesize receptors for antigens that commit them to a single antigenic specificity for the rest of their life span. When these cells encounter their specific antigen, in the appropriate context, they become activated and proliferate, giving rise to differentiated effector cells.

T lymphocytes are activated by specific binding of their T

cell receptors (TCR) to peptides associated with major histocompatibility complex (MHC) antigens on the surface of antigen-presenting cells (APCs). This stimulates the proliferation of two different types of effector T-cells: T helper cells that induce the proliferation and differentiation of specific APCs; and CTLs that lyse specific TCs.

The screening of monoclonal antibodies for their ability to inhibit cell mediated cytolysis has led to the identification of 4 cell-surface molecules essential for the interaction of CTLs with their TCs. These antibodies recognize either the TCR (CD8) or one of three of these molecules termed lymphocyte function associated (LFA) antigens: LFA-1, LFA-2 (CD2), and LFA-3. The LFA antigens are now known to account for the antigen-independent adhesion of CTLs to prospective TCs. Monoclonal antibodies against any of them, or the TCR (CD8), inhibits cytolytic activity, demonstrating that TC recognition and binding is a highly complex process requiring cooperation between several different cell-surface molecules (Springer, 1990). What is much less clear is the identity of the structures that participate in the delivery of the 'lethal hit' by the CTL to the TC. Also, it is not known if these structures are constitutive components of CTLs or synthesized after TC binding.

By immunofluorescence staining with antibodies to tubulin, Geiger et al. (1982) examined the microtubule organization in CTLs bound to their targets. They reported that the centrosome of the CTL, but not that of the target cell, was found in the region of

cell-cell contact and suggested that the CTL plasma membrane proximal to the centrosome was particularly active in forming intercellular contacts. However, a random orientation of the centrosome was observed in conjugates formed between CTLs and lysis resistant target cells, providing evidence that cell binding does not occur at a polarized site on the plasma membrane of CTLs (Kupfer and Dennert, 1984). In addition when conjugates were formed in the absence of Ca^{++} the centrosome of the CTL was randomly located with respect to the target cell (Kupfer et al., 1985). Orientation of the centrosome was restored within minutes by the addition of Ca^{++}, demonstrating that orientation can occur subsequent to target cell binding. More recent work by Gray et al. (1987) has shown a Ca^{++} influx in CTL in response to target cell binding, suggesting that centrosome orientation would normally be effected after cell binding. Therefore, the rapid orientation of the centrosome within CTLs to face bound target cells provides a suitable model system for studying the role of microtubule organization in the establishment of cell polarity and its function in cell-cell interactions.

As discussed earlier, in lymphocytes the Golgi apparatus and centrosome are closely associated. This has led to the suggestion that the orientation of the centrosome could be involved in the targeting of Golgi derived vesicles to the area of cell contact with the target cell. Vesicular granules containing one or more cytotoxic components such as perforins or pore-forming proteins, serine proteases and tumour necrosis factors have been isolated

from a wide range of CTL cell lines (for reviews see Clark et al., 1988; Young et al., 1988; Brunet et al., 1988).

Experiments with CTLs in which the reorientation of the centrosome towards the TC was reversibly inhibited, by removal of Ca^{++} from the medium, or by treatment of the cells with microtubule disassembling drugs, indicated that the orientation of the centrosome is a prerequisite for the effective lysis of the target cell and supported the directed secretion model (Kupfer et al., 1983; Kupfer et al.,1985). However, there is now evidence that CTL lysis of certain target cells can occur in the absence of Ca^{++} and without any appreciable degranulation (Ostergaard and Clark, 1989). It has also been reported that many TCs attacked by CTLs undergo violent blebbing followed by a fragmentation of the nucleus and cytoplasm into initially sealed vesicles which is characteristic of cells undergoing programmed cell death or apoptosis (Martz and Howell, 1989). This has led to the suggestion that several different lytic pathways are used by CTLs in target cell killing. Regardless of the pathway used, target cell lysis in the absence of centrosome orientation has never been observed (Ostergaard and Clark, 1987). Therefore, while the function of the centrosome orientation remains uncertain, it appears to be required for a successful interaction between the CTL and the target cell.

RATIONALE OF THE EXPERIMENTAL APPROACH

As stated previously the main goal of this thesis was to identify the role of the microtubules and intermediate filaments in

the orientation of the MTOC/GA. The selective stabilization model proposed by Kirschner and Mitchison (1986) suggested that the stabilization of a subset of microtubules following TC binding may result in orientation of the MTOC. This hypothesis was tested using immunofluorescence microscopy with antibodies specific for tubulin isoforms that are associated with stable subsets of microtubules.

The capping studies of Paulin-Levasseur and Brown (1987a,b) suggested that the intermediate filament system may be involved in transducing spatial information between surface receptors and the microtubules and therefore also play a role in MTOC orientation. Using double immunofluorescence microscopy with antibodies to tubulin and vimentin a reorganization of the intermediate filaments was found to accompany the reorientation of the MTOC. In an attempt to determine whether the reorganization of the intermediate filaments occurred as the result of, or was required for, the movement of the MTOC the effect of experimentally altered cytoskeletal distributions on the cytolytic ability of CTLs was tested.

The effects of hyperthermia on cytoskeletal organization and cytolytic activity were first explored in the hope that it might provide a means to manipulate the intermediate filament organization without affecting the microtubule system. Instead of having an effect on intermediate filament organization, hyperthermia was found to result in a reversible disorganization of the radial microtubule array. As a consequence of this result

hyperthermia was used to test the role of the radial array of microtubules in cytolytic activity. Hyperthermia was also found to be a useful experimental tool to investigate the structures involved in the maintenance of microtubule organization, MTOC orientation and cytolytic activity. For this reason experiments involving hyperthermia treatments constitute the majority of the thesis.

MATERIALS AND METHODS.

CELL CULTURES

Animals and cell lines. 6-8 week old BALB/c female mice were purchased from Charles River (Montreal, Que.). The C57BL/6 T-cell leukaemia cell line, EL4, was obtained from the American Type Culture Collection (Rockville, MD). The cell line was maintained as a suspension culture in α-minimal essential medium (αMEM) supplemented with 10% heat inactivated fetal calf serum (FCS) and penicillin-streptomycin (1,000 U/ml of each). When a saturation density of 1 x 10^6 cells/ml was obtained the cells were diluted to 1-2 x 10^5 cells/ml in fresh medium.

Cell Viability Counts. Cells were diluted 1:5 with a 0.85% (w/v) NaCl solution containing 0.04% (w/v) trypan blue (Matheson, Coleman and Bell Manufacturing Chemists, Norwood, OH). The number of trypan blue impermeable cells was determined with a heamocytometer (Reichert Sci. Instr., Buffalo, NY).

Generation of CTLs. Nine to twelve days after an intraperitoneal injection of 3 x 10^7 EL4 leukaemia cells, the allogeneic BALB/c host mice were killed by cervical dislocation and their spleens removed. Splenocytes were obtained by pressing the spleens through

a 100 mesh screen. The cell suspension was then forced through a 22G needle to break up any clumps. Erythrocytes were lysed by resuspension in cold hypotonic saline (0.2% NaCl wt./vol.) for 20 seconds. The remaining cells were referred to as effector cells. T-cell preparations were made by removing B-cells with magnetic goat anti-mouse IgG(H&L) antibodies and a BioMag Separator purchased from Advanced Magnetics Inc. (Cambridge, MA).

Before use the Biomag slurry was washed 3 times with PBS (PBS used in tissue culture, 4g NaCl, 0.1g KCl, 0.1g KH_2PO_4, 0.575g Na_2HPO_4, in 500 mL). Splenocytes were suspended in αMEM + 5% FCS at a concentration of 1 x 10^7 cells/mL in 15 mL disposable conical centrifuge tubes (Corning, Canlab, Mississauga, Ont.). To each mL of splenocytes 0.5 mL of goat anti-mouse Biomag slurry was added. The solution was then mixed well, centrifuged at 30 x g for 5 min, and incubated for 10 min at 4^0C. Following this incubation the cells were gently resuspended, incubated an additional 10 min at 4^0C, and then transferred to a 75 cm^2 polystyrene tissue culture flask (Corning). Enough media was added to make the volume at least 8 mL. The flask was then placed over the BioMag Separator, taped in position, and gently rocked at room temperature for 5-10 min. Then the flask, with the magnet still attached, was placed upright, and the unbound cells harvested. This procedure resulted in the removal of all cells in the splenocyte population normally detected by immunofluorescence microscopy following staining with an antibody to mouse IgG. T-cell preparations not immediately used in an experiment were cultured in αMEM supplemented with 10% FCS

penicillin-streptomycin (1,000 U/ml of each) at a concentration of 2.5×10^6 cells/mL.

HYPERTHERMIA TREATMENTS.

Effector cell suspensions (<2 ml) at concentrations of 2.5-5.0 $\times 10^7$/ml were placed in conical centrifuge tubes and then placed in water baths. The water was heated with a Julabo VC immersion circulator that was able to maintain the set temperature within $\pm 0.02^0$C (Canlab, Mississauga, Ont.).

DRUG TREATMENTS AND con A STIMULATION

Stock solutions of 0.4 mg/mL colchicine (Sigma) 1 mg/mL con A (Calbiochem) and 50 mM cycloheximide (Sigma) stock solutions were prepared in αMEM. A stock solution of 10 mM taxol (Natural Products Branch, Division of Cancer Treatment, National Cancer Institute) was made up in DMSO. All stock solutions were kept frozen in aliquots at -70^0C (Con A) or -20^0C until used.

The extent of stimulation was assayed by measuring the incorporation ^{3}H-Thymidine (Amersham) (2 μCi/mL, 1 h) as described by Rudd et al., 1979.

CYTOLYTIC ACTIVITY ASSAYS.

TCs were labelled with 100 μCi $Na_2{}^{51}CrO_4$ (NEN, Dupont Canada Inc., Mississauga, Ont.) per 10^7 cells for 45 min in αMEM medium containing 5% FCS, washed once in PBS, resuspended in a large volume of PBS (10^5 cells/mL) and incubated at 37^0C for 30 min before being centrifuge and resuspended at $1x10^6$ cells/mL in αMEM + 5% FCS. Labelled TC (1×10^5/well) were incubated in a total volume of 200 μl with CTL ($25-50 \times 10^5$) in αMEM supplemented with 5% FCS in 96-well round bottom microtiter plates. All samples were done in triplicate. The plates were centrifuged at 50 x g for 1 min to enhance cell-cell contact at the start of the assay. After incubation for 3-4 h at 37^0C the plates were centrifuged for 10 min at 250 x g and 100 μl of the sample supernatants harvested with a Pipetman and counted in a Beckman 8000 gamma counter. Each sample was counted once for one min on one channel with the lower limit set to 10 and the upper limit set to 760 KeV. Maximal release was calculated by lysing labelled TC with 0.5% Triton X-100. Spontaneous release was calculated by incubating the labelled TC with complete medium for the duration of the assay. Cytolytic activity was calculated according to the formula:

% cytotoxicity = 100 x(cpm experimental release - cpm spontaneous release)/(maximal cpm - cpm spontaneous release).

Cytolytic activity was normalized to cytolytic activity of controls according to the formula:

relative cytolytic activity = 100 x (treated cytolytic activity/ control cytolytic activity).

For an example of the calculations of a typical experiment investigating the effect of hyperthermia on cytolytic activity see Appendix 1.

IMMUNOFLUORESCENCE MICROSCOPY.

Microscopy. Conventional fluorescence microscopy was carried out with a Zeiss Universal photomicroscope equipped for epifluorescence and phase contrast optics. Confocal microscopy was carried out with a Nikon photomicroscope equipped with the Lasersharp MRC-500 confocal fluorescence imaging system (courtesy of Bio-Rad Laboratories of Canada Ltd., Mississauga, Ont.) using an argon laser for illumination.

Antibodies. The following primary antibodies were used: 5A6, a mouse monoclonal that recognizes α-tubulin used as a general tubulin stain (Aitchison and Brown, 1986); a rabbit autoimmune serum that labels a centrosome-associated protein was the gift of Dr. V. Kalnins (Tursken et al., 1982); the rabbit polyclonal antibodies specific for Glu and Tyr α-tubulin were the gift of Dr. G. Gundersen (Gundersen et al., 1984); 6-11B-1, a mouse monoclonal antibody specific for acetylated α-tubulin was the gift of Dr. G.

Piperno (Piperno and Fuller, 1985); Vim-2, a rabbit antiserum to vimentin that was the gift of Dr. V. Kalnins (Fedoroff et al., 1983); Lyt 2.2, a mouse monoclonal IgM (Cedarlane Lab. Ltd., Hornby, Ont.); YL½, a rat monoclonal antibody specific for Tyr α-tubulin (Dimension Lab. Inc., Mississauga, Ont.);

The following secondary antibodies were used: fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Miles-Yeda Ltd., Elkhart, IN); FITC-conjugated goat anti-mouse IgM (Kirkgaard and Perry, Gaitherburg, MD); tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG (Jackson Immunoresearch Lab., West Grove, PA); FITC-conjugated rabbit anti-sheep (Cedarlane Laboratories LTD., Hornby, Ont.); TRITC-conjugated rabbit anti-mouse cross-absorbed to rat antiserum and FITC-conjugated rabbit anti-rat cross-absorbed to mouse antiserum (Zymed Laboratories Inc., Dimension Laboratories Inc., Mississauga, Ont.).

Conjugate Formation. CTL/TC conjugates were formed by mixing equal volumes of each at 4 x 10^5 cells/ml and centrifuging at 300 x g for 5 min. The cells were resuspended using a 1 ml Pipetman and then incubated for 10 min at 37^0C. Following the incubation 300-400 μL of the cell suspension was layered onto half a 22 x 22 mm glass coverslip coated with poly-L-lysine. In experiments where the orientation of the MTOC was to be assayed the coverslips were prewarmed to 37^0C and the cells once layered onto the coverslips were incubated at 37^0C for the 10 min settling period. T cell samples to be processed for immunofluorescence microscopy were

layered onto poly-L-lysine coated cover slips at room temperature and allowed to settle for 10 min.

Coating Coverslips. Cover slips were coated with poly-L-lysine (Mol. Wt. 30,000-70,000, Sigma Chemical Co., St. Louis, MO) by layering a 1 mg/mL poly-L-lysine (in H_2O) solution over them and incubating at room temperature for 20 min. The solution was then recovered (it can be reused) and the cover slips were washed by dipping them in double distilled water ≈10 times and then allowed to air dry. Once coated the cover slips will be good for several days.

Immunofluorescence Staining. Cells to be stained with any combination of 5A6, 6-11B-1, YL ½, and the rabbit antisera to TYR, GLU, and vimentin were fixed in PEM (80 mM Pipes, 5 mM EGTA, 1 mM $MgCl_2$, pH 6.8) buffer containing 0.3% gluteraldehye (v/v) and 1% Triton X-100 (both purchased from Sigma) for 7 min, reduced with $NaBH_4$ (1 mg/ml) in PBS (PBS used in staining, 7.65g NaCl, 0.725g Na_2HPO_4, 0.212g KH_2PO_4 in 1 L) for 7 min, and then further extracted with 0.1% Tween-20 (J.T. Baker Chemical Co., Phillipsburg, NJ) in PBS for 15 min. The primary antibodies were diluted in PBS + 0.1% Tween-20 and ≈70 μL of this solution was applied to each half coverslip. The coverslips were incubated at room temperature for 45 min with the primary antibodies, washed 3 times for 4 min in PBS, and then incubated for 45 min with the secondary antibodies (diluted in PBS), washed 3 times for 4 min in PBS, counterstained

for 1 min with 1 μg/mL Hoescht 33258, and then mounted in a 50% glycerol/PBS solution containing 0.1% (w/v) phenylenediamine. When double labellings were done the two primaries and then the two secondaries were applied simultaneously.

Cells that were double labelled with 5A6 and the rabbit autoimmune serum to the centrosome-associated protein were fixed in cold (-20^{0}C) methanol for 5 min. The cells were then washed 3 times in PBS for 4 min and the antibodies, diluted in PBS, were applied as described above.

Conjugates to be stained with Lyt 2.2 were formed as described above. Following the 10 min incubation at 37^{0}C the cells were placed in 1.5 mL eppendorf tubes, centrifuged for 30 sec at 13,000 rpm, gently resuspended in 50 μL of ice-cold media containing the primary antibody, incubated on ice for 30 min, diluted by the addition of 1.4 mL of ice-cold medium not containing antibody, and centrifuged again. This procedure was repeated with the secondary antibody after which the cells were layered onto ice-cold poly-L-lysine coated cover slips and allowed to settle for 10 min, washed by dipping in PBS, and fixed with 3.7% formaldehyde in PEM buffer for 10 min.

ELECTRON MICROSCOPY.

T cells. T cells were fixed for electron microscopy by resuspension in 0.1M phosphate buffer (pH 7.2) containing 4%

glutaraldehyde for 30 min at room temperature. Once fixed the cell suspension was placed in 50 μL disposable capillary tubes (Dade Diagnostics Inc., Miami, Fl.). The tubes were plugged with plasticene and centrifuged in an IEC clinical centrifuge with haematocrit head at 650 x g for 20 min. The cell pellets produced were removed from the capillary tubes, post-fixed in 1% OsO_4 in 0.05M phosphate buffer (pH 7.2) for 20 min on ice, dehydrated through alcohol series, propylene oxide (BDH Inc., Toronto, Ont.) and embedded in Epon-Araldite (30g DDSA, 11.1g Araldite, 15.5g Epon, 1.4g DMP-30, J.B. EM Services Inc., Dorval, Que).

Conjugates. Conjugates were formed as described above, layered onto poly-L-lysine coated support film (Bellcon Glass Inc., Vineland, NJ), and then fixed and dehydrated as described above. The support film was then inverted on a glass microscope slide that had been sprayed and polished 3 times with Slipcone Release Spray before being overlayed with Epon-Araldite. After polymerization the thin sheet of Epon-Araldite containing the cells was detached from both the support film and the slide, conjugates were located by bright field microscopy, and then cut out and glued onto Epon-Araldite blocks with Krazy glue (Krazy Glue Inc., Chicago, IL).

Microscopy. Sections were stained for 2 h in uranyl acetate (1% aqueous) and for 7 min in lead citrate (Reynolds, 1963) before being examined in a Philips 201C electron microscope.

GEL ELECTROPHORESIS AND AUTORADIOGRAPHY.

30 μCi/ml ^{35}S-methionine (> 100 mCi/mM, NEN Dupont Canada Ltd.) was added to control and treated cells at a concentration of 2 x 10^6/mL. The cells were then incubated at 37^0C in 5% CO_2 for 6 h and washed twice in PBS (pH 7.0) prior to resuspension in 0.06 M Tris-HCl, pH 6.8, containing 2% (w/v) SDS. To reduce their viscosity these samples were each given three 5 sec sonications with a Braun-Sonic 2000 sonicator fitted with a probe 3 mm in diameter. The protein concentration of each sample was determined by the method of Sandermann and Strominger (1972). Equal amounts of total cell protein were added to each well and then separated by uniform-concentration polyacrylamide gels as described by Laemmli (1970) using a 'Mini-Protein II' dual slab cell apparatus (Bio-Rad Laboratories Ltd., Richmond, CA). The gels were stained with 0.1% Coomassie Brilliant Blue R-250 (Bio-Rad) in 25% methanol and 10% acetic acid and then dried between two pieces of 'BioGelWrap' (BioDesign Inc., Carmel, NY) and exposed to X-ray film (Kodak XAR-5).

STATISTICS

The cytolytic activity assays generated continuous, normally distributed data points that were amenable to analysis by one-way analysis of variance tests. The MTOC orientation data was

generated by placing the cells scored into one of two mutually exclusive groups. That is, the cells were either scored as oriented towards or away from the TC. Chi-square tests were used to determine if the frequency of the cells oriented towards was significantly altered by the different treatments. Both tests were performed using the 'Epistat Statistical Package' written by T.L. Gustafson, 1705 Gattis School Rd., Round Rock, Texas 78664.

CHAPTER 1

INTRODUCTION

The small unactivated lymphocytes that continuously circulate throughout the body are each committed to a single antigenic specificity. When a foreign antigen is presented a proliferation and expansion of the clones capable of responding to that particular antigen occurs, resulting in the generation of cytotoxic or other effector cells. The proliferative response is characterized by large increases in the volume of the nucleus and cytoplasm (Setterfield et al., 1983; 1985; Bladon et al., 1988), an increase in the number of microtubules assembled from the centrosome (Waterhouse et al., 1983), and an extensive reorganization of the intermediate filament system (Paulin-Levasseur and Brown, 1987a).

The function of the particular effector cells used in this study, CTLs, is to recognize and lyse the specific TCs that elicited their generation. Upon binding to an appropriate TC a rapid and coordinate orientation of the MTOC/GA occurs within the CTL so that the two organelles face the TC. Experiments in which the MTOC/GA was reversibly prevented from orienting towards the TC, by treatment of the cells with microtubule disrupting drugs or by the removal of Ca^{++} from the medium (Kupfer et al., 1983; Kupfer et al., 1985), suggested that the orientation of the MTOC/GA was microtubule and Ca^{++} dependent and a prerequisite for TC lysis. The rapid movement of the MTOC described and the suggested link to

an easily assayable function, TC lysis, made CTLs an attractive system in which to study cytoskeletal dynamics and functions.

Kirschner and Mitchison (1986) have proposed a model for alterations in microtubule organization based on the selective stabilization of a subset of microtubules within a dynamic array. The production of antibodies that specifically recognize two posttranslationally modified forms of α-tubulin (Gundersen et al., 1984; Piperno and Fuller, 1985) has facilitated the testing of this model. These modifications have no known function, but are preferentially associated with, and can be used as markers for, stable microtubules (Gundersen et al., 1987; Piperno et al., 1987).

Recently it has become clear that intermediate filaments in several cell types are highly dynamic components of the cytoskeleton (for reviews see Steinert and Liem, 1990; and Stewart, 1990). The results of Paulin-Levasseur and Brown (1987a,b) indicate that the vimentin intermediate filaments of lymphocytes also are highly dynamic. The function(s) of such dynamic reorganizations are not known in lymphocytes or for any other cell type. It has been suggested that the functions of intermediate filaments, whose subunits exhibit remarkable tissue-specific expression, may be readily observable only in cells that still perform their differentiated functions (Gawlitta et al., 1981; Klymkowsky et al., 1983). The primary cultures of CTLs used in this study provide such a system.

Before this system could be exploited it was first necessary

that I characterize the CTLs generated by the method used in this study and the conditions required by them for efficient TC lysis. Morphological studies by EM, immunofluorescence examination of the microtubule and intermediate filament systems, and the increase in cell size and microtubule number in response to mitogenic stimulation all indicate that the effector cells isolated are no longer proliferating. Immunofluorescence staining with antibodies specific to posttranslationally modified tubulins showed that the two forms associated with stable microtubules, detyrosinated (Glu) and acetylated, are not prevalent in freshly isolated effector cells; however, treatment with the microtubule stabilizing drug taxol showed that the cells possess the enzymatic machinery to perform both posttranslational modifications. Cytolytic activity assays demonstrated that the effector cells are able to recognize and lyse their specific TC and that TC lysis is dependent upon the reorientation of the MTOC and the presence of Ca^{++}. The selective stabilization model was then tested in CTLs by immunofluorescence staining with antibodies to Glu and acetylated α-tubulin as markers for stable microtubules. The selective stabilization model would have been supported by the appearance of a subset of stable microtubules following, or concurrent with, MTOC/GA orientation. The results presented here do not support the model. Conjugate formation was found to result in a significant increase in the number of filamentous vimentin aggregates observed in CTLs.

RESULTS

Ultrastructure of the T cell populations obtained. The T cell populations isolated from a Balb/C mouse injected intraperitoneally 10-12 days earlier with $3x10^7$ EL4 cells will be referred to as effector cells. These effector cell populations consist of uniformly small cells of 5-8 μm in diameter. Figure 1 is an electron micrograph of a CTL bound to an EL4 which will be referred to as TCs. This cell has a diameter of 7 μm and a thin layer of dense, granular cytoplasm. The nucleus, which occupies most of the cell volume, has highly aggregated peripheral chromatin and a small undifferentiated nucleolus. The centrioles of the centrosome are visible and located adjacent to a nuclear cleft. The few organelles found in these cells are usually located near the centrosome.

The microtubule array of the effector cell populations. Immunofluorescence staining with antibody to tubulin reveals a sparse microtubule system which forms a radial array extending from the centrosome and around the periphery of the cell near the plasma membrane (Fig. 2a). It has been reported that stimulation of purified populations of unactivated mouse splenic T lymphocytes by the mitogen con A results in a large increase in cell volume, cellular tubulin content, and up to a 5-fold increase in the numbers of assembled microtubules (Waterhouse et al., 1983). A

Figure 1.1 Electron Micrograph of a CTL Conjugated to a TC. The CTL is identified on the basis of its smaller size and regular shape. The nucleus (N) of the CTL occupies most of the cell volume, has highly aggregated peripheral chromatin and a small undifferentiated nucleolus (Nu). The centrosome (C) is situated beneath the portion of the plasma membrane in contact with the TC. Magnification 12,000x.


Nu
C

similar increase in cell size and microtubule content is seen in the effector cell populations of this study following mitogenic stimulation (Fig. 2d,g). Using an antibody to tyrosinated (Tyr) α-tubulin as a marker for dynamic microtubules, and antibodies to Glu and acetylated α-tubulin as markers for stable microtubules (Gundersen et al., 1987; Piperno et al., 1987), the composition of the microtubule array of these T cell populations was examined.

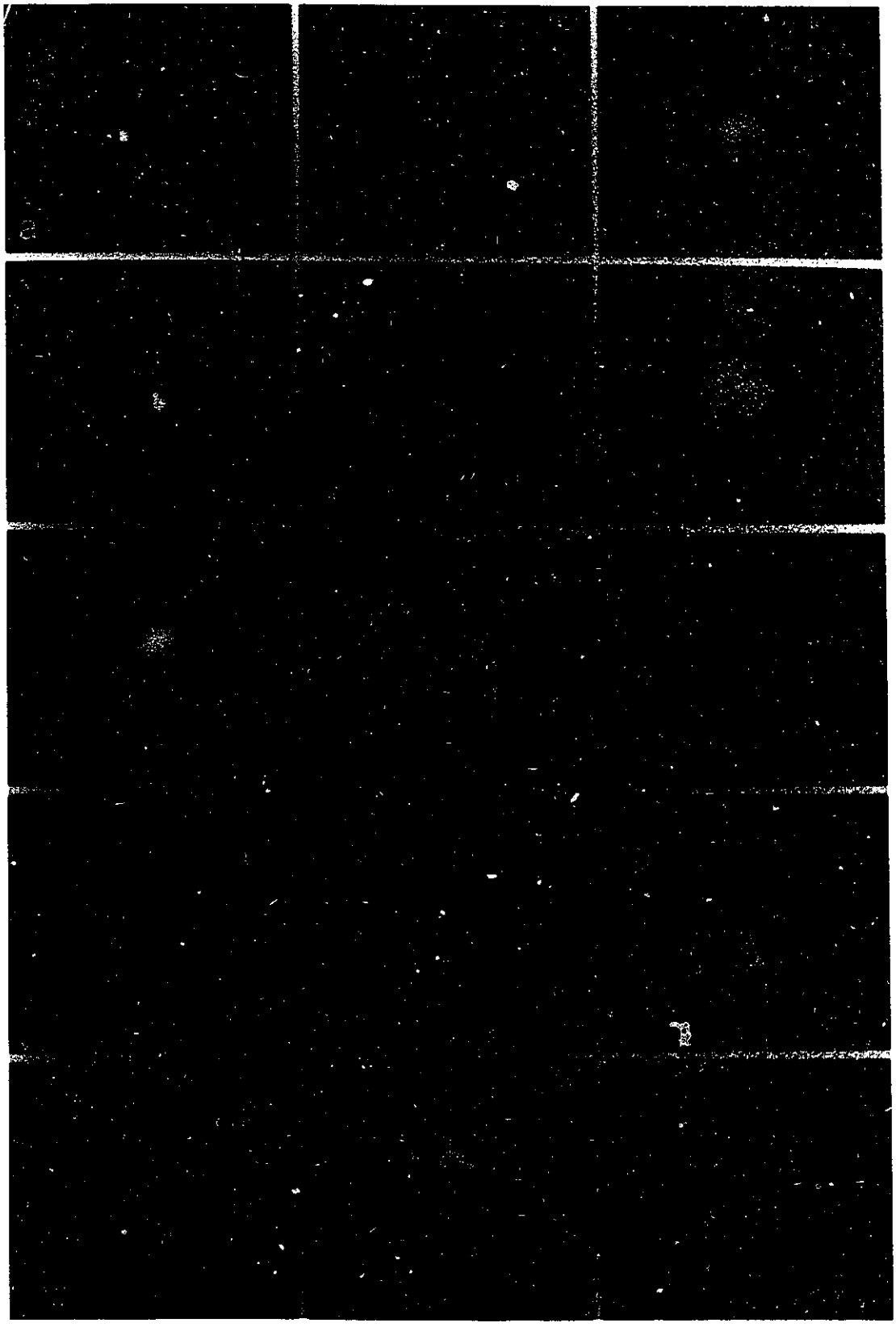
Figure 2 demonstrates that most, if not all, microtubules in freshly isolated (Fig. 2b), 24 h stimulated (Fig. 2e), and 48 h stimulated (Fig. 2h) effector cells are recognized by the antibody to Tyr α-tubulin. In Figure 3 it can be seen that the centrosome and some microtubules in the centrosomal region are stained by the antibody to Glu α-tubulin in freshly isolated effector cells (Fig. 3b). Culturing these cells in αMEM supplemented with 10% FCS and 1000 U/ml of penicillin and streptomycin (Fig. 3e) or stimulating them with mitogen (Fig. 3h) leads to a loss of microtubule staining by this antibody. To determine the extent to which these cells are capable of the posttranslational modification of α-tubulin the 24 h stimulated cells were treated with 10 μM of the plant alkaloid taxol. A 4 h taxol treatment results in the stabilization and then aggregation of microtubules to form one to several microtubule bundles in ≥ 90% of the cells (Brown et al., 1985) and a reappearance of microtubules recognized by the antibody to Glu α-tubulin (Fig. 3k). 18 h after the addition of taxol nearly all the microtubules are recognized by this antibody (Fig. 3n).

No microtubules in freshly isolated (Fig. 4b) or 24 h

Figure 1.2. Distribution of Tyrosinated α-Tubulin in Effector Cell Populations. Double immunofluorescence staining of cells from freshly isolated (a-c), 24 h (d-f), and 48 h (g-i) con A-stimulated effector cell populations showing the large increase in the numbers of assembled microtubules that accompanies stimulation. The micrographs a,d,g show the distribution of all microtubules detected with the mouse monoclonal antibody 5A6. The corresponding micrographs b,e,h show the distribution of Tyr α-tubulin detected using rabbit antiserum. Most, if not all, the microtubules are recognized by the antiserum to tyrosinated α-tubulin. The Hoechst 33258 staining in the right column shows the increase in nuclear size that accompanies stimulation. Magnification 1,500x.

a
b
c
d
e
f
g
h
i

Figure 1.3. Distribution of Detyrosinated α-Tubulin in Effector Cell Populations. Double immunofluorescence staining of effector cells from populations that were freshly isolated (a-c), cultured 24 h in αMEM + 10% fetal calf serum (d-f), stimulated with con A for 24 h (g-i), stimulated with conA for 24 h and then treated with 10 μM taxol for 4 h (j-l), or 18 h (m-o) in the continued presence of con A. The micrographs of the left column (a,d,g,j,m) show the distribution of all microtubules detected with the mouse monoclonal, 5A6, those of the middle column (b,e,h,k,n) show the distribution of microtubules recognized by rabbit antiserum specific for detyrosinated α-tubulin, and those of the right column (c,f,i,l,o) shows the nuclei of these cells detected by Hoechst 33258 staining. It is evident that culturing these cells or stimulating them with mitogen leads to a loss, and stabilization of the microtubules with taxol leads to a recovery, of microtubule staining by the antibody to detyrosinated α-tubulin. Magnification 1,500x.


a
b

stimulated (Fig. 4e) effector cells are recognized by the antibody to acetylated α-tubulin; however, the treatment of the 24 h stimulated cells with taxol leads to the appearance of microtubules stained with this antibody within 4 h (Fig. 4h) and the recognition of most microtubules 18 h after the addition of taxol (Fig. 4k).

Vimentin intermediate filaments in effector cell populations. The vimentin intermediate filaments form a complex pattern that often extends from the MTOC and is partially coincident with the radial pattern of the microtubules (Paulin-Levasseur and Brown, 1987a,b; Fig. 5a,d); however, the distribution of the intermediate filaments, unlike that of the microtubules, is unpredictable and varies markedly from one cell to the next. Thick coils of intermediate filaments that are not coincident with the microtubules are regularly seen (Fig. 5b,e) and in 20-30% of the effector cells intermediate filaments are not detectable at the light microscope level (Fig. 5c,f).

The fine structure of the vimentin intermediate filaments and their variable distribution suggested that intermediate filaments may have been present even in those cells where I was unable to detect them. Treatment of freshly isolated effector cells with 10 μM taxol for 4 h leads to the formation of one to a few large microtubule bundles (Fig. 6a,b). The taxol treatment also results in the colocalization of the vimentin filaments with the microtubule bundles extending from the centrosome (Paulin-Levasseur and Brown, 1987a,b; Fig. 6e,f). The concentration of the vimentin

Figure 1.4. Distribution of Acetylated α-Tubulin in Effector Cell Populations. Double immunofluorescence staining of effector cells from populations that were freshly isolated (a-c), stimulated with con A for 24 h (d-f), stimulated with con A for 24 h and then treated with 10 μM taxol for 4 h (g-i), or 18 h (j-l) in the continued presence of conA. The micrographs of the left column (a,d,g,j) show the distribution of microtubules recognized by the rabbit antiserum specific for tyrosinated α-tubulin, those of the middle column (b,e,h,k) show the distribution of microtubules recognized by the mouse monoclonal antibody, 6-11B-1, specific for acetylated α-tubulin, and those of the right column (c,f,i,l) show the nuclei of these cells detected by Hoechst 33258 staining. No microtubules are recognized by the 6-11B-1 antibody in freshly isolated or in stimulated effector cells. Treatment of 24 h stimulated cells with taxol leads to the appearance of microtubules stained with this antibody within 4 h and the recognition of most microtubules within 18 h. Magnification 1,500x.

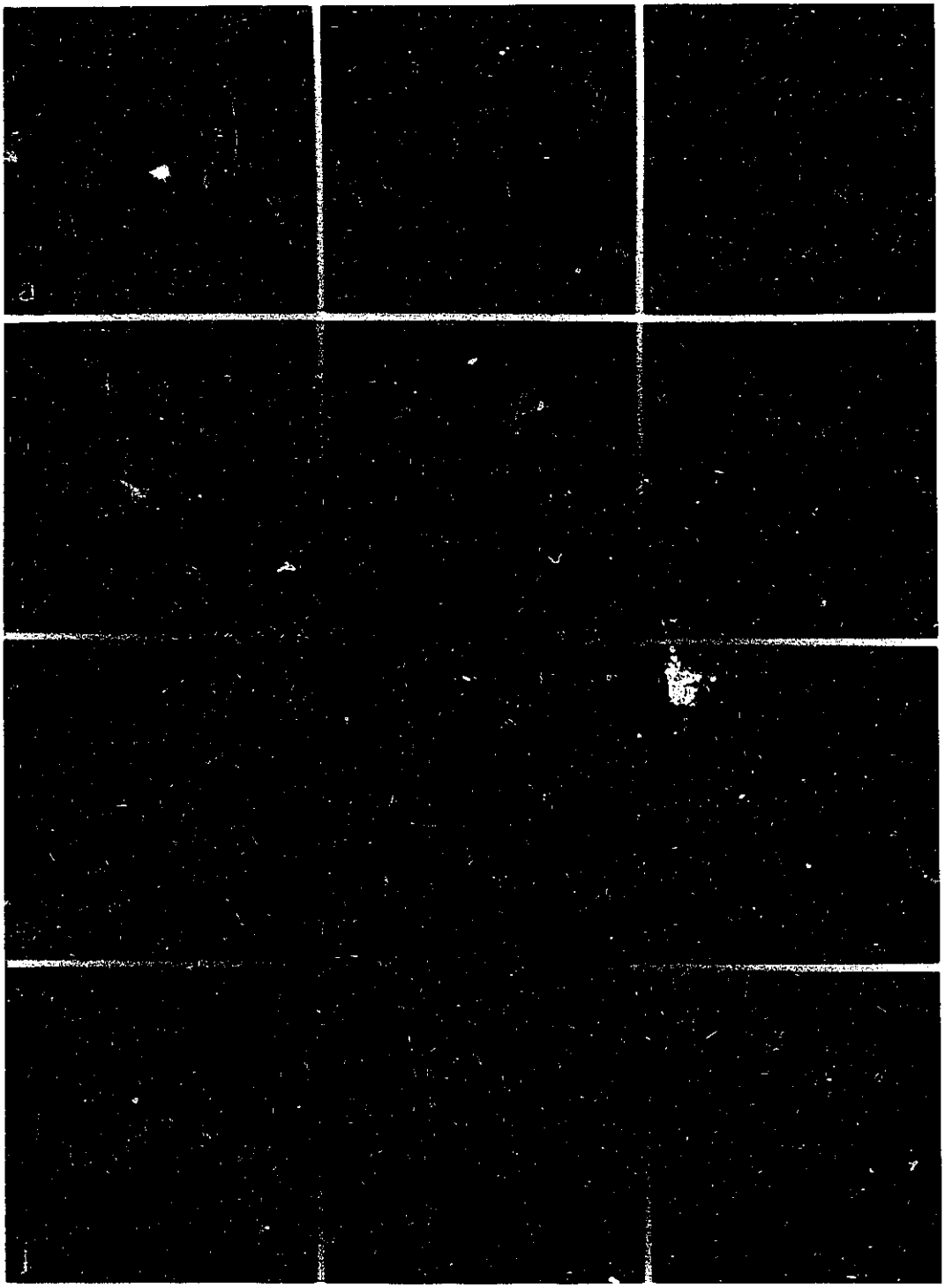
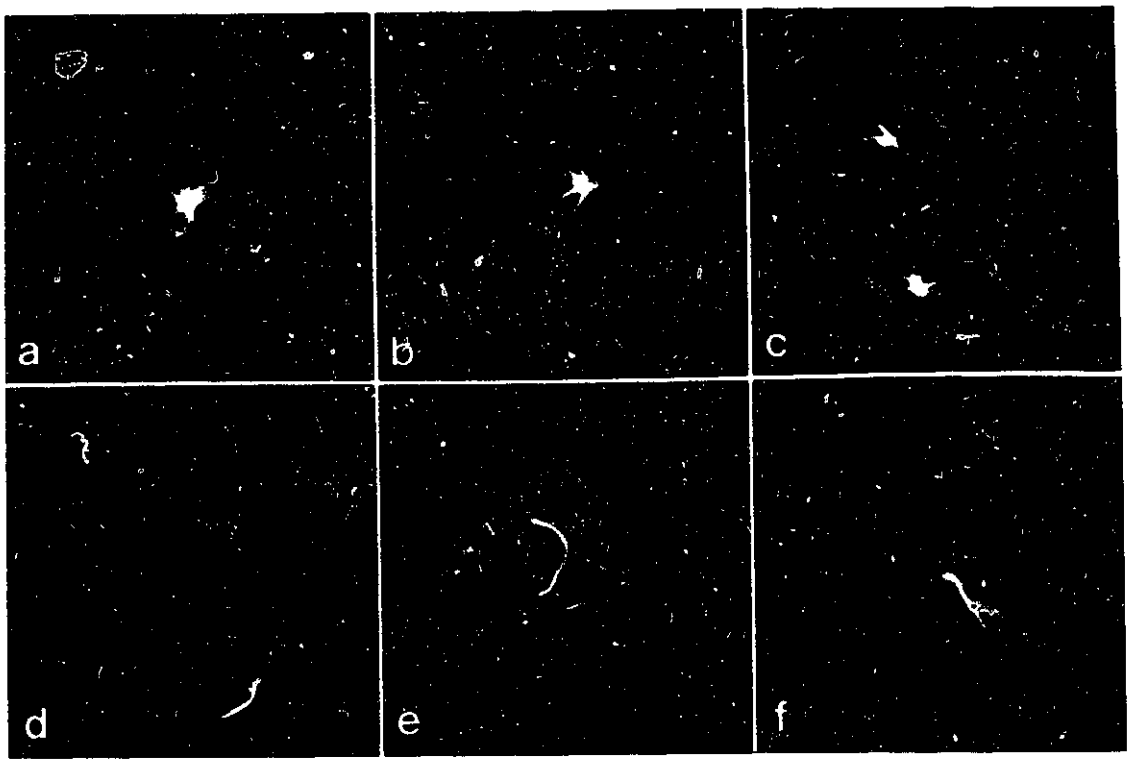


Figure 1.5. Distribution of Intermediate Filaments in Freshly Isolated Effector Cell Populations. Double immunofluorescence staining with the mouse monoclonal 5A6 (a-c) and a rabbit antiserum to vimentin (d-f) of freshly isolated effector cells showing the variability in distribution of vimentin intermediate filaments in relation to the microtubule distribution. Magnification 1,500.


a
b
c
d
e
f

intermediate filaments in the area of an easily detectable structure, the taxol-induced microtubule bundle, facilitates their detection in most cells (Paulin-Levasseur and Brown, 1987a,b). However, in effector cell populations there are still cells in which there is no detectable vimentin staining following taxol treatment.

Treatment of the freshly isolated T cells with 4 μg/mL of the microtubule disassembling drug colchicine for 4 h results in the disassembly of most microtubules, leaving only a few short microtubules associated with the centrosome (Fig. 6c). In these cells vimentin forms a ring-like aggregate (Paulin-Levasseur and Brown, 1987a,b; Fig. 6g). Infrequently ring-like aggregates are also seen in untreated cells that have normal microtubule networks (Fig. 6d,h).

Specific target cell recognition by CTLs. To demonstrate that the intraperitoneal injection of EL4 cells results in the generation of CTLs that specifically recognize EL4 cells as targets, the cytolytic activities of splenocytes isolated from injected and control mice were tested against ^{51}Cr loaded EL4 or YAC-1 cells. Figure 7 clearly shows that the CTLs generated are able to lyse EL4 cells; whereas, there is no increase in cytolytic activity against the YAC-1 cells. This indicates that the cells generated by this procedure are specific for EL4 cells. The cytolytic activity observed increases until an effector cell to TC ratio of nearly 50:1 is reached after which further increases in

Figure 1.6. Effect of Microtubule Specific Drugs on the Distribution of Intermediate Filaments in Effector Cells. Freshly isolated effector cells were double stained using a the mouse monoclonal 5A6 (a-d) and a rabbit antiserum to vimentin (d-f) to show the distribution of vimentin following taxol (a,e and b,f) and colchicine treatment (c,g). The micrographs d,h demonstrate that ring-like vimentin aggregates can also be observed in control cell populations. Magnification 1,500x.

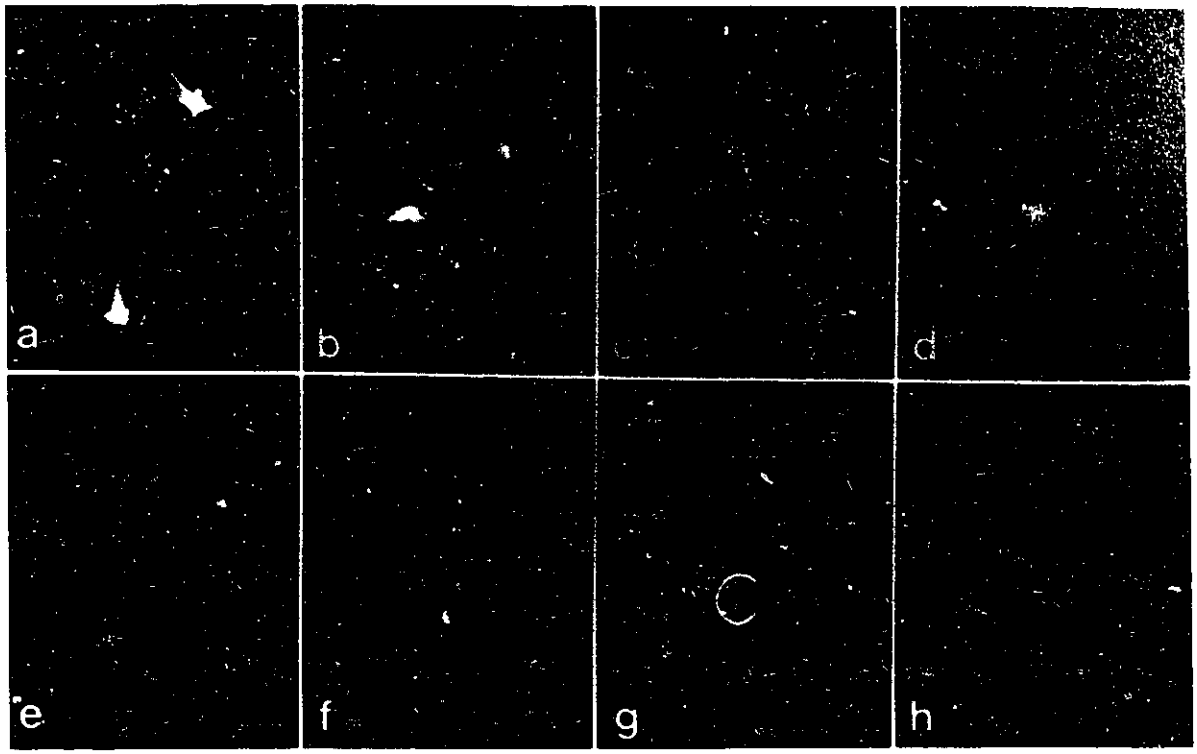
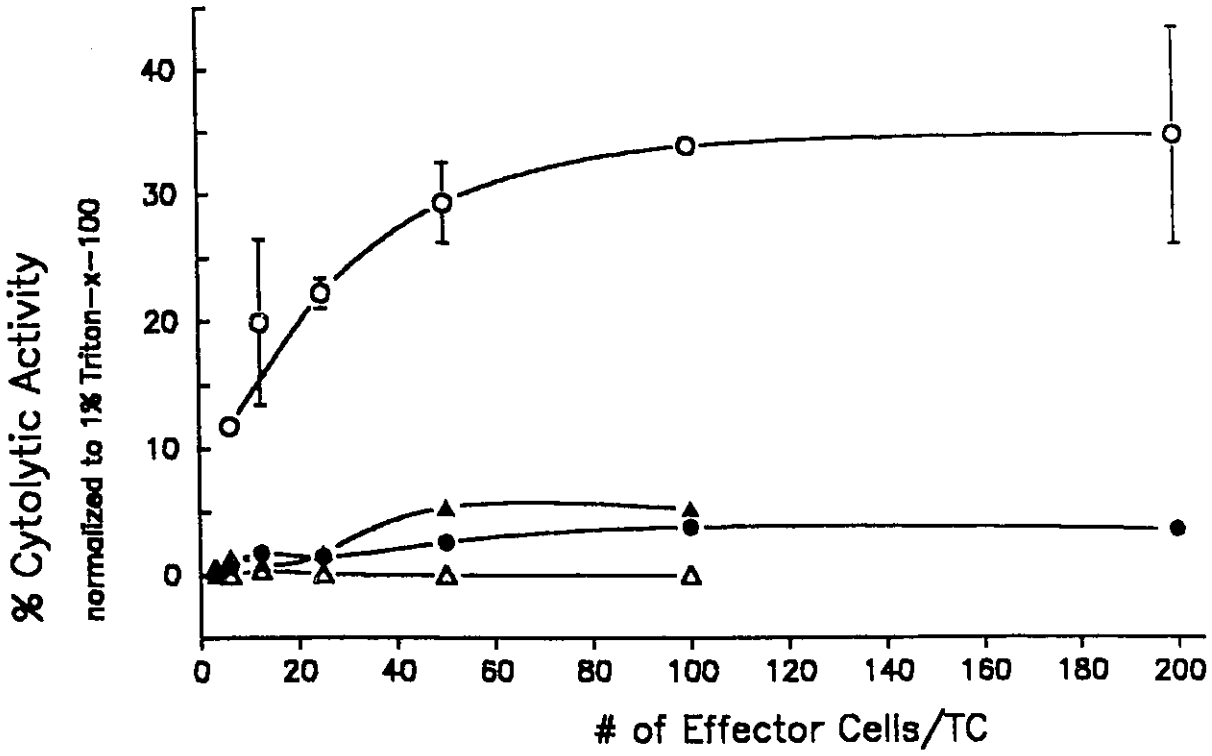

a
b
c
d
e
f
g
h

Figure 1.7. Injection of Allogeneic Tumour Cells Results in the Generation of CTLs. The graph compares the relative cytolytic activity of the cells isolated from control mice (filled symbols) and mice injected intraperitoneally 10-12 days earlier with $3x10^7$ EL4 cells (open symbols) against ^{51}Cr-loaded EL4 (circles) and YAC-1 (triangles) cells. All samples were done in triplicate and the values shown are the mean ±SD of a typical experiment.


% Cytolytic Activity
normalized to 1% Triton-x-100
40
30
20
10
0
0
20
40
60
80
100
120
140
160
180
200
# of Effector Cells/TC

the effector cell to TC ratio do not result in markedly higher cytolytic activity.

Ca^{++} requirement for orientation of the MTOC and cytolytic activity. Since both Ca^{++}-dependent and Ca^{++}-independent cytolytic pathways have been reported (Ostergaard and Clark 1987; Young et al., 1988) I examined the requirements for Ca^{++} in MTOC orientation and cytolytic activity by the effector cells of this study. The effect of Ca^{++} on MTOC orientation was determined by forming conjugates in complete medium (DMEM + 0.5% BSA) or complete medium supplemented with either 2.5 mM EGTA + 2.5 mM $MgCl_2$ to deplete free Ca^{++} or 2.5 mM EGTA + 2.5 mM $MgCl_2$ + 5.0 mM $CaCl_2$ as a control (see Table 1).

Table 1. The free Ca^{++} levels of the indicated medium formulations were measured with a Ca^{++}-selective electrode (Orion Research, #932000).

Medium	Free Ca^{++} (mM)
Complete Medium (DMEM + 0.5% BSA)	1.8
Complete Medium + 2.5 mM EGTA + 2.5 mM $MgCl_2$	0.7×10^{-6}
Complete Medium + 2.5 mM EGTA + 2.5 mM $MgCl_2$ + 5.0 mM $CaCl_2$	3.2

To determine the percentage of CTLs having oriented their MTOC towards the TC, conjugates were formed in the presence or absence of Ca^{++}, stained with anti-tubulin, and examined by epifluorescence

microscopy. The small spherical uniformly-sized lymphocytes are easily distinguished from the much larger variably-shaped TCs. Figure 8 shows that when conjugates are formed in complete medium >90% of the CTLs orient their MTOC to face the TC by the time the cells are fixed 25 min after mixing the two cell populations. In contrast, when conjugates are formed in the absence of Ca^{++} only ≈50% of CTLs have their MTOC oriented towards the TC, which is the percentage expected from random binding of the CTLs. Orientation towards the TC is unaffected by the presence of EGTA and Mg^{++}, provided that free Ca^{++} is available. Furthermore, the addition of Ca^{++} to conjugates formed in the absence of Ca^{++} results in the rapid orientation of the MTOC in the CTLs, to the levels seen in control cells, within 5 min.

To determine whether or not, once oriented, the continued presence of Ca^{++} was required to maintain the orientation of the MTOC towards the TC, conjugates were formed in complete medium, allowed to orient, and then fixed up to 40 min after the removal of Ca^{++}. Figure 9 illustrates that once it is oriented to face the TC the subsequent removal of Ca^{++} from the medium has no effect on the position of the MTOC.

Ca^{++} requirement for cytolytic activity. The effect of Ca^{++} on cytolytic activity was determined by mixing CTLs with ^{51}Cr-labelled TCs in the presence or absence of Ca^{++}. The results of these experiments are shown in Figure 10. Clearly, the removal of Ca^{++} has a strong inhibitory effect on cytolytic activity, reducing

Figure 1.8. Effect of Ca^{++} on MTOC Orientation. The histogram compares the effect of the presence or absence of Ca^{++} on the percentage of CTLs with their MTOC towards a bound TC. The control cells (A) were incubated in complete medium. The addition of 2.5 mM EGTA and 2.5 mM $MgCl_2$ does not have a significant effect on the orientation of the MTOC provided that 5.0 mM $CaCl_2$ is also present in the medium (B) or added 5 min prior to the preparation of the conjugates for immunofluorescence observation (C) ($X=4.3329$, $p=0.1146$). The addition of 2.5 mM EGTA and 2.5 mM $MgCl_2$ alone (D) does have a significant effect on the orientation of the MTOC ($X=40.1569$, $p \leq 1 \times 10^{-8}$). Triplicate counts of $\geq$ 50 cells found in random fields were made of each sample and the values shown are the average of the mean of two experiments ±SE.

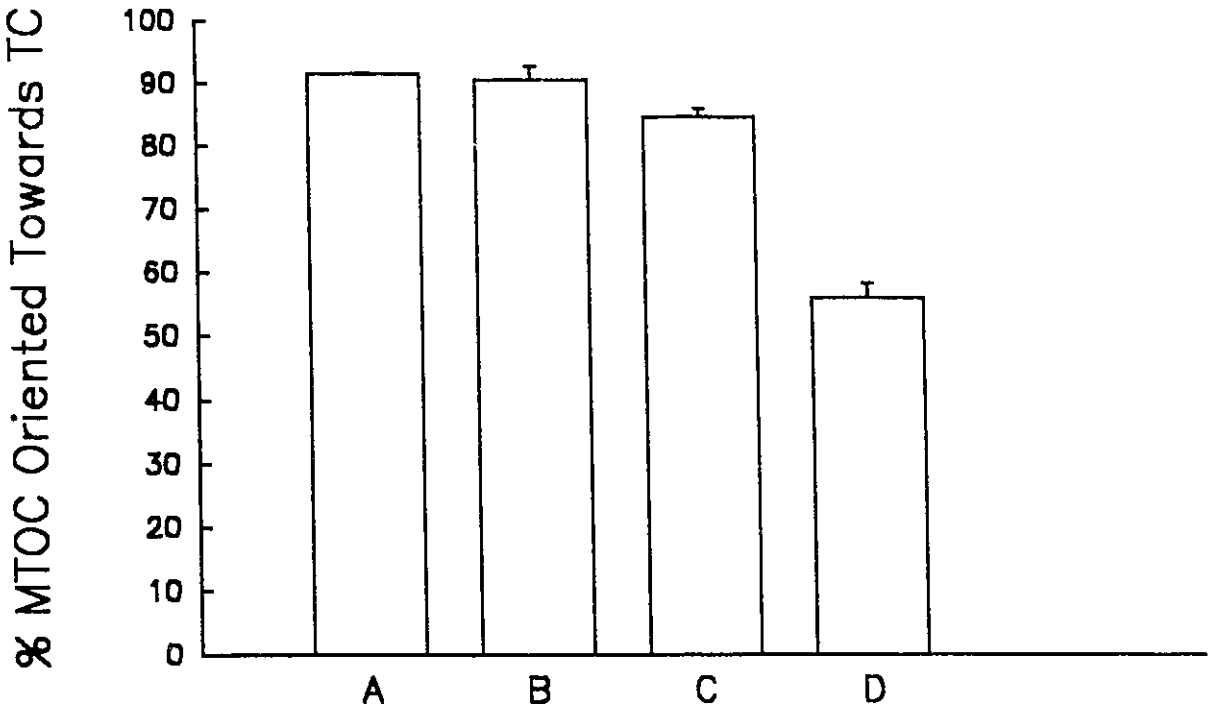
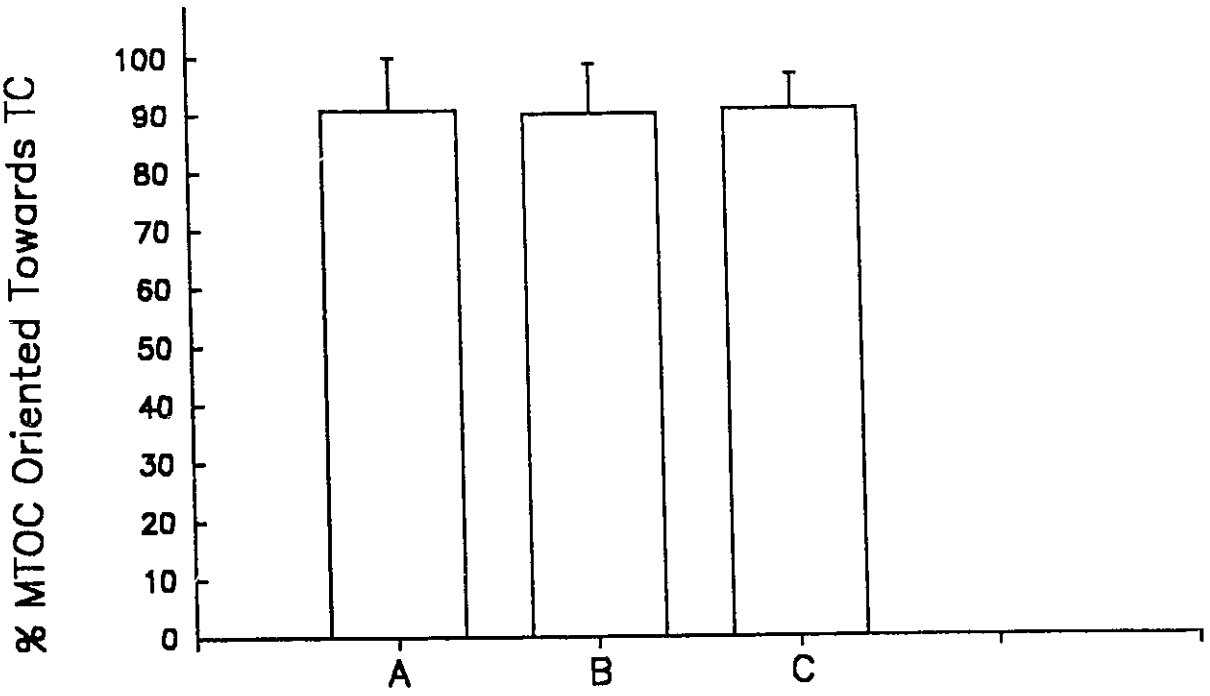

% MTOC Oriented Towards TC
100
90
80
70
60
50
40
30
20
10
0
A
B
C
D

Figure 1.9. Effect of the Removal of Ca^{++} on the Orientation of the MTOC. Conjugates formed in complete medium, allowed 25 min to orient their MTOC towards a bound TC, were then either promptly prepared for immunofluorescence observation (A), or the medium was replaced with modified medium containing 2.5 mM EGTA and 2.5 mM $MgCl_2$ and the conjugates were incubated an additional 20 min (B), or 40 min (C) before fixation. Removal of Ca^{++} from the medium after conjugate formation has no effect on the orientation of the MTOC ($X=0.0756$, $p=0.9629$). Triplicate counts of $\geq$ 25 cells were made of each sample and the values shown are the mean of two experiments $\pm$SE.


% MTOC Oriented Towards TC
100
90
80
70
60
50
40
30
20
10
0
A
B
C

it to 12% of control levels. As was found with orientation, cytolytic activity is unaffected by the presence of EGTA and Mg^{++} provided that free Ca^{++} is available.

The coincident loss of the ability to orient and of cytolytic activity caused by the removal of Ca^{++} supports the suggestion that orientation of the MTOC is a prerequisite for lysis. Having found that this orientation towards the TC persists in the absence of Ca^{++}, it was of interest to examine the effect of removing Ca^{++} on cytolytic activity after orientation had occurred. These results (Fig. 11) demonstrate that, for significant cytolytic activity, Ca^{++} is required beyond the initial 25 min of the incubation period. When a cytolytic assay is carried out in the presence of Ca^{++} the release of ^{51}Cr from the labelled TCs into the supernatant occurs at a constant rate during the initial 2 h of the assay period (Fig. 12).

Effect of conjugate formation on the CTL cytoskeleton. The working hypothesis of this study was that selective stabilization of a subset of microtubules is the mechanism of orientation of the MTOC towards the TC. Most, if not all, microtubules in effector cells are composed of Tyr α-tubulin (Fig. 2). Therefore, finding a subset of microtubules composed of Glu or acetylated α-tubulin following conjugation would suggest that that particular subset is being selectively stabilized and support the hypothesis. To determine the effect of conjugate formation on microtubule composition, conjugates were stained with antibodies to Tyr, Glu,

Figure 1.10. Ca^{++} Requirement for Cytolytic Activity. The histogram compares the effect of the presence or absence of free Ca^{++} on cytolytic activity. A. Complete medium. B. The presence of EGTA has no effect on cytolytic activity provided there is free Ca^{++} in the medium ($F=2.1020$, $DF_1=1$, $DF_2=10$, $p=0.2207$). C. Removal of most of the free Ca^{++} in the medium by the addition of 2.5 mM EGTA results in significant loss of cytolytic activity ($F=469.5426$, $DF_1=1$, $DF_2=10$, $p=2.69\times10^{-5}$). All samples were done in triplicate and the values shown are the average of the mean of two experiments ±SE.

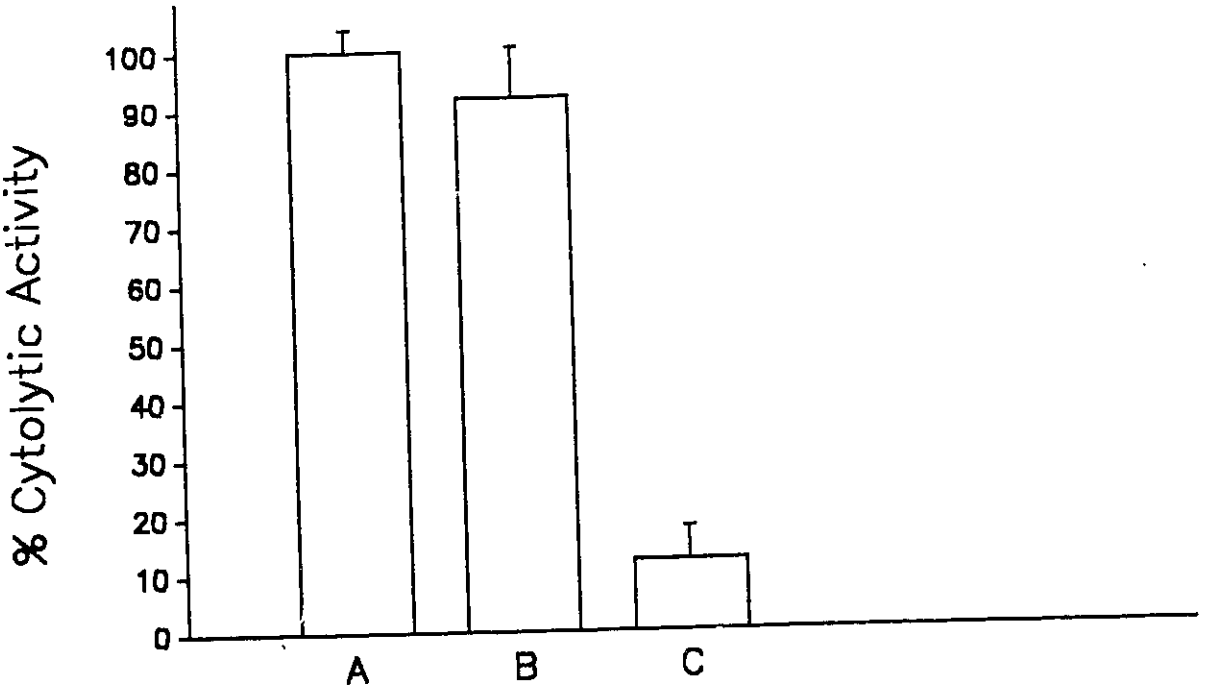

% Cytolytic Activity
100
90
80
70
60
50
40
30
20
10
0
A
B
C

Figure 1.11. Effect on Cytolytic Activity of Removing Ca^{++} After Allowing Orientation to Occur. After a 10 min incubation at $37^{0}C$ the cytolytic assay plates were removed from the incubator and the complete medium withdrawn and replaced with either complete medium (B), or medium supplemented with 2.5 mM EGTA and 2.5 mM $MgCl_2$ (C). These plates were then returned to the incubator and the effect of the medium replacement on cytolytic activity was compared to that of cells that were incubated in complete medium throughout (A). The removal of free Ca^{++} after allowing orientation caused a significant inhibition of cytolytic activity (B=C, F=139.8624, DF_1=1, DF_2=9, $p=7.03x10^{-6}$) while the replacement of the medium did not (A=B, F=5.0258, DF_1=1, DF_2=10, p=0.0599). All samples were done in triplicate and the values shown are the average of the mean of two experiments ±SE.

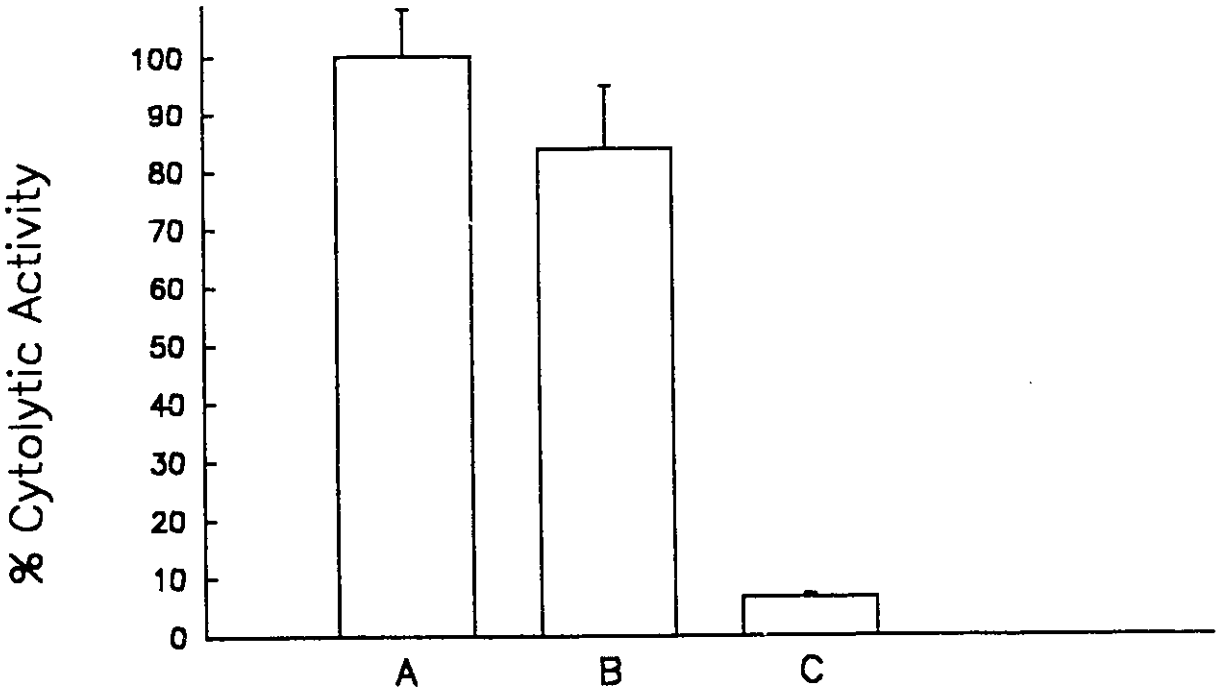
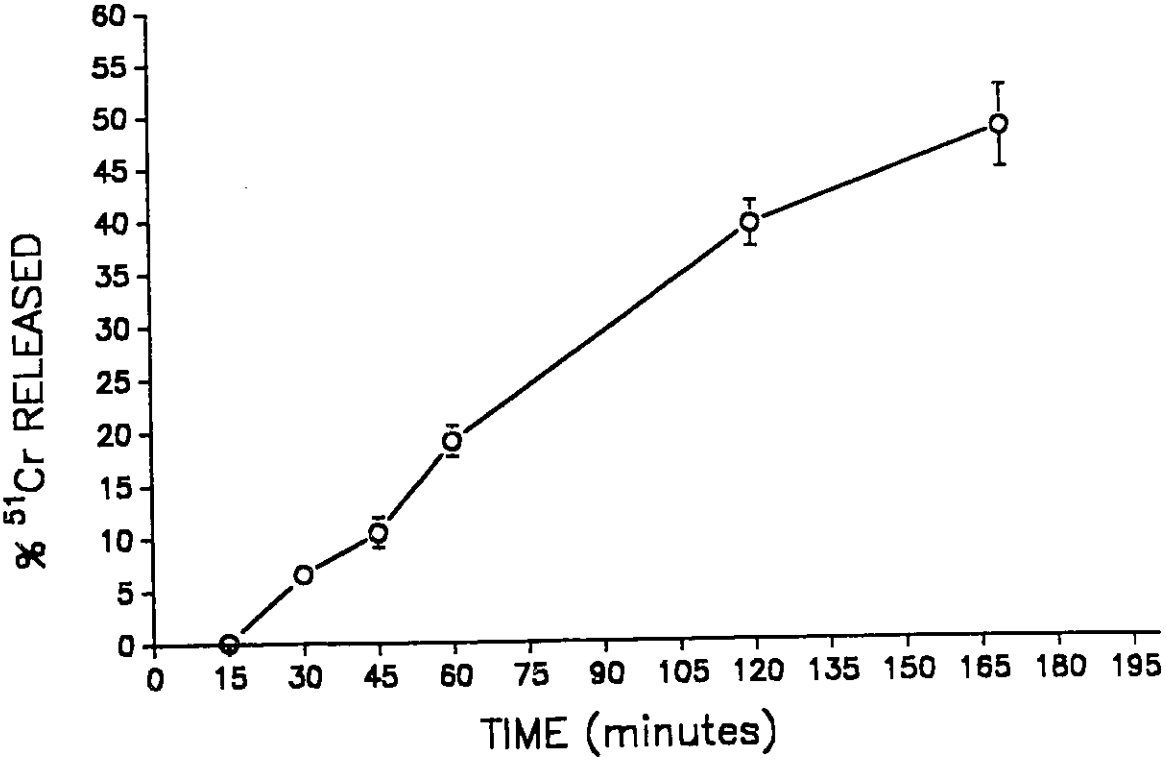

% Cytolytic Activity
100
90
80
70
60
50
40
30
20
10
0
A
B
C

Figure 1.12. Time Course of ^{51}Cr Release. After the start of a cytolytic assay plates were removed at the times indicated and the amount of ^{51}Cr released into the supernatant determined. The graph shows that ^{51}Cr is released from the labelled TCs into the supernatant at a constant rate during the first 3 h of the assay period. The effector cell:TC ratio used was 25:1. All sample were done in triplicate and the values shown are the mean ±SD of a typical experiment.


% 51Cr RELEASED
60
55
50
45
40
35
30
25
20
15
10
5
0
0
15
30
45
60
75
90
105
120
135
150
165
180
195
TIME (minutes)

and acetylated α-tubulin. Most, if not all, of the microtubules in conjugated CTLs are labelled with the mouse monoclonal antibody,5A6 (Fig. 13a,d), or the rat monoclonal antibody to Tyr α-tubulin, YL 1/2 (Fig. 13g). Double staining with rabbit antiserum specific for Tyr α-tubulin demonstrates that this antibody recognizes all the microtubules labelled with 5A6 (Fig 13b). Only the centrosome and a few microtubules in the centrosomal region are labelled by the rabbit antiserum to Glu α-tubulin (Fig. 13e), and no microtubules are stained by the antibody to acetylated α-tubulin (Fig. 13h).

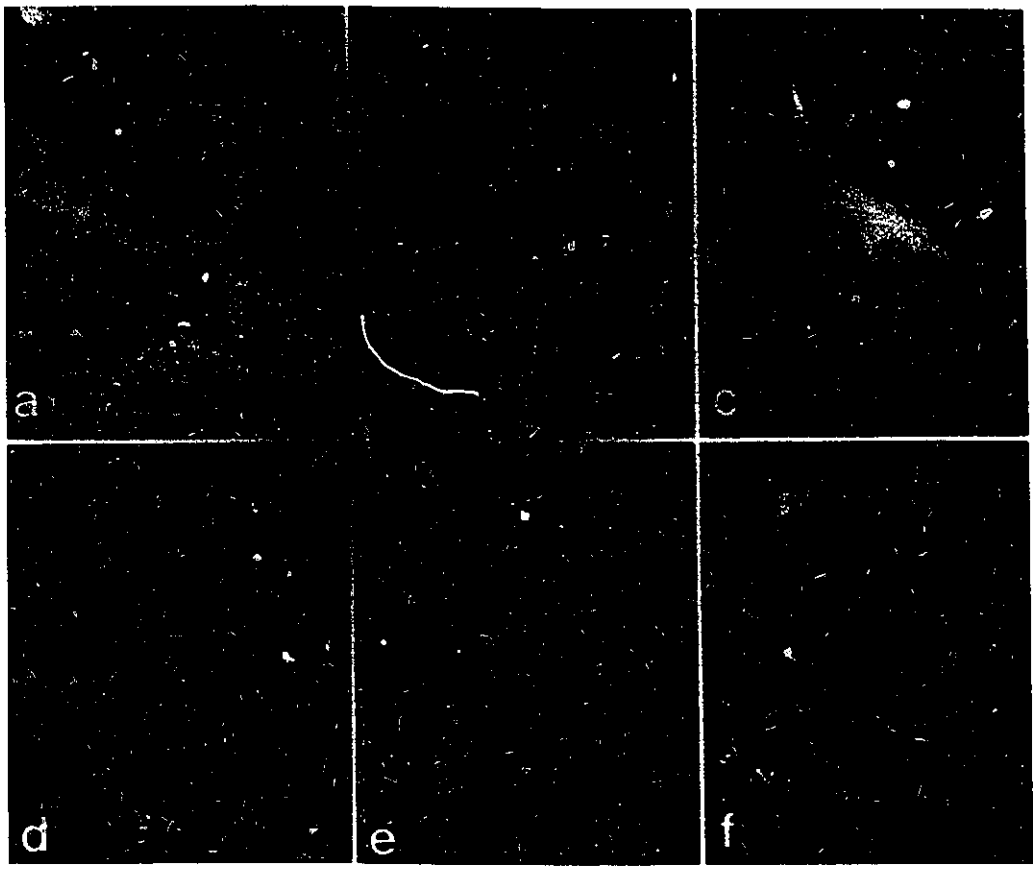
The effect of MTOC orientation on the intermediate filament distribution was investigated by double immunofluorescence staining using antibodies to tubulin and vimentin. The most striking change observed is the presence of ring-like vimentin aggregates (Fig. 14a,d) analogous to those formed following colchicine-induced microtubule disassembly (Fig. 6g). In addition to these ring-like structures other filamentous vimentin aggregates such as those shown in Figure 14e are observed. Vimentin aggregates are also observed in taxol treated cells that orient their MTOC towards the TC (Fig. 14c,f).

Not all conjugated CTLs contain vimentin aggregates; nor are vimentin aggregates peculiar to conjugated CTLs, since they are also observed in unconjugated effector cells (Fig. 6h). To determine if conjugate formation or orientation increases the number of vimentin aggregates, the frequency of vimentin aggregate formation in unconjugated effector cells and CTLs conjugated in the

Figure 1.13. Effect of Conjugate Formation on Microtubule Composition. Double immunofluorescence staining with the mouse monoclonal 5A6 (a,d) or the rat monclonal YL 1/2 (g) to show all microtubules and rabbit antiserum specific for Tyr (b), and Glu (e) α-tubulin, or the mouse monoclonal 6-11B-1 specific for acetylated (h), α-tubulin. Hoechst 33258 staining shows the nuclei of these cells (c,f,i). Magnification 1500x.

c
f
g
h
i

Figure 1.14. Effect of Conjugate Formation on Intermediate Filament Distribution I. Freshly isolated (a,b,d,e) and taxol-treated (10 μM, 4 h) effector cells were mixed with cultured EL4 cells to form conjugates and then prepared for double immunofluorescence staining with the murine monoclonal 5A6 (a-c) and a rabbit antiserum to vimentin (d-f). Magnification 1500x.


a
c
d
e
f

presence and absence of Ca^{++} was determined (Fig. 15). Analysis of this data indicates that there are significantly more vimentinaggregates formed in CTLs conjugated in the presence of Ca^{++}; whereas, the frequency of vimentin aggregates found in conjugates formed in the absence of Ca^{++} is no different from that of unconjugated effector cells.

Taxol treatment significantly reduces the number of intermediate filament aggregates observed in unconjugated cells and prevents an increase in the number vimentin aggregates similar to that seen in control cells following conjugation (Fig. 15). Pretreatment of effector cells with 10 μM taxol for 4 h before conjugate formation also results in a significant reduction in the percentage of conjugated CTLs that orient their MTOC towards the TC (Fig. 16).

Figure 1.15. Effect of Conjugate Formation on Intermediate Filament Distribution II. The percentage of cells having visible intermediate filaments in unconjugated effector cells (A), is not affected by conjugation in the presence (B) or absence (C) of Ca^{++} (A=B=C, X=1.2850, p=0.5260). Taxol treatment (10 μM, 4 h) does not alter the percentage of conjugated (D) or unconjugated (E) cell populations having visible intermediate filaments (D=B=C, X=3.5394, p=0.1704; E=A, X=3.0501, p=0.0807).

Conjugation in the presence of Ca^{++} causes an increase in the number of vimentin aggregates formed (A=B, X=13.2511, p=0.0002) while conjugation in the absence of Ca^{++} does not (A=C, X=0.0932, p=0.7601). Taxol treatment inhibits the formation of vimentin aggregates (E=A, X=23.4886, $p=1.3709 \times 10^{-6}$) and prevents the increase in number of vimentin aggregates observed to occur in untreated cells following conjugation (D=E, X=1.1044, p=0.2933). Triplicate counts of ≥50 cells were performed on each sample and the values shown are the mean of the counts obtained from two or more experiments ±SD.

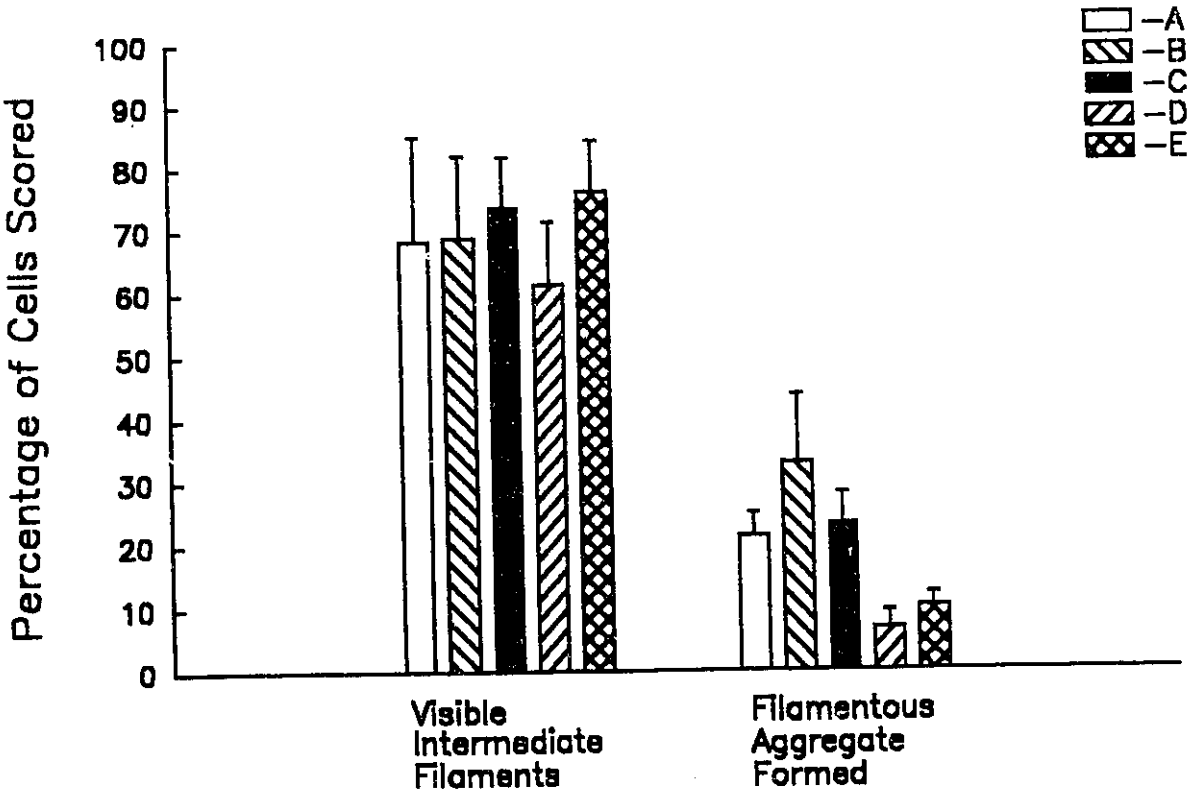
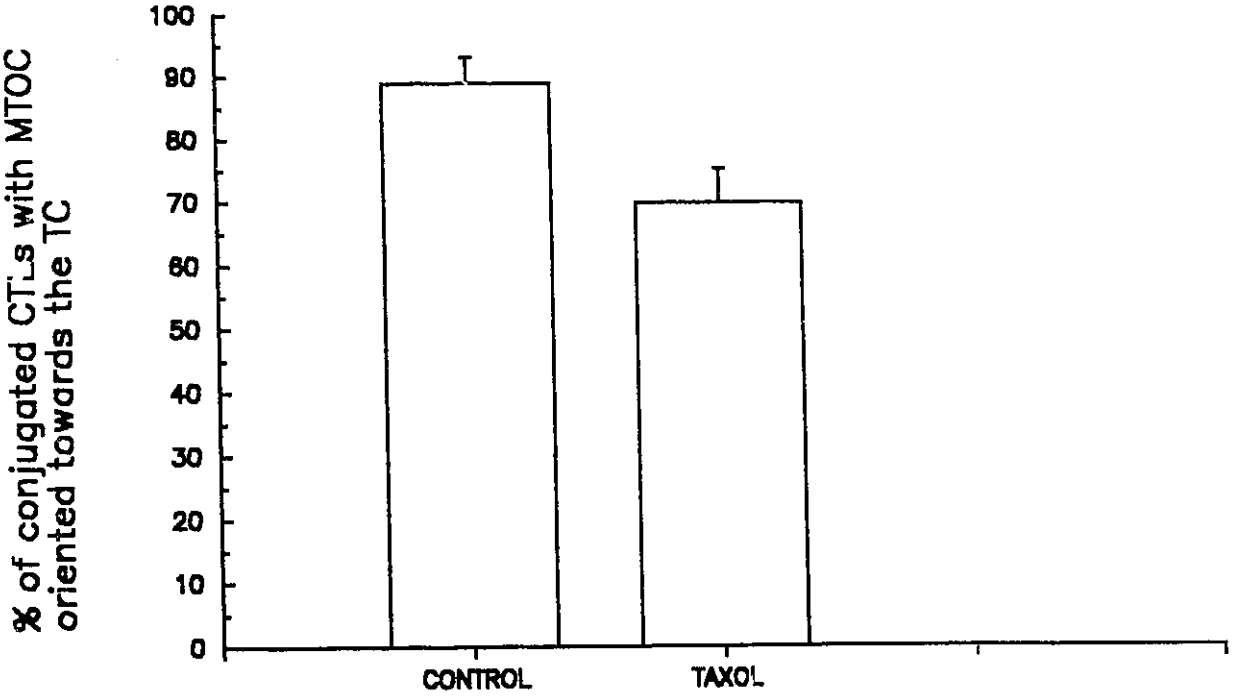

Percentage of Cells Scored
100
90
80
70
60
50
40
30
20
10
0
A
B
C
D
E
Visible
Intermediate
Filaments
Filamentous
Aggregate
Formed

Figure 1.16. Effect of Taxol on MTOC Orientation. Pretreatment of effector cells with taxol (10 μM, 4 h) prior to conjugate formation results in a significant reduction in the percentage of the CTLs able to orient their MTOC towards a bound TC (X=0.4487, p=0.5029).


% of conjugated CTLs with MTOC oriented towards the TC
100
90
80
70
60
50
40
30
20
10
0
CONTROL
TAXOL

DISCUSSION

The microtubule network (Waterhouse et al., 1983), intermediate filament organization (Paulin-Levasseur and Brown, 1987a; 1987b), and ultrastructure (Setterfield et al., 1983) of unactivated lymphocytes have all been previously described. Clearly by these morphological criteria the effector cell populations of this study are 'unactivated' lymphocytes. The ability of the effector cells to undergo an increase in cell volume and microtubule number in response to mitogen (Fig. 2) is also characteristic of unactivated cells.

It is also clear that the intraperitoneal injection of the allogeneic tumour cells results in the generation of effector cells that specifically recognize and lyse the allogeneic tumour cells (Fig. 7). The generation of TC specific CTLs stimulated by allogeneic tumours is dependent upon the clonal expansion of host cells able to recognize the tumour cells as foreign (Denizot et al., 1986). The unactivated appearance of the effector cells isolated indicates that the clonal expansion initiated by the intraperitoneal injection of the EL4 cells is completed within 10 days and that the CTLs isolated are non-dividing cells.

The precise role of Ca^{++} in the lytic process remains obscure. Indeed, while a transient rise in the concentration of free Ca^{++} in the CTL is always observed when CTLs bind their specific TC (Poenie et al., 1987; Ostergaard and Clark, 1987), there is evidence that the requirement for Ca^{++} in cytolytic assays

is dictated by the TC and not by the CTL (Tirosh and Berke, 1985; Ostergaard et al., 1987; Ostergaard and Clark, 1989). Regardless of the specific site of action of the Ca^{++}, the results presented here demonstrate that in specific conjugates formed between primary cultures of non-dividing CTLs and EL4 cells, Ca^{++} is required for the orientation of the MTOC within the CTL to face the TC and for efficient TC lysis.

Once established, the orientation of the MTOC of the CTL towards the TC persists in the absence of Ca^{++}. Cytolytic activity, on the other hand, is greatly inhibited by the removal of Ca^{++} after conjugate formation. This indicates that while MTOC orientation towards the TC may be necessary for cytolytic activity, it is not sufficient for TC lysis. Therefore, in addition to being necessary for MTOC orientation, Ca^{++} is also apparently required for an event that occurs more than 25 min after conjugation.

The steady release of ^{51}Cr throughout the cytolytic assay indicates that 'lethal hit' delivery begins soon after conjugation. In these experiments there is always a large excess of effector cells, making it likely that all the labelled target cells are conjugated with one or more CTL at the start of the assay. Therefore, the ability of the CTLs to conjugate with a TC, deliver a 'lethal hit', and then recirculate is probably not the rate limiting step in these assays. The requirement of Ca^{++} at a time later than 25 min after conjugation indicates that in addition to MTOC orientation Ca^{++} is involved in the potentiation of TC lysis.

Events thought to be involved in TC lysis, requiring Ca^{++}, and

occurring after MTOC orientation include the secretion of cytolytic granules or molecules by the CTL (Trenn et al., 1987), the assembly and insertion of complement factor 9-like cytolytic molecules into the TC membrane (Tschopp et al., 1989), and the potentiation of CTL-induced apoptosis of the TC (Martz and Howell, 1989). Some reports (e.g. Berke and Rosen, 1987) have raised doubts as to whether primary cultures of CTLs actually possess lytic granules or complement-like proteins. These reports and the absence of electron dense granules in the effector cell populations of this study make granule secretion and perforin assembly unlikely candidates for the Ca^{++}-dependent lytic event required for TC lysis and occurring after MTOC orientation in this study. It is important to note that this does not preclude the involvement of a lytic component secreted by the CTLs in TC lysis.

The model that best explains the behaviour of microtubules is dynamic instability (Mitchison and Kirschner, 1984). According to this model the whole microtubular array is very dynamic, probing many regions of the cell at random. In such a cell stabilization of a particular subset of microtubules in response to a stimulus received at the plasma membrane would cause the whole array to become rapidly transformed (Kirschner and Mitchison, 1986). The working hypothesis of this study was that the selective stabilization of a subset of microtubules in the CTLs following conjugate formation was the mechanism by which the reorientation of the MTOC is effected.

Tyr α-tubulin is the translation product of α-tubulin mRNA.

The tubulin specific carboxy-peptidase and acetylase enzymes that remove the carboxy-terminal tyrosine residue of α-tubulin to transform Tyr tubulin into Glu tubulin and acetylate α-tubulin, both act preferentially on polymerized tubulin. Conversely, the tyrosine ligase and deacetylase enzymes act preferentially on tubulin dimers (Gundersen et al, 1987; LeDizet and Piperno, 1986). Therefore, the pool of tubulin dimers within the cell consists primarily of Tyr α-tubulin and the extent to which a microtubule is detyrosinated and acetylated can be used as an indirect measure of its stability (Piperno et al., 1987; Gundersen et al., 1987). Gundersen and Bulinski (1988) have used immunofluorescence staining with an antibody to Glu α-tubulin as a marker for stable microtubules to test the selective stabilization model in fibroblast cells orienting towards an experimental wound. They showed that the formation of a polarized array of stable microtubules preceded the onset of cell migration into the wound and closely paralleled the orientation of the centrosome towards the wound edge. This result led them to suggest that the selective stabilization of microtubules may be involved in the repositioning of the centrosome.

In contrast, as shown in Figure 14, no subset of posttranslationally modified microtubules is formed in CTLs conjugated to TCs. This indicates that, if a more stable subset of microtubules is formed during the orientation of the centrosome towards the TC, it does not persist long enough for the posttranslational modifications to occur. This result does not

support, but does not rule out the selective stabilization hypothesis.

Although I was able to demonstrate that murine T cells are able to generate Glu and acetylated α-tubulin, this only occurred after prolonged treatment with the microtubule stabilizing drug taxol. Therefore, unless conjugation increased the activity of the enzymes required to make the posttranslational modifications relative to that observed in the unconjugated effector cell populations it is unlikely that one would be able to detect stabilized microtubules by this method.

Intermediate filament proteins are almost entirely assembled into filaments in the cytoplasm and newly synthesized subunits are rapidly incorporated into this network (Blikstad and Lazarides, 1983). Whether the newly synthesized vimentin proteins are assembled vectorially from the nuclear surface or incorporated throughout the existing vimentin network at multiple sites distant from the nuclear surface via an incorporation/exchange process is not yet known, although, data consistent with both of these possibilities has been reported (Steinert and Liem, 1990). The lack of a soluble pool of subunits indicates that the intermediate filaments cannot be rearranged in the cytoplasm by disassembly-reassembly reactions as the other two major filament systems, the microtubules and microfilaments, are (Eckert et al., 1984); however, dynamic rearrangements of the intermediate filaments, the mechanism and speed of which are unknown, do take place (Steinert and Liem, 1990).

In this study it was demonstrated that conjugation of CTLs results in the formation of significantly more vimentin aggregates than are seen in unconjugated effector cells. Conjugation in the absence of Ca^{++} leads to the coordinate loss of the increase in intermediate filament aggregation, and lytic activity, which indicates that the rearrangement of intermediate filaments is associated with an event that occurs during TC lysis. However, the observation that only ≈50% of the lytically active CTLs contain an aggregate implies that its formation is not required for lytic activity.

A close association between the intermediate filaments and microtubules has been reported in various cell lines (Celis et al., 1983, Paulin-Levasseur and Brown, 1987a). Consistent with this apparent interaction is the observation that drugs that affect the microtubular organization also affect intermediate filament organization. Microtubule disassembly in lymphocytes (Paulin-Levasseur and Brown, 1987a;) and in other cell types (Bloom et al., 1985) results in the formation of intermediate filament aggregates. This suggests that the microtubules normally function as a scaffold for the maintenance of the intermediate filament system. In contrast, taxol treatment of lymphocytes induces the parallel reorganization of the microtubules and intermediate filaments into thick bundles that extend from the centrosome. Consistent with this apparent increased interaction between the two systems, taxol treatment has been shown to prevent the rearrangement of vimentin intermediate filaments associated with surface immunoglobulin cap

formation (Paulin-Levasseur and Brown, 1987b). On the other hand, taxol treatment does not block the series of intermediate filament rearrangements associated with mitogenic stimulation (Paulin-Levasseur and Brown, 1987a).

Conjugation results in the formation of vimentin aggregates similar to those induced by microtubule disassembling drugs. The observation that conjugation in the absence of Ca^{++} prevents both the increase in intermediate filament aggregate formation and MTOC orientation suggests that in some cells during MTOC orientation a disassociation of the microtubules and intermediate filaments takes place. Consistent with this suggestion taxol treatments (10 μM, 4 h), which increase the interaction between the microtubules and intermediate filaments and inhibit MTOC orientation, also inhibit aggregate formation.

CHAPTER 2

INTRODUCTION

The clinical use of hyperthermia in the treatment of cancer has raised questions about the effects of heat on the immune response of the host (Lowenthal, 1984). Previous investigations have shown that the cytolytic activity mediated by CTLs is severely impaired by hyperthermia treatments (40^0C-43^0C) that do not significantly affect T cell viability (Harris, 1976; MacDonald, 1977; Harris and Meneses, 1978; Roberts et al., 1985). Several of these studies demonstrated that a partial recovery of cytolytic activity occurs following the heat treatment (MacDonald, 1977; Harris and Meneses, 1978; MacDonald and McFarlane, 1978), but the particular CTL functions that are inhibited by heat have not yet been identified. A factor complicating their identification is the pleiotropic effects of heat.

The means by which CTLs lyse their TCs has been the subject of intensive study (for reviews see Henkart, 1985; Clark et al., 1988; Young et al., 1988; Brunet et al., 1988). CTL mediated killing can be divided into several discrete steps including: the recognition and binding of the TC (Spits et al., 1986; Tschopp and Jongeneel, 1988); the rapid orientation of the GA and the MTOC within the CTL so that the two organelles face the TC (Kupfer et al., 1983; Kupfer et al., 1985); and delivery of a 'lethal hit' to the TC followed by

recirculation of the CTL (Clark et al., 1988; Young et al., 1988; Kupfer and Singer, 1989a). Early experiments in which the MTOC/GA was reversibly prevented from orienting towards the TC, by treatment of the cells with microtubule disrupting drugs or by removal of Ca^{++} from the medium (Kupfer et al., 1983; Kupfer et al., 1985), demonstrated that the orientation of the MTOC/GA was microtubule and Ca^{++} dependent and that this orientation was a prerequisite for lysis.

In this study the ability of heated CTLs to perform each of the above steps was examined in order to determine the killing step, or steps, affected by hyperthermia. The results presented show that moderate hyperthermia treatments result in a marked inhibition, followed by a transient recovery, of cytolytic activity. The same treatments do not affect the ability of CTLs to recognize and bind to TCs, but cause a reversible disruption of the radial distribution of microtubules about the MTOC. The disruption and reorganization of the microtubule system correlates with the loss and recovery of cytolytic activity. This suggests that a radial array of microtubules is required for CTL-mediated cytolysis. Although the microtubules recover completely from the effects of hyperthermia, the recovery of cytolytic ability is incomplete and transient, indicating that the hyperthermia treatment has other effects. It is proposed that the initial loss of cytolytic activity is due to the disruption of microtubule organization. The inhibition of protein synthesis that occurs as a result of hyperthermia is discussed as a possible explanation for the

observation that recovery of cytolytic activity is only partial and transient.

RESULTS

Effect of hyperthermia on cytolytic ability. Figure 1 shows that exposure of CTL to increasing temperatures for 30 min causes an inhibition of cytolytic activity that is particulary evident at temperatures over 41^0C. A 42^0C treatment, which reduces cytolytic activity by 80% while still permitting significant recovery (see below), was chosen for subsequent experiments. A trypan blue exclusion assay, used as a measure of T cell viability, shows no significant difference between the permeability of cells exposed to 42^0C for 30 min and unheated control cells in the two days following the hyperthermia treatment (Fig. 2).

Effect of hyperthermia on protein synthesis. The application of a heat stress is known to result in the enhanced production of a specific set of proteins, the heat shock proteins, and an accompanying generalized repression of the synthesis of other cellular proteins (Pelham, 1986; Burdon, 1986). The extent of the change in the normal pattern of protein synthesis, however, depends on the cell type, temperature, and the length of hyperthermia exposure.

The gel shown in Figure 3 compares the proteins synthesized in control and hyperthermia-treated T cells. The histogram in Figure 4 shows the reduction in ^{35}S-methionine incorporation caused by the hyperthermia treatment. Clearly, the hyperthermia treatment results in a reduction in the total amount of protein synthesis and

Figure 2.1. Effect of Hyperthermia on the Cytolytic Activity of CTLs. Immediately following a 30 min hyperthermia treatment at the various indicated temperatures 25 x 10^5 CTLs were mixed with 1 x 10^5 ^{51}Cr loaded TCs in a total volume of 200 μL in round bottomed 96 well plates. The assay plates were centrifuged at 50 x g for 1 min before incubation for 4 h at 37^0C. After this incubation the plates were centrifuged for 10 min at 250 x g and 100 μL of supernatant collected and radioactivity counted. The histogram compares the relative cytolytic activity of the samples following the different hyperthermia treatments. All samples were done in triplicate and the values shown are the average of the mean of 5 experiments ± SE.

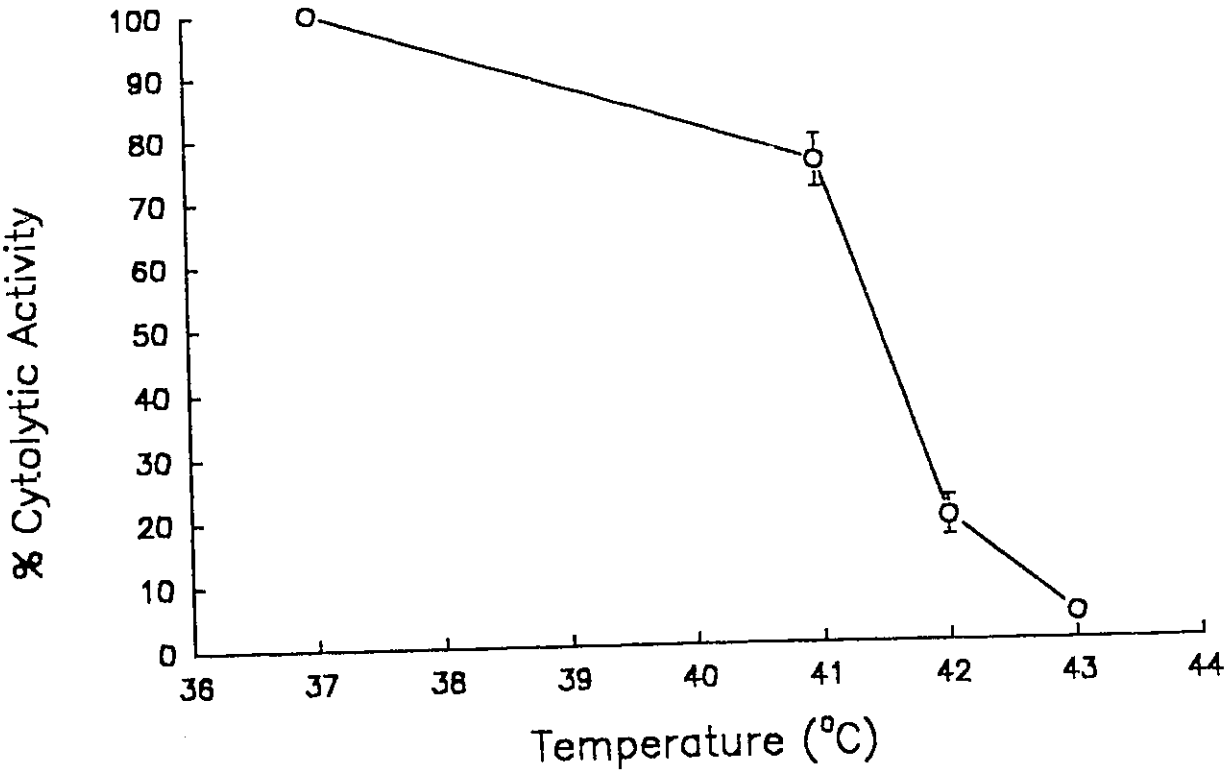

% Cytolytic Activity
100
90
80
70
60
50
40
30
20
10
0
36
37
38
39
40
41
42
43
44
Temperature (°C)

Figure 2.2. Effect of Hyperthermia (42^{0}C, 30 min) on T Lymphocyte Viability. Isolated T cells were resuspended at 2.0 x 10^6 cells/mL. Half the sample was hyperthermia-treated (filled circles) while controls (open circles) were kept at 37^{0}C. After the treatment all samples were incubated at 37^{0}C in 5% CO_2. At the times indicated aliquots of both samples were taken and diluted 1:4 in 0.16% trypan blue dissolved in 0.85% saline, and using a haemacytometer (Reichert Sci. Instr., Buffalo, NY) and an inverted microscope the number of trypan blue impermeable cells counted at the times indicated. Quadruplicate counts of each sample were made and the values are the average of the mean of two experiments ±SE.

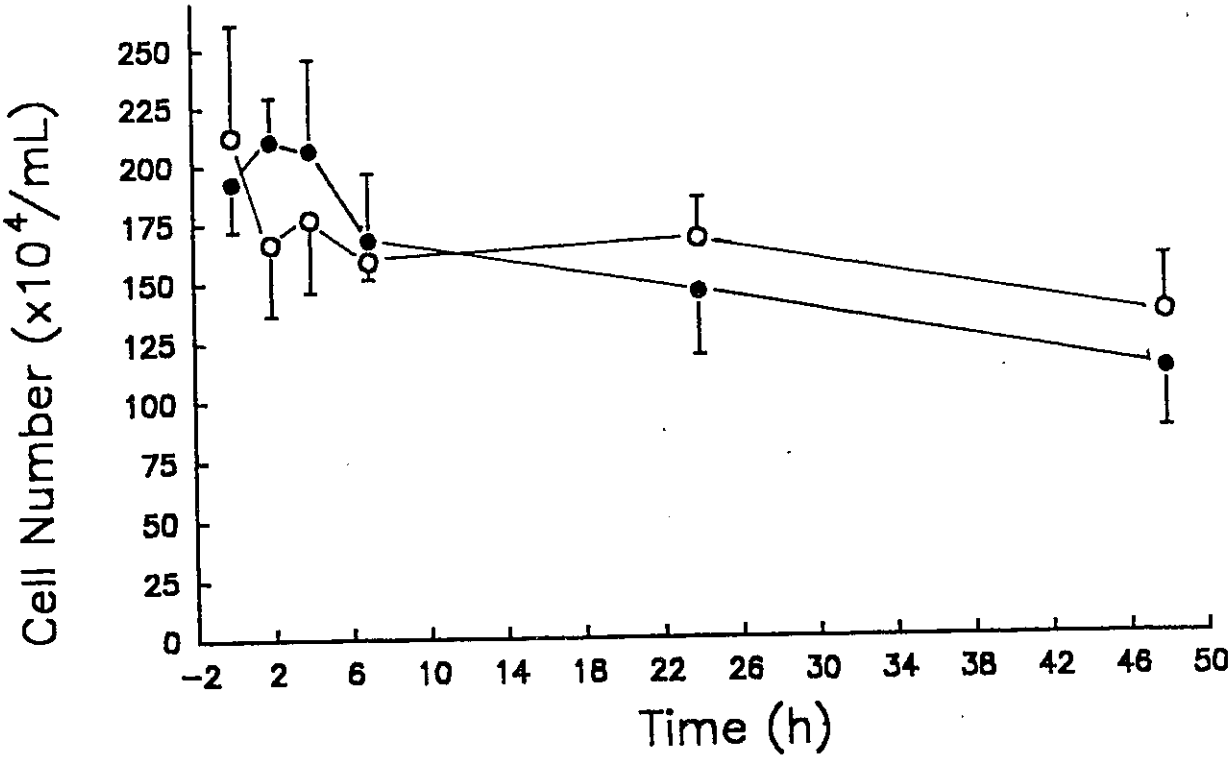
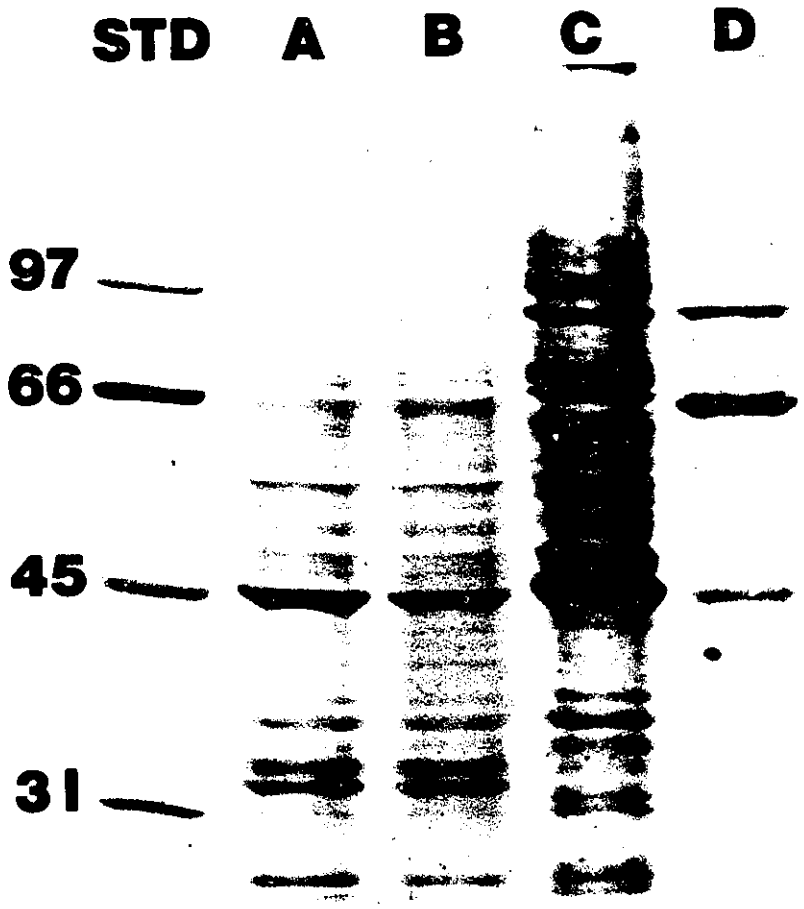

Cell Number (x10⁴/mL)
250
225
200
175
150
125
100
75
50
25
0
−2
2
6
10
14
18
22
26
30
34
38
42
46
50
Time (h)

Figure 2.3. Effect of Hyperthermia (42^0C, 30 min) on Protein Synthesis. Control (A) and hyperthermia-treated (B) cells were incubated for 6 h at a concentration of $2.0x10^6$/mL in media containing 30 μCi/mL of ^{35}S-methionine. Equal amounts of total cell protein obtained from these samples were run on an 8.5% polyacrylamide gel and then stained with Coomassie blue. The corresponding autoradiograph shows the pattern of ^{35}S incorporation in control (C) and hyperthermia-treated (D) cells.


STD
A
B
C
D
97
66
45
31

the major protein products have molecular weights corresponding to those of the major heat shock proteins reportedly expressed in human lymphocytes (Haire et al., 1988).

Effect of hyperthermia on TC recognition and binding. Lyt 2.2 is a cell surface antigen specific for the cytotoxic and suppressor T cell populations. Random fields of conjugates that had been stained with anti-Lyt 2.2 antibody were examined by epifluorescence and then by phase contrast microscopy and the percentage of Lyt 2.2^+ cells having bound a TC determined. Figures 5a and 5b show one example of a labelled Lyt 2.2^+ CTL bound to an unlabelled TC and Figure 5c shows that for a population of conjugates the percentage of Lyt 2.2^+ cells having bound a TC is unaffected by the hyperthermia treatment.

Effect of hyperthermia treatment on microtubule organization. Having established that the recognition and binding of TC is not affected by hyperthermia, immunofluorescence microscopy, with monoclonal antibodies to tubulin, was used to determine if hyperthermia affects the movement of the MTOC to face the TC. An unexpected finding, however, was that a hyperthermia treatment sufficient to inhibit 80% of the cytolytic activity of CTL also results in a disruption of microtubular organization in approximately 75% of the CTL. This disorganization is easily seen when making through focus observations on a conventional fluorescence microscope, but is difficult to record

Figure 2.4. The Effect of Hyperthermia (42^{0}C, 30 min) on the Rate of ^{35}S-Methionine Incorporation. Control (C) and hyperthermia-treated (HS) cells were incubated for 6 h at a concentration of 2.0x10^{6}/mL in media containing 30 μCi/mL of ^{35}S-methionine. The histogram compares the activity in equal amounts of total cell protein obtained from these samples. Hyperthermia treatment significantly reduces the amount of ^{35}S-methionine incorporation ($F=21.5220$, $DF_1=1$, $DF_2=4$, $p=0.0097$). All samples were done in triplicate and the values shown are the means of a typical experiment ±SD.

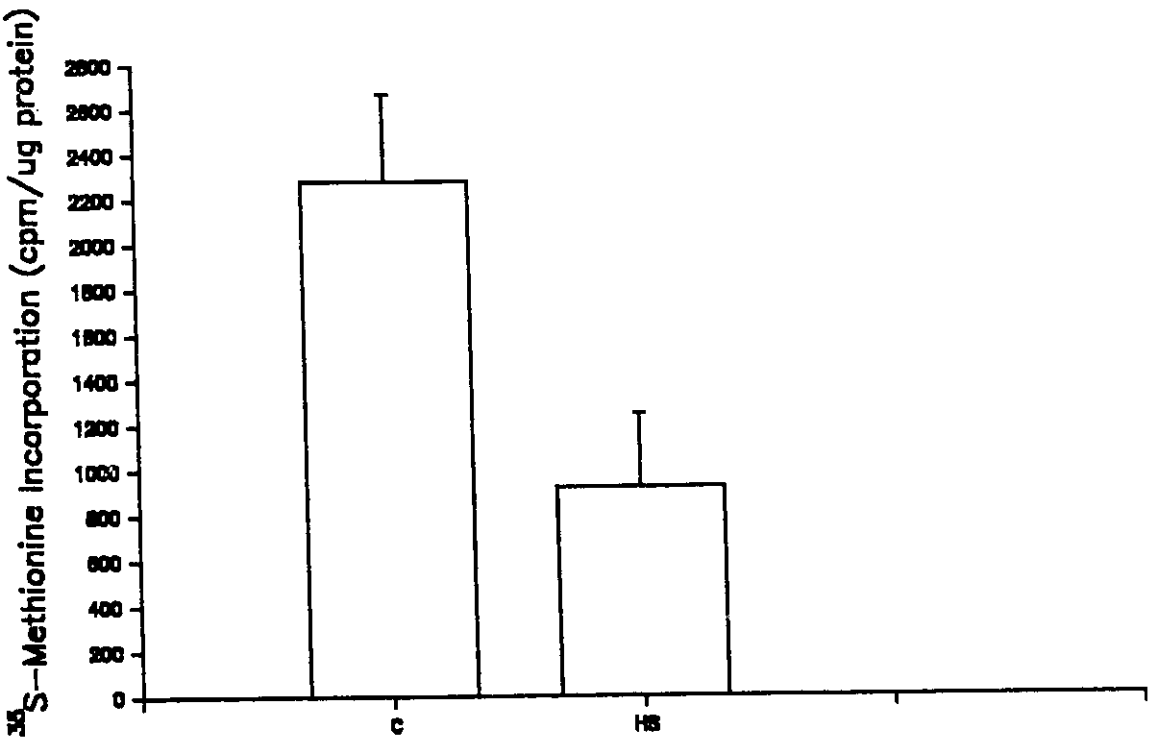
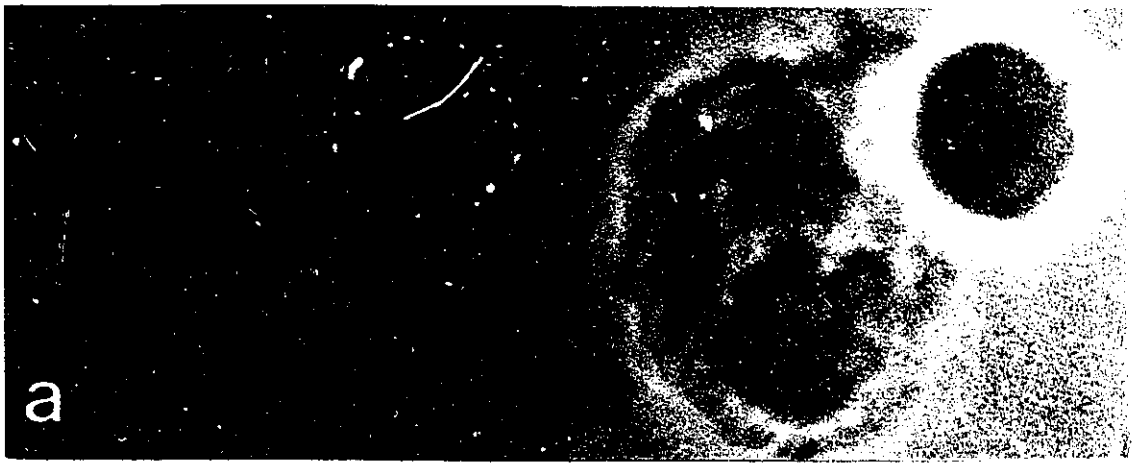
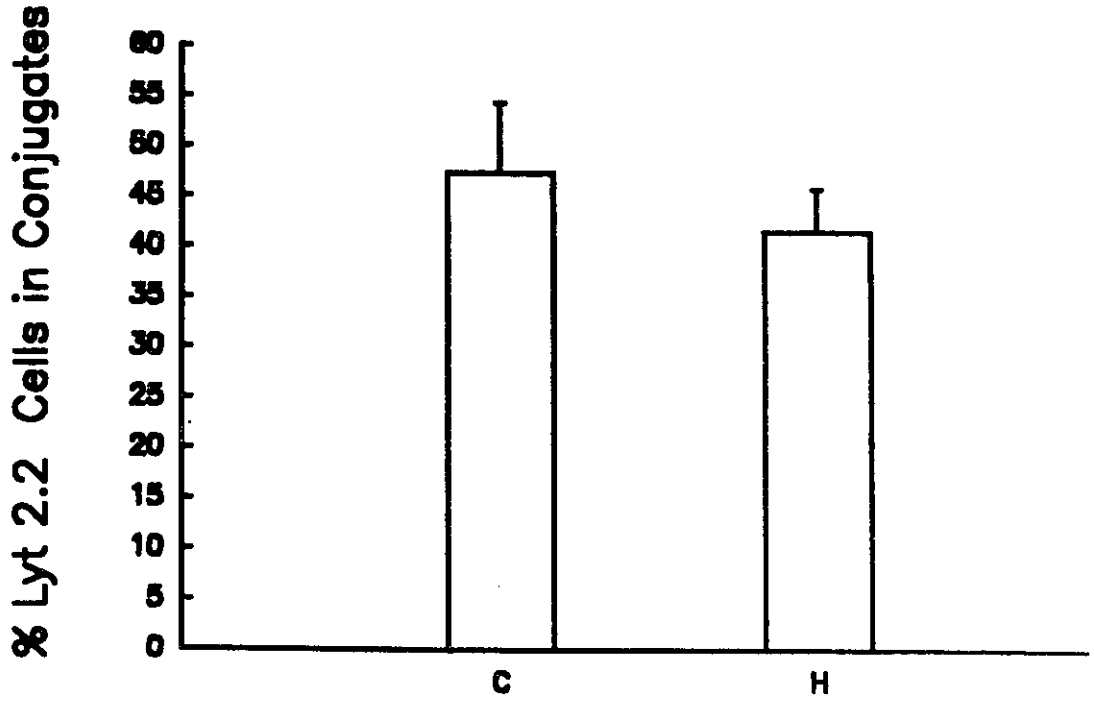

35S-Methionine Incorporation (cpm/ug protein)
2600
2800
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
C
HS

Figure 2.5. The Effect of Hyperthermia (42^0C, 30 min) on the Ability of CTLs to Recognize and Bind to their specific TCs. Immunofluorescence (a) and phase contrast (b) micrographs showing a Lyt 2.2^+ cell bound to a TC. The cell to which the Lyt 2.2^+ cell is bound is identified as a TC on the basis of its much greater size and irregular shape. Magnification 2,500x. CTL/TC conjugates were formed using either hyperthermia-treated (H) or control (C) effector cell samples. The coverslips were scored blind and the percentage of Lyt 2.2^+ cells bound to TC plotted (c). The hyperthermia treatment did not significantly affect the ability of the CTLs to recognize and bind their specific TCs ($X=0.8564$, $p=0.3548$). Triplicate counts of ≥50 cells of each sample were made and the values presented are the average of the mean of two experiments ±SE.

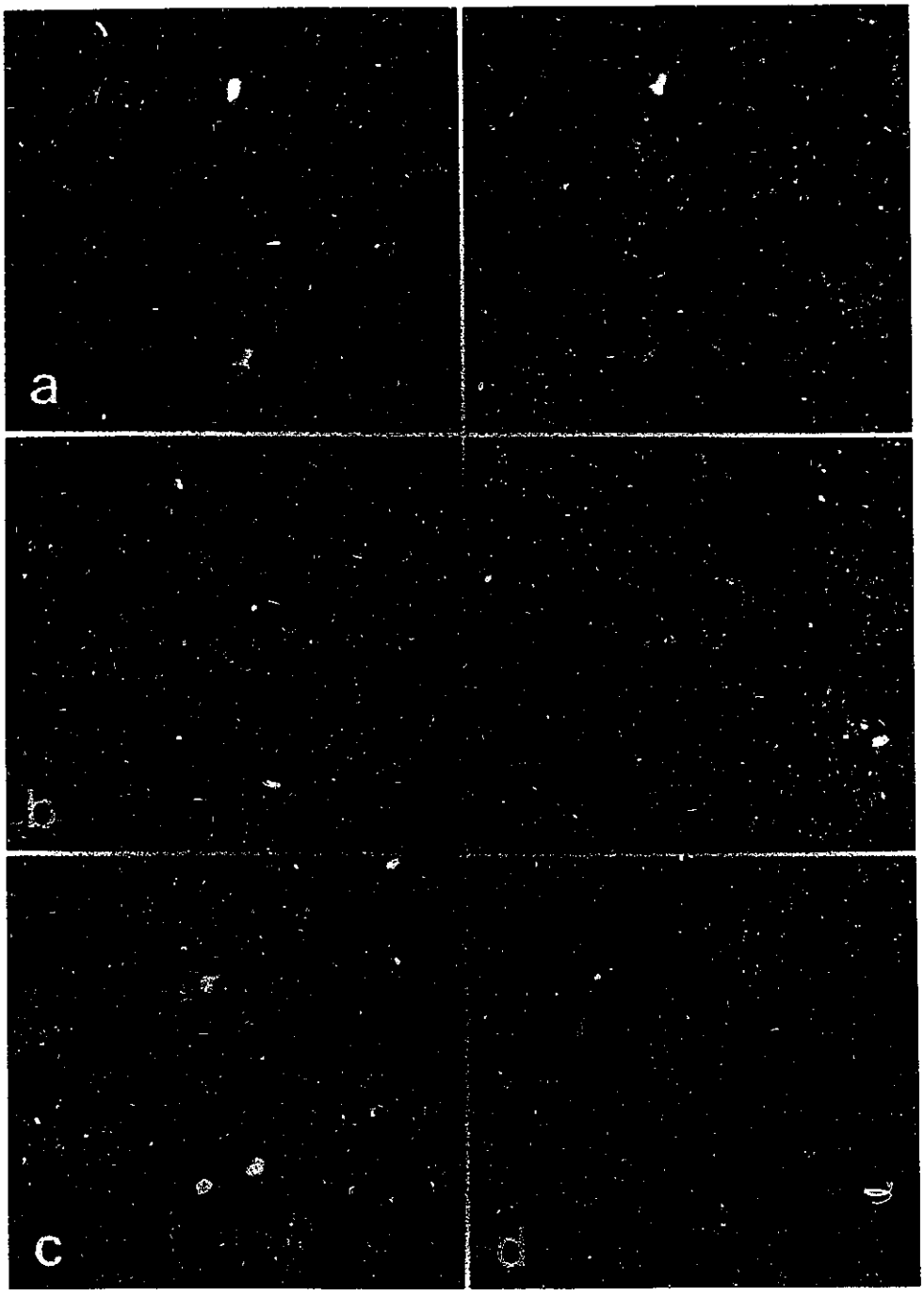

a


% Lyt 2.2 Cells in Conjugates
0
5
10
15
20
25
30
35
40
45
50
55
60
C
H

photographically. To clearly illustrate the effect of heat on the microtubule organization of these cells a confocal microscope was used to optically section heated and control cells. Projections of all 12 of the 0.5 μm sections were then photographed for stereoscopic imaging. Figure 6a shows the characteristic radial array of microtubules (Schweitzer and Brown, 1984) extending from the single MTOC, the centrosome, and around the margins of the cell. In contrast, in the heated cells the microtubules do not appear to extend from a single site, but form an intersecting network (Fig. 6b). The total amount of polymerized tubulin observed by immunofluorescence microscopy does not appear to be altered by the heat treatment. In both control and heated cells microtubules are restricted to the thin layer of cytoplasm between the nucleus and plasma membrane, giving these cells the appearance of hollow balls.

The extension of microtubules from a single site in control cells permits the position of the MTOC of a CTL with respect to a bound target cell to be determined by anti-tubulin immunofluorescence (see Geiger et al., 1982; Kupfer et al., 1983; and Figs. 11a and 11b). The disruption of microtubule organization by heat, however, made it impossible to detect the position of the MTOC in the affected CTL by this method. As an alternate method to detect the position of the MTOC in heated cells a rabbit autoimmune serum that recognizes centrioles (Turksen et al., 1982) was obtained. This antiserum does detect the MTOC at the centre of the radial microtubule array in control cells (Figs. 6c, 6d); however,

Figure 2.6. The Effect of Hyperthermia on the Microtubule Organization of Effector Cells. Figures (a) and (b) are stereo pairs of projections of 12, 0.5 μm thick, optical sections produced by confocal microscopy of cells immunofluorescently stained with anti-tubulin. (a) shows the characteristic radial distribution of microtubules about the MTOC in control T lymphocytes and (b) the disruption of this pattern in hyperthermia treated (42^{0}C, 30 min) cells. Double immunofluorescence of anti-tubulin (c) and the rabbit autoimmune serum (d) demonstrating that this serum does detect the MTOC at the centre of the radial array of microtubules in control cells. Magnification 2,000.


a
b
c
d

in heated cells the antiserum no longer detected any structure. Therefore, by these methods it was impossible to determine whether or not heat affects the ability of CTL to orient their MTOC towardsa bound TC.

Earlier reports that hyperthermia treatments of other cell types caused microtubule disassembly (Malawista and de Boisfleury Chevance, 1982; Coss et al., 1982) and a change in centrosome structure (Barrau et al., 1978; Malawista and de Boisfleury Chevance, 1982), as well as the observation of the loss of immunodetection with the autoimmune serum presented here, prompted us to examine the effect of heat on the structure and organization of the CTL centrosome at the EM level. As shown in Figure 7, we also found that the only obvious effect of hyperthermia treatment on the CTL centrosome is an aggregation of the pericentriolar material (PCM) about the centrioles. The structure of the centrioles themselves, their location near the nucleus, and their association with the Golgi apparatus do not appear to be affected by the hyperthermia treatment.

Recovery of CTL from hyperthermia. CTL hyperthermia-treated for 30 min at 42^{0}C and then incubated at 37^{0}C show a partial recovery of cytolytic activity. This recovery of activity is transient, with a peak at 6 h followed by a steady decline over the next 18 h (Fig. 8).

CTL recovering at 37^{0}C from a 42^{0}C, 30 min hyperthermia treatment also showed the re-establishment of the normal

Figure 2.7. Electron Micrographs Showing the Aggregation of the PCM Caused by the Hyperthermia Treatment (42^0C, 30 min). (a) cross section of a control centriole. (b) cross section of a centriole of a hyperthermia-treated cell. (c) longitudinal section of a centriole of a control cell. (d) longitudinal section of the centrosome of a hyperthermia-treated cell. Satellite bodies can be seen associated with the centrioles of hyperthermia-treated and control cells (arrows). Magnification 60,000x.

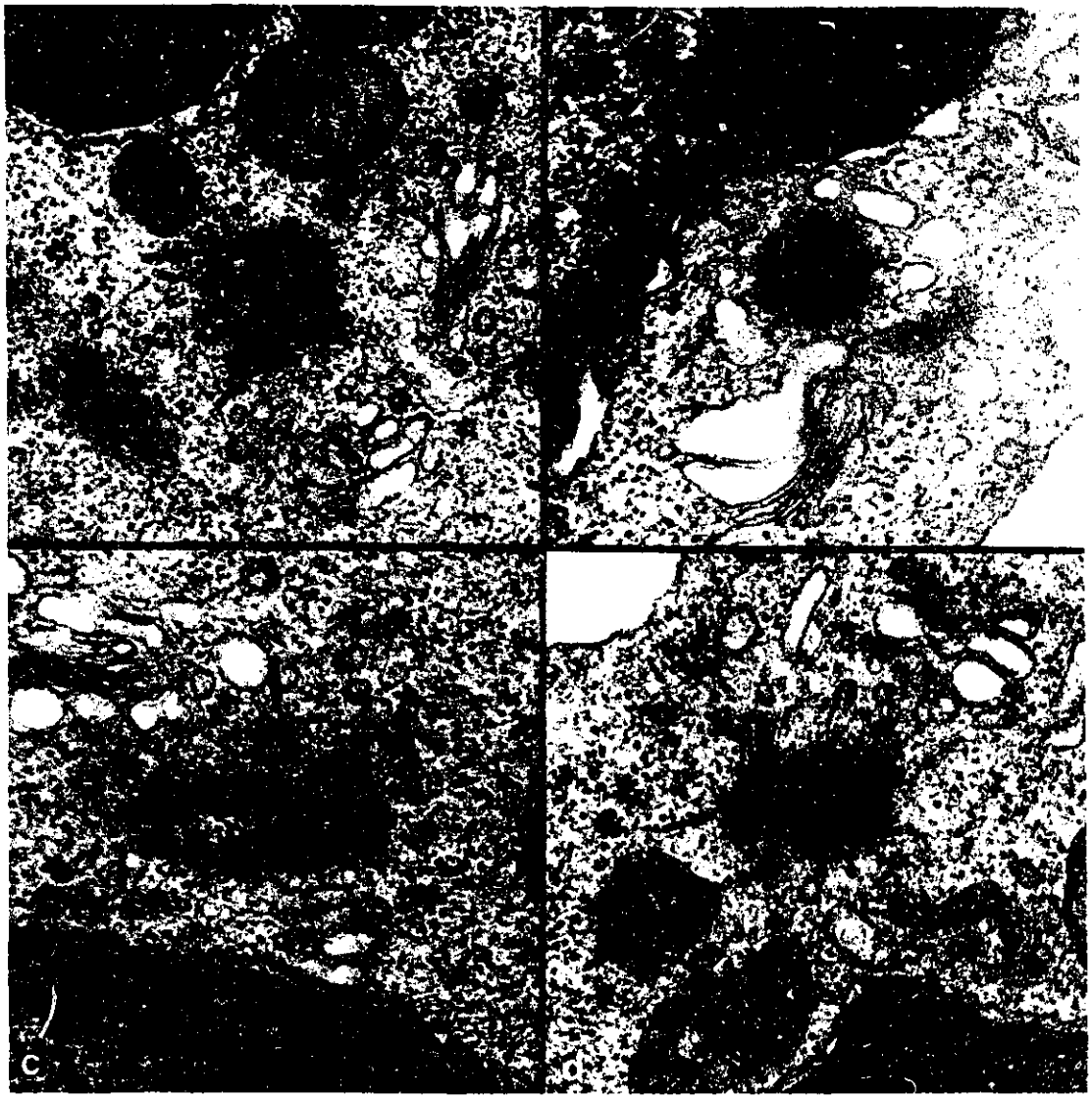
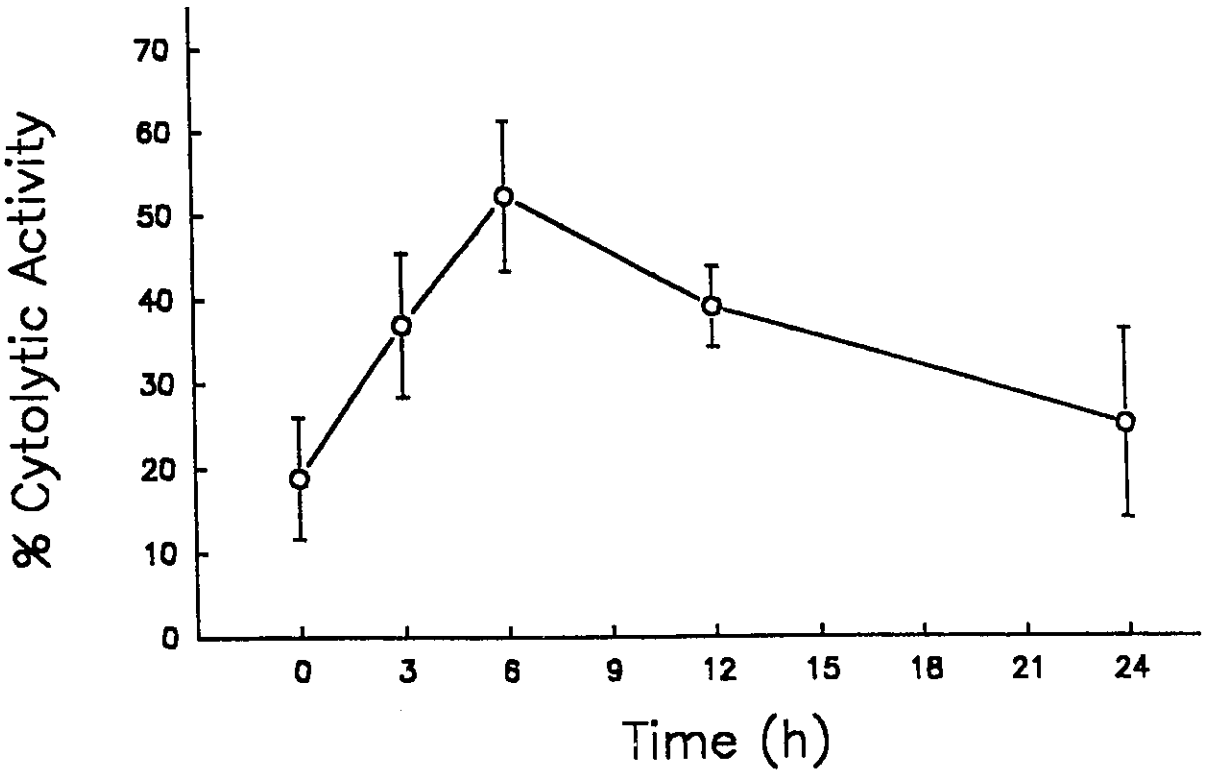

C

Figure 2.8. The Recovery of Cytolytic Activity Following Hyperthermia (42^0C, 30 min). Aliquots of hyperthermia treated effector cells were incubated at 37^0C for the indicated times before being mixed with ^{51}Cr-labelled TCs and their cytolytic activity assayed. Values are normalized to the cytolytic activity of unheated control cells that were assayed at each time point and are the average of the mean of seven experiments ±SE.


% Cytolytic Activity
70
60
50
40
30
20
10
0
0
3
6
9
12
15
18
21
24
Time (h)

microtubule organization (Fig. 6a) in which all microtubules appear to radiate from the single MTOC. As shown in Figure 9, the MTOC is detectable in most cells after 3 to 4 h of recovery and by 6 h the microtubule organizations in the control and recovering samples are indistinguishable. Staining of the centrosome with the rabbit autoimmune serum (see Fig. 6,d) reappeared coincident with microtubule reorganization (not shown).

The recovery of an observable MTOC made it possible to determine if there were any long lasting effects of hyperthermia on the ability of the CTL to orient its MTOC towards the bound TC (Fig. 10a, 10b). A comparison between heated cells allowed to recover for 6 h at 37^0C and unheated control cells shows no detectable difference in their ability to orient their MTOC towards a bound TC (Fig. 10c).

Figure 2.9. The Recovery of Microtubule Organization Following Hyperthermia (42^{0}C, 30 min). T-cells, following a hyperthermia treatment (filled circles), and unheated controls (open circles), were incubated at 37^{0}C. Samples were removed at the times indicated and prepared for immunofluorescence staining with the murine monoclonal antibody 5A6 to determine the duration of the heat shock effect on their microtubular organization. The scoring was done blind. Triplicate counts of ≥50 cells were made of each sample and the values shown are the average of the mean of three experiments ±SE.

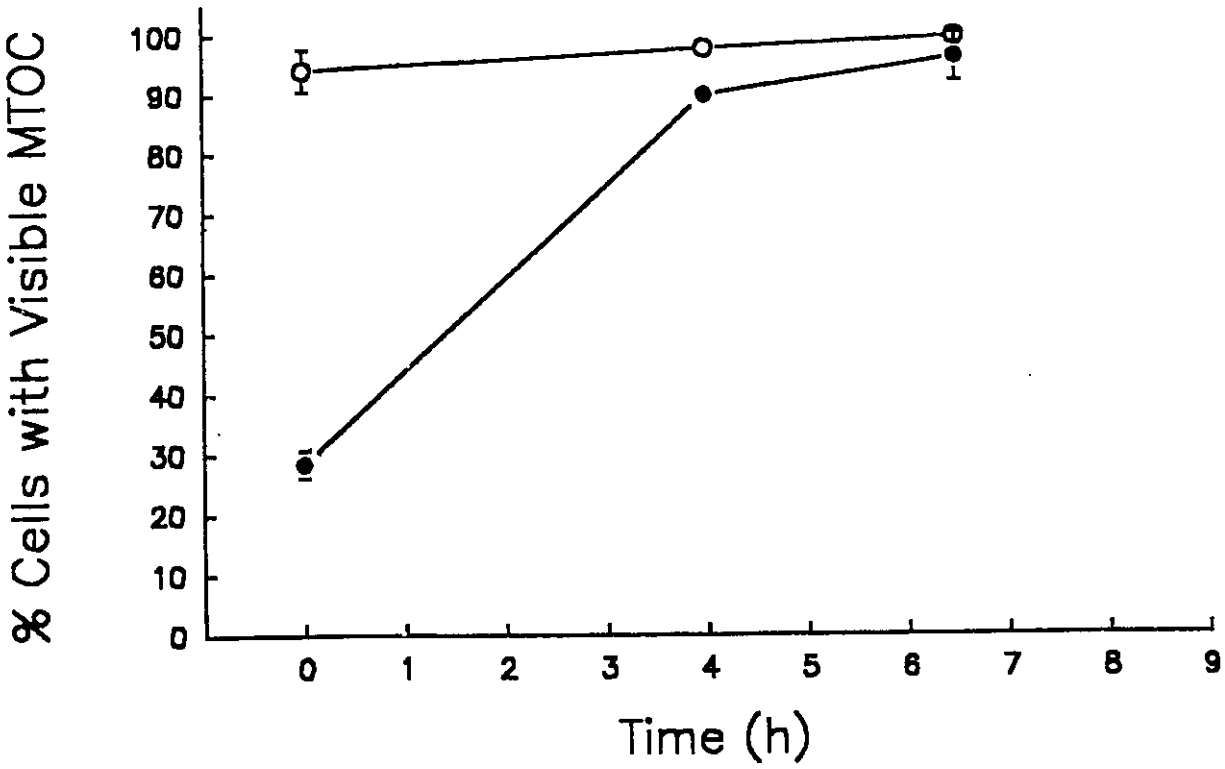
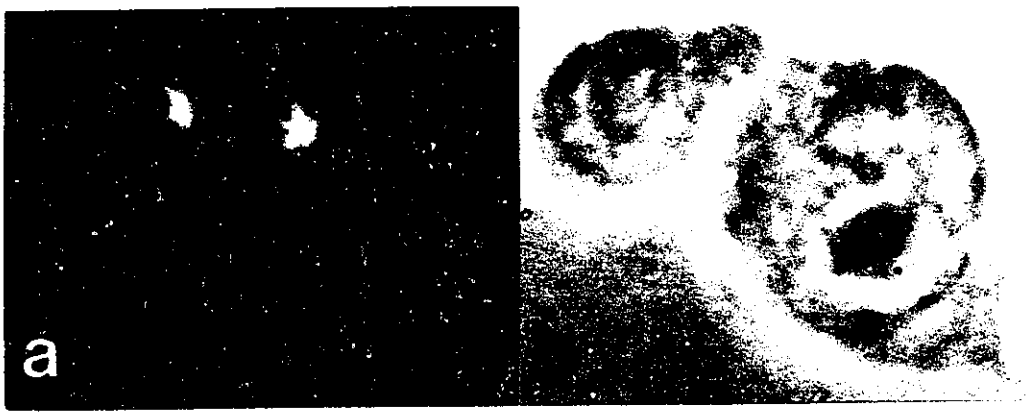
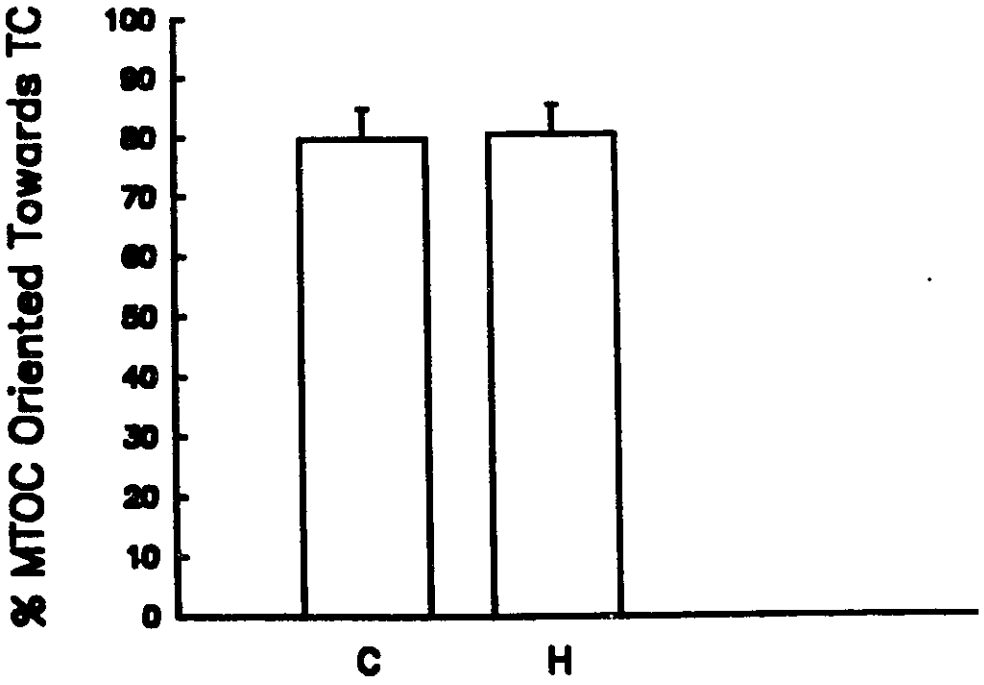

% Cells with Visible MTOC
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
Time (h)

Figure 2.10. Ability of the MTOC to Orient after the Recovery of Microtubule Organization. Immunofluorescence (a) and phase contrast (b) micrographs of a CTL-TC conjugate stained with anti-tubulin. The MTOC of the CTL on the left is clearly oriented towards the much larger TC, whose MTOC is also visible. Magnification 2,500x. Following hyperthermia treatment (42^{0}C, 30 min) the cells were incubated in medium for 6 h at 37^{0}C. The histogram (c) compares the ability of hyperthermia-treated (H) and control (C) cells that had been similarly incubated in medium to orient their MTOC towards a bound TC. No significant difference in the ability of control and hyperthermia-treated CTLs to orient their MTOC towards a bound TC was observed ($X=0.0249$, $p=0.8745$). The scoring was done blind. Duplicate counts of ≥25 cells were made of each sample and the values shown are the average of the mean of two experiments ±SE.


a


% MTOC Oriented Towards TC
100
90
80
70
60
50
40
30
20
10
0
C
H

DISCUSSION

The purpose of this study was to identify the specific mechanisms by which hyperthermia inhibits CTL-mediated cytolytic activity. Hyperthermia is known to produce deleterious alterations in protein structure, causing alterations in cytoskeletal organization (Wachsberger and Coss, 1990) and disrupting numerous cellular processes including protein synthesis (Napolitino et al., 1987; Anathan et al., 1986), and phosphoinositide metabolism (Stevenson et al., 1986). Any, or all, of these effects could be involved in the loss of cytolytic activity. While the ability of a cell to exclude trypan blue is not definitive proof, our results (Fig. 2), in accordance with other published reports (Harris, 1976; Roberts et al., 1985), indicate that the moderate hyperthermia treatments used in this study do not affect T cell viability.

Rather than cataloguing the effects of heat on CTL we approached this problem by examining the ability of heated CTL to perform each of a documented sequence of events that lead up to the lysis of the TC. This sequence, which includes the recognition and binding of the TC, the orientation of the MTOC and GA to face the TC, and the delivery of the 'lethal hit', has been well studied and structures that participate in each step of the process are known. Therefore, the identification of the event blocked by hyperthermia would suggest which structures should be examined further.

Alterations in cytoskeletal organization in response to

hyperthermia have been shown for many cell types in culture; however, variable effects have been reported for each of the cytoskeletal components (see Wachsberger and Coss, 1990 for a recent discussion). Microfilament stress fibres, for example, have been observed to remain unchanged (Collier and Schlesinger, 1986), decrease (van Bergen en Henegouwen and Linnemans, 1987; Glass et al., 1985) or increase (Welch and Suhan, 1985) in number in response to hyperthermia. Based on immunolocalization (Ryser et al., 1982) and inhibitor (Katz et al., 1982) studies, microfilaments in cytotoxic lymphocytes have been implicated in TC binding. Although we have not directly examined microfilament organization in this study, the observation that the hyperthermia treatments used did not adversely affect the ability of CTL to form conjugates with the appropriate TC (Fig. 4) indicates that microfilaments were not affected. This observation also suggests that the heat labile event in cytolysis occurs subsequent to TC recognition and binding.

The next easily assayable event in the lytic process is the orientation of the MTOC/GA to face the TC. This orientation, initially reported by Geiger et al. (1982), has been shown to occur after TC binding and to be a prerequisite for efficient TC lysis (Kupfer et al., 1983; Kupfer et al., 1985). The proposed function of the orientation is to direct the secretion of lytic granules into the tight intercellular junction formed between the CTL and the TC. This remains the dominant model for CTL-mediated cytolysis; however, there is evidence which suggests that CTL

lysis of certain TC can occur in the absence of Ca^{++} and any appreciable degranulation (Young et al., 1988; Kupfer and Singer, 1989; Ostergaard and Clark, 1989). This has led to the suggestion that there are two, and possibly three, different lytic pathways used by CTL in TC killing (Ostergaard and CLark, 1989). Regardless of the pathway, TC lysis in the absence of MTOC orientation has never been observed (Clark et al., 1988; Ostergaard and Clark, 1987).

Although some studies have suggested that microtubules are not affected by heat treatments that result in collapse of the intermediate filament system (Welch and Suhan, 1985; Welch et al., 1985), others have reported disassembly (Malawista and de Boisfleury-Chevance, 1982; Wachsberger and Coss, 1990) or disorganization (van Bergen en Henegouwen and Linnemans, 1987; Lobreau et al., 1988) of the microtubule system in response to hyperthermia. The hyperthermia treatment used in this study does not result in microtubule disassembly, but does lead to a loss of the characteristic radial MTOC-microtubule array in most CTLs. It has been shown previously that 4 h treatments of lymphocytes either with taxol, which promotes the assembly and alters the organization of microtubules, or with colcemid which leads to microtubule disassembly, result in marked changes in the intermediate filament distribution (Paulin-Levasseur and Brown, 1987a). In contrast, there is no readily apparent effect of a 30 min hyperthermia treatment on the organization of the vimentin intermediate filament system. The majority of the hyperthermia treated cells contain

visible intermediate filaments and their distribution is just as unpredictable and variable as that seen in control cells (see Fig. 1.5). Although it is possible that a careful study would reveal a significant effect of the hyperthermia treatment on intermediate filament organization it is clear that in effector cells, unlike the situation in chicken or rat embryo fibroblasts (Collier and Schlesinger, 1986; Welch and Suhan, 1985), the microtubule system is more labile to hyperthermia treatment.

The hyperthermia treatment does not cause any apparent reduction in the amount of polymerized tubulin at the immunofluorescence level, but an obvious MTOC can no longer be detected. Similar observations of a loss of a detectable MTOC, rather than the absence of microtubules, have been reported following hyperthermia treatment of other cell types (van Bergen en Henegouwen and Linnemans, 1987; Lobreau et al., 1988). In one of these studies (Lobreau et al., 1988) it was suggested that the MTOC was disrupted by heat, leading to the formation of multiple microtubule initiation sites distributed through the cytoplasm. This is reminiscent of the loss of MTOC activity and the concurrent random initiation of microtubule assembly in response to the microtubule assembly-promoting drug taxol (De Brabander et al., 1981).

The disorganization of the microtubules in heated effector cells is accompanied by a loss of centriole immunodetection by the rabbit autoimmune serum. At the EM level the only obvious effect of the hyperthermia treatment is an aggregation of the PCM about

the centrioles. The aggregation of the PCM is not accompanied by a disappearance of the sattelite bodies (SBs) and some microtubules are still observed to end on them. Although it appears that the number of microtubules in the centrosomal region is reduced, EM of serial sections would be necessary to confirm this. When heat shock effector cells are allowed to recover for 6 h at 37^{0}C before being processed for immunofluorescence or electron microscopy a reappearance of centrosome staining and redistribution of the PCM is seen.

Earlier studies of Chinese hamster ovary cells heated for 15 min at 45.5^{0}C (Barrau et al., 1978, Coss et al., 1982) and of human blood leucocytes heated for 9 min at 44.5-45.5^{0}C (Malawista and de Boisfleury-Chevance, 1982), showed that such treatments which resulted in the disassembly of most microtubules also caused an aggregation of the PCM about the centrosome. Since hyperthermia was shown not to prevent the polymerization of brain microtubule proteins in vitro, it was suggested that the reduced number of microtubules present in heated cells was due to the loss of the ability of the centrosome to initiate microtubules (Coss et al., 1982). Whether or not similar centrosome disruption/inactivation is responsible for the altered microtubule organization observed in heated effector cells has not yet been determined.

There are several possible explanations for the loss of immunodetection of the centrosome by the rabbit autoimmune serum in hyperthermia-treated effector cells. For example, the aggregation of the PCM may make the 50,000 kDa protein (Turksen et al., 1982)

recognized by the serum inaccessible to the antibody. Alternatively the hyperthermia treatment may directly alter the conformation of the antigen so that it is no longer recognized. A third possibility is that the antigen disperses to multiple sites in the cytoplasm that are below the detection limit of immunofluorescence. Whatever the explanation for the loss of centrosome staining by the autoimmune serum, this loss correlates with the disorganization of the microtubule array and aggregation of the PCM. In addition, the reorganization of the microtubule array about the MTOC and redistribution of the PCM during recovery from hyperthermia treatment is accompanied by a reappearance of centrosome staining. These results raise the possibility that the 50,000 kDa polypeptide may function in maintaining the structure/function of the centrosome.

Kupfer et al., (1983), in experiments utilizing the microtubule depolymerizing drug nocodazole, demonstrated that the orientation of the MTOC was dependent upon the presence of cytoplasmic microtubules. The disorganization of the microtubules and the loss of centriole immunodetection made it impossible to determine the position of the MTOC in most heated CTLs. While the possibility that the signal to orient is blocked by the hyperthermia treatment can not be excluded two observations suggest that these cells are still receiving the signal to orient their MTOCs. Firstly, the ability to orient their MTOCs towards a bound TC is not impaired in the approximately 25% of heated cells whose microtubule organization is unaffected by the hyperthermia

treatment. These cells may account for the approximately 25% of cytolytic activity observed after hyperthermia. Secondly, as soon as microtubule organization recovers the CTLs are able to orient their MTOCs towards the TC. Therefore, if the signalling mechanism is affected it is no more heat labile than the microtubule organization.

The close correlation observed between the loss and recovery of microtubule organization and the loss and recovery of cytolytic activity suggests that the initial inhibitory effect of hyperthermia on cytolytic activity is due to the disruption of MTOC-microtubule organization. It is possible that orientation is dependent not only upon the presence of cytoplasmic microtubules but upon the radial MTOC-microtubule array and that hyperthermia inhibits cytolytic activity by blocking orientation. This interpretation of the results would support the hypothesis that MTOC orientation is a prerequisite for lysis. Alternatively, the disruption of microtubule organization might have an effect similar to the disassembly of microtubules by colcemid or colchicine in lymphocytes and other cell types (Thyberg and Moskalewski, 1985) leading to a loss of cell polarity and Golgi organization that may be required for cytolytic activity. However, the maintenance of Golgi organization and its association with the centrosome in the nuclear cleft in effector cells examined following hyperthermia treatment suggest that the microtubule disorganization has an effect unlike microtubule disassembly by colcemid or colchicine.

The observation that the recovery of cytolytic activity is

incomplete and transient indicates that hyperthermia has effects in addition to those on the microtubule system. It has been demonstrated that the moderate hyperthermia treatments used in this study cause a generalized suppression of protein synthesis and the enhanced synthesis of a subset of proteins, the heat shock proteins. This is the classic heat shock response that has been demonstrated in many different cell types (Pelham, 1986; Burdon, 1986; Anathan et al., 1986). The inhibition of protein synthesis suggests that the depletion of a preformed pool of a lytic component may be responsible for the limited recovery and subsequent loss of cytolytic activity observed following hyperthermia treatment. The drop in cytolytic activity reported in several studies (Brunner et al., 1968; Thorn and Henney, 1976; Landon et al., 1990) following the addition of protein synthesis inhibitors support this hypothesis.

There are however, other reports in the literature where the inhibition of protein synthesis has no short term (under 8 h) effect on cytolytic activity (Duke et al., 1983; Ostergaard and Clark, 1989). These reports do not necessarily contradict the hypothesis because it is possible that, as a result of the hyperthermia treatment, the turnover of some component of the lytic pathway is accelerated. The increased turnover rate would make the effect of the loss of production of this component apparent in under 6 h and its continued depletion after this time would account for the continued loss of cytolytic activity.

CHAPTER 3

INTRODUCTION

When murine CTLs are heated at 42^0 for 30 min their ability to lyse their specific TCs becomes severely impaired. This loss of cytolytic activity is followed by a partial recovery of activity that peaks within 6 h. It was shown in the previous chapter that CTL viability is not affected by such treatments and that the killing step, or steps, affected by hyperthermia occur subsequent to TC recognition and binding. It was also demonstrated that the organization of the MTOC-microtubule array is disrupted by the hyperthermia treatments and that the partial recovery of cytolytic activity is coincident with the reorganization of the MTOC-microtubule array. These results led to the suggestion that the initial inhibitory effect of hyperthermia on CTL function results from the disruption of microtubule organization.

In contrast to what is observed in other cell types (De Brabander et al., 1981; Sandoval et al., 1984), taxol treatment of lymphocytes promotes the assembly of microtubules only from the centrosome and does not disrupt the organization of the Golgi apparatus or its association with the centrosome (Brown et al., 1985). The microtubule bundles formed in lymphocytes (Brown et al.,1985) and in other cell types (Manfredi et al.,1982) in response to taxol treatment are relatively resistant to

microtubule-depolymerizing drugs and low temperature. Thus, taxol can antagonize the effects of microtubule disrupting agents. In particular, the inhibition of cytolytic activity caused by microtubule disrupting agents is reportedly limited by low concentrations (0.05 μM) of taxol (Wolberg et al., 1984). These results suggested that taxol may be a useful agent to test the relationship between the hyperthermia-induced disorganization of the microtubule network and its effect on cytolytic activity.

RESULTS

Effect of taxol on microtubule organization. Effector cells were exposed to different concentrations of taxol for 4 h and then prepared for immunofluorescence microscopy. Treatment of lymphocytes with 10 μM taxol for 4 h leads to the formation of one or a few large bundles of microtubules. It has been previously reported that while taxol doses of under 10 μM have the same effect on the microtubule organization of murine lymphocytes, longer times of exposure are required (Brown et al., 1985). After a 4 h treatment 0.01 or 0.1 μM doses did result in significantly less microtubule bundling in the effector cells (Fig. 1); however, no difference was seen between the 1 and 10 μM taxol treatments. In both the 1 and 10 μM taxol treatments nearly 95% of the cells exposed exhibited a few large microtubule bundles. The taxol-induced bundles in effector cells extend through the cytoplasm near the surface of the nucleus, distant from the submembranous position of the microtubules in untreated cells. The taxol treatment does not lead to a disruption of the Golgi apparatus which maintains its association with the centrosome (Fig. 2 and Brown et al., 1985).

Effect of taxol on MTOC orientation and cytolytic ability. Figure 3 shows the effects of the 1 and 10 μM taxol treatments on

Figure 3.1. Effect of Taxol on Microtubule Organization. Effector cells were exposed to the indicated concentrations of taxol for 4 h and then prepared for immunofluorescence microscopy with antibody to tubulin. The 0.01 μM dose resulted in clearly less microtubule bundling than that caused by the 10.0 μM dose. The 0.1 μM taxol dose also resulted in significantly less microtubule bundling ($X=6.8709$, $p=0.0322$). No such difference was observed between the amount of microtubule bundling caused by the 1.0 or 10 μM doses ($X=6.9755$, $p=0.9334$).

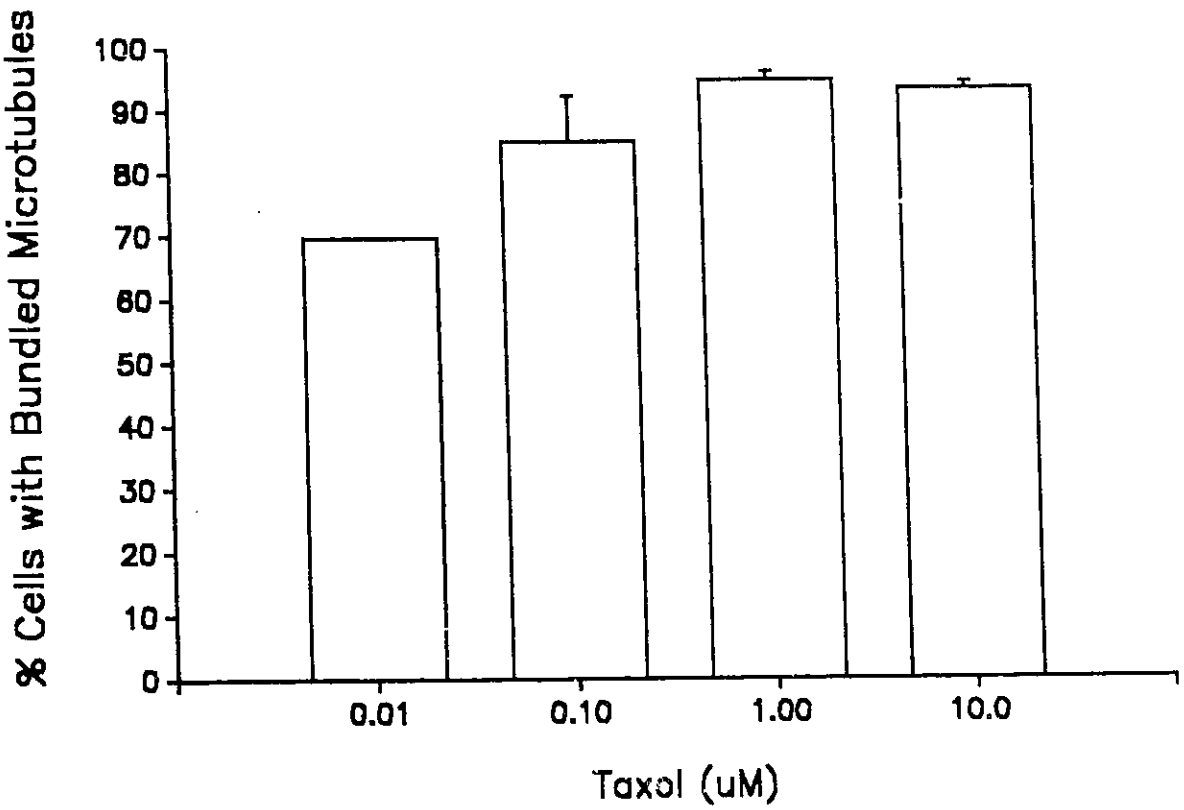
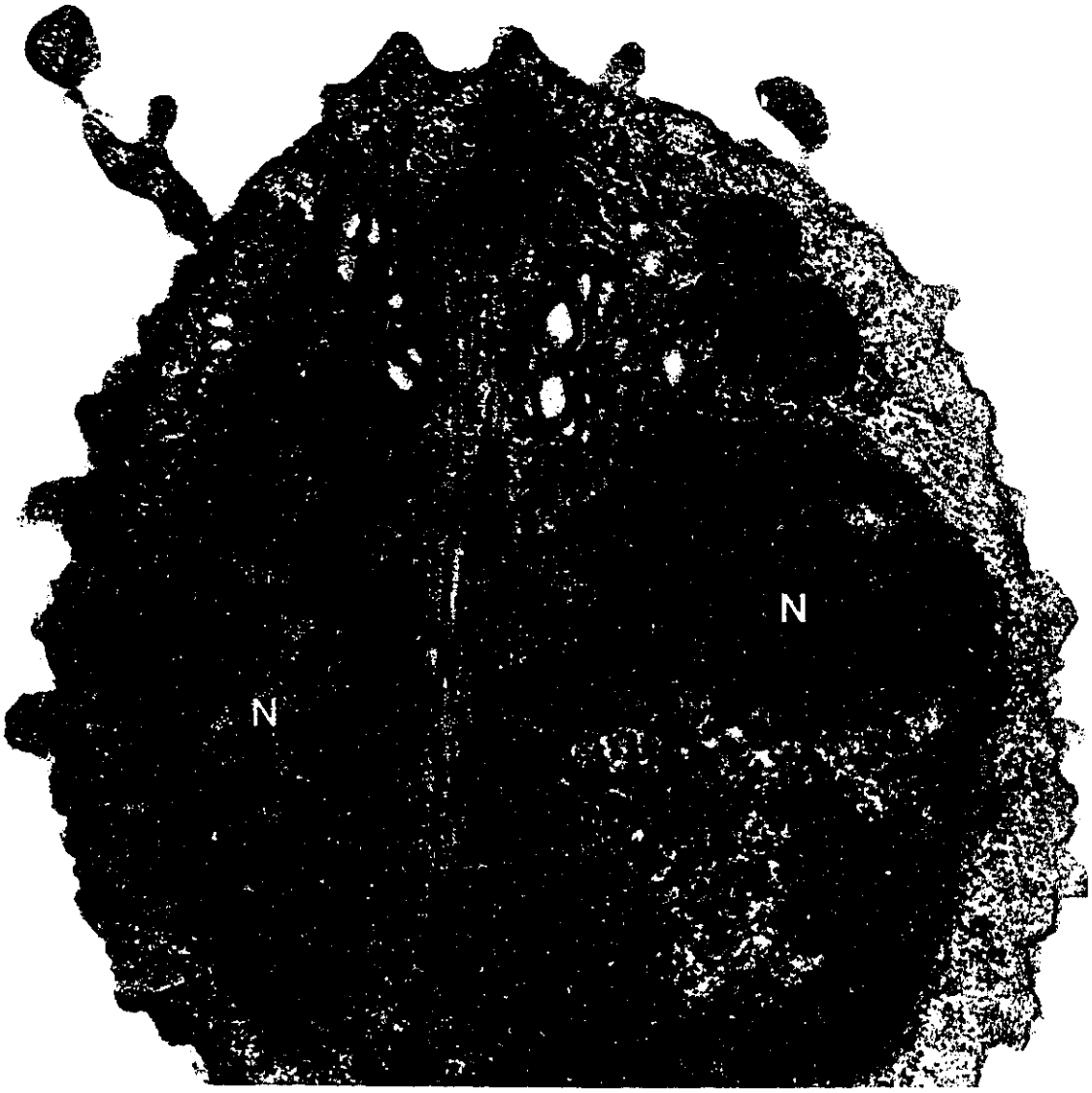

% Cells with Bundled Microtubules
100
90
80
70
60
50
40
30
20
10
0
0.01
0.10
1.00
10.0
Taxol (uM)

Figure 3.2. Effect of Taxol on Microtubule Organization. Electron micrograph of an effector cell exposed to 1 μM taxol for 4 h. A microtubule bundle (arrow) can be seen to extend from the centrosome (C) and pass by the surface of the nucleus (N). It is evident that the Golgi apparatus (Ga) is not disrupted by this treatment and that it maintains its association with the centrosome. Magnification 40,000x.


N
N

the ability of conjugated CTLs to orient their MTOC towards the TC. Surprisingly, the 1 μM taxol dose has no effect on the ability of the CTLs to become polarized towards the TC while the 10 μM taxol dose, as was previously observed (Chapter 1), causes a small but, statistically significant inhibition of orientation (Fig. 3). When the effect of these same two taxol treatments on cytolytic ability was assayed it was found that neither treatment significantly affects this CTL function (Fig. 4).

Taxol protection of microtubule organization from the effects of hyperthermia. In taxol-treated (1 μM, 4 h) effector cells the microtubule bundles extend from a single site that is recognized by the rabbit autoimmune serum that detects centrioles (Fig. 5a,b). At the EM level it can be seen that the microtubule bundles remain associated with the centrosome and many of the microtubules end on the SBs. Hyperthermia treatment does not disrupt taxol-induced microtubule bundles and these bundles still extend from a single site (Fig. 5g; Fig. 6); however, taxol treatment does not prevent the loss of immunodetection of the centrosome by the rabbit autoimmune serum (Fig. 5h). EM of similarly treated cells confirms that the bundles remain associated with the centrosome and that the organization of the Golgi and its association with the centrosome is not disrupted (Fig. 5e,f). These micrographs also demonstrate that while the microtubules remain associated with the centrosome the aggregation of the PCM about the centrioles, like the loss of immunodetection of the centrosome, is not prevented by the taxol

Figure 3.3. Effect of Taxol on the Ability of Conjugated CTLs to Orient their MTOC Towards the TC. CTL/TC conjugates were formed and processed for immunofluorescence microscopy with antibody to tubulin. Immunofluorescence (a) and phase contrast (b) micrographs show a taxol-treated CTL with its MTOC oriented towards the TC. Magnification 2,000x. The histogram compares the ability of control cells and cells that had been pretreated with either 1.0 μM or 10.0 μM taxol for 4 h prior to conjugate formation to orient their MTOC towards a bound TC. Pretreatment with 1.0 μM taxol did not significantly affect the ability of the CTLs to orient their MTOC ($X=0.4487$, $p=0.5029$) while pretreatment with 10.0 μM did ($X=21.4061$, $p=0.000004$). Triplicates counts of $\geq$ 50 cells were made of each sample and the values shown are the average of the mean of two experiments.


a
b

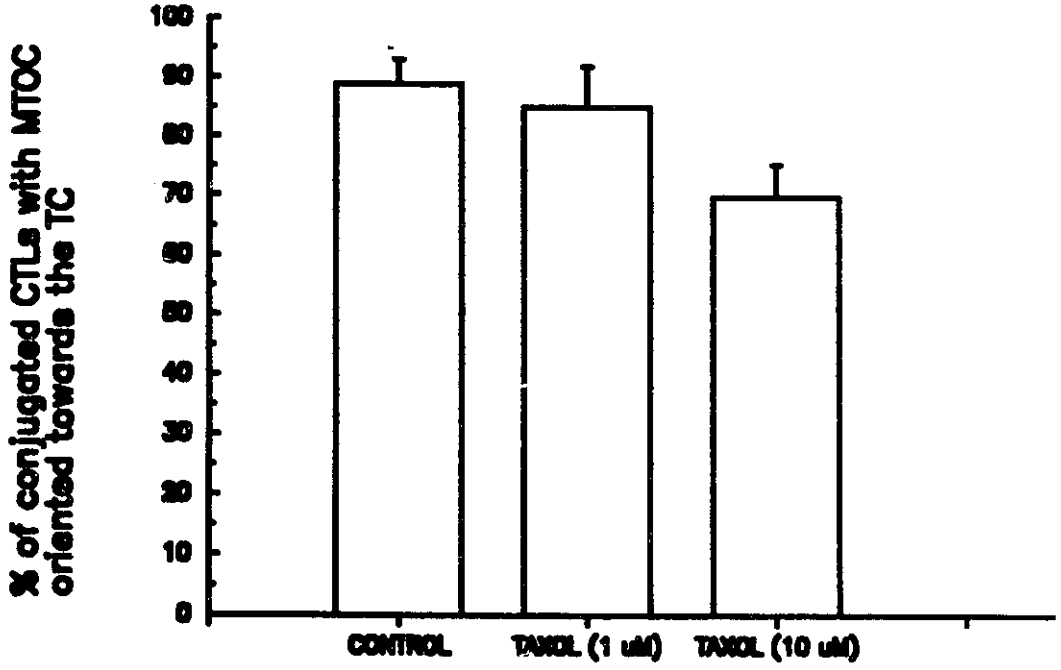

% of conjugated CTLs with MTOC oriented towards the TC
0
10
CONTROL
TAXOL (1 uM)
TAXOL (10 uM)

Figure 3.4. Effect of Taxol on Cytolytic Ability. Effector cells were pretreated for 4 h with the indicated dose of taxol and then mixed with ^{51}Cr loaded TCs in the continued presence of the drug. The histogram compares the cytolytic activity of the taxol treated cells to control cells. Neither dose of taxol was found to significantly affect cytolytic activity ($F=1.5566$, $DF_1=2$, $DF_2=75$, $p=0.2176$). All samples were done in triplicate and the values shown are the average of the means of ≥5 experiments ±SE.

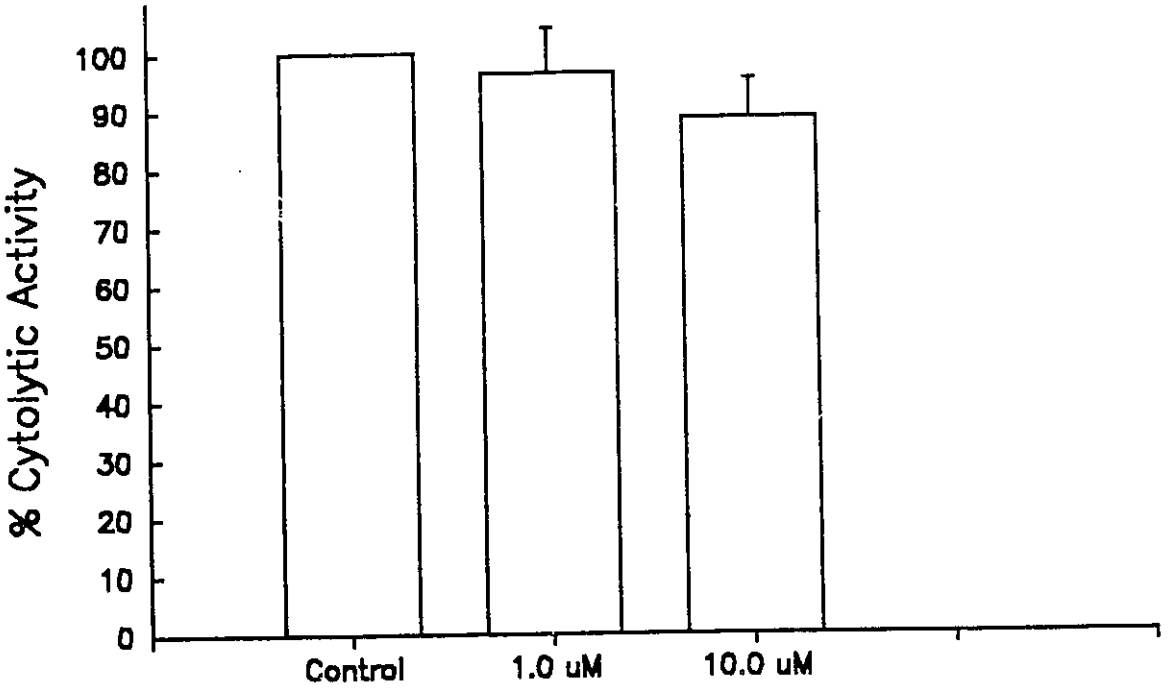

% Cytolytic Activity
100
90
80
70
60
50
40
30
20
10
0
Control
1.0 uM
10.0 uM

Figure 3.5. Effect of Hyperthermia on the Taxol-induced Microtubule Bundles. Double immunofluorescence with an antibody to tubulin (a) and a rabbit autoimmune serum that recognizes the centriole (b) demonstrates that the treatment of effector cells leads to the formation of a few centrosome associated microtubule bundles. The association of the microtubule bundles with the centrosome is confirmed by electron microscopy (c,d). Following hyperthermia treatment EM shows that the microtubule bundles remain associated with the centrosome, but that the taxol treatment does not prevent the aggregation of the pericentriolar material (e,f). That the microtubules remain organized as bundles can also be seen by immunofluorescence (g) but there is a loss of immunodetection of the centrioles by the autoimmune serum (h). Magnification: Immunofluorescence 1,500x; EM 50,000x.

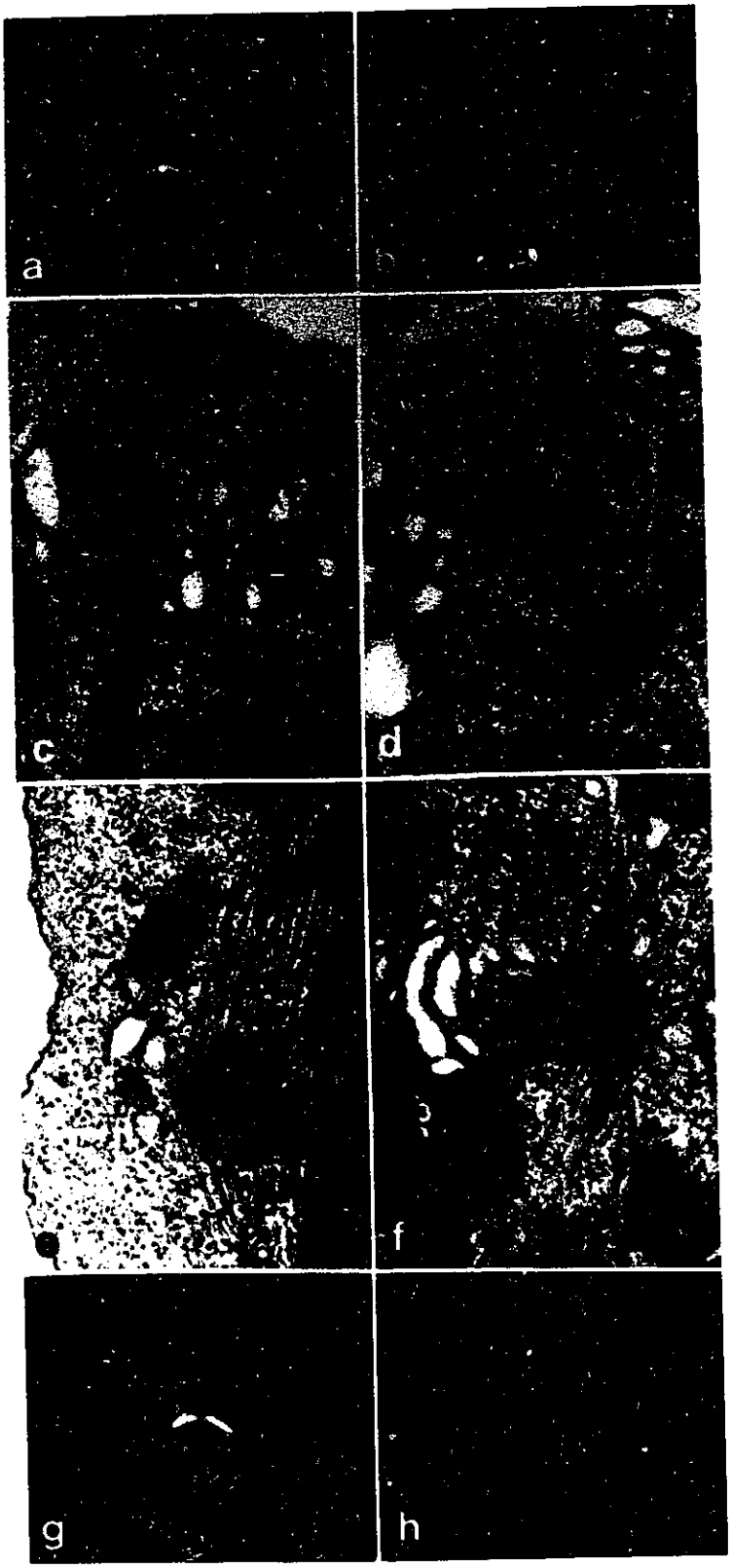
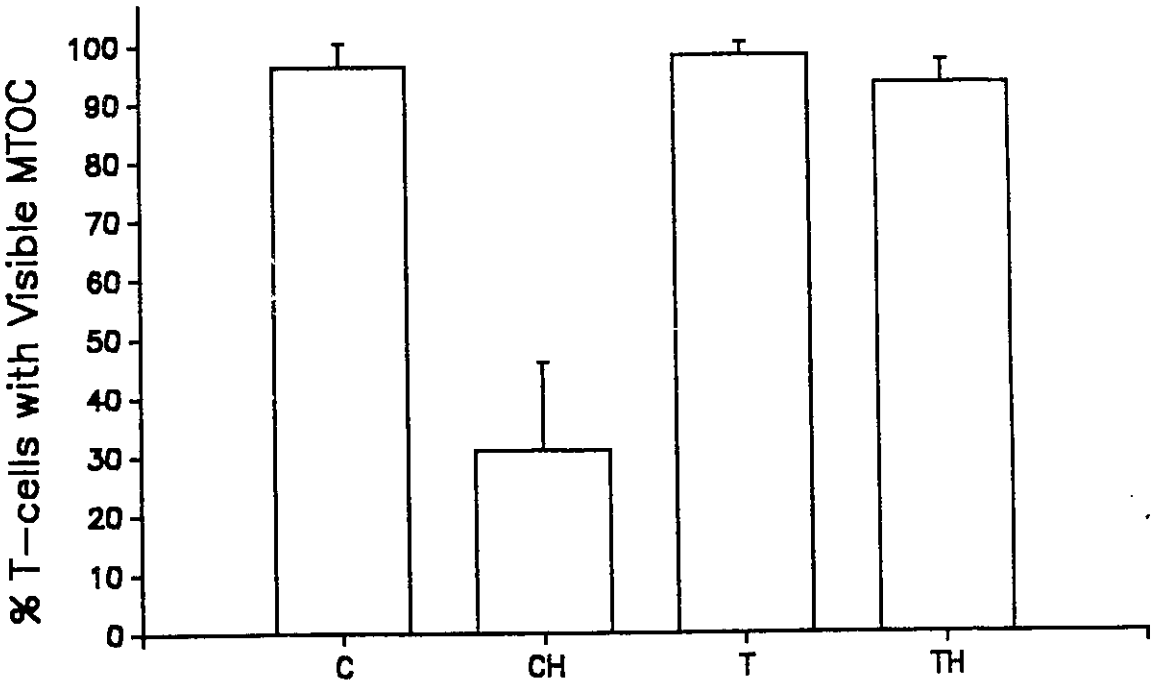

a
c
d
f
g
h

Figure 3.6. Ability of Taxol to Prevent Hyperthermia-induced Microtubule Disruption. Effector cells were harvested and divided into two aliquots. One aliquot was treated with 1.0 μM taxol for 4 h. The taxol-treated and control cells were then exposed to 42^0C for 30 min. Immediately following the hyperthermia treatment all the cells were fixed and prepared for immunofluorescence microscopy. The histogram compares the effect of the hyperthermia treatment on the microtubule organization of taxol-treated and control cells. The hyperthermia treatment clearly disrupts the microtubule organization of the control heated (CH) cells. No significant difference in the number of cells with a discernable MTOC was seen between the control (C) and taxol-treated (T) cells ($X=2.6463$, $p=0.1038$) or between the control (C) and hyperthermia-treated cells containing taxol-induced microtubule bundles (TH) ($X=2.9926$, $p=0.0836$). Triplicate counts of ≥ 50 cells were made of each sample and the values shown are the average of the mean of 4 experiments $\pm$SE.


% T–cells with Visible MTOC
100
90
80
70
60
50
40
30
20
10
0
C
CH
T
TH

treatment.

Effect of hyperthermia on MTOC orientation and cytolytic activity of taxol treated cells. 1 μM taxol treatments have no effect on orientation and, unlike the situation in control cells, the position of the MTOCs of heated taxol-treated effector cells can be detected by immunofluorescence microscopy with antibodies to tubulin. This made it possible to determine the effect of hyperthermia treatment on MTOC orientation in taxol-treated CTLs bound to TCs. Figure 7 shows that hyperthermia treatment causes an inhibition of MTOC orientation. ^{51}Cr release assays demonstrate that taxol treatment does not prevent the loss, or affect the recovery, of cytolytic activity following hyperthermia treatment (Fig. 8).

Figure 3.7. Effect of Hyperthermia on the Ability of Taxol-Treated Cells to Orient their MTOC. Effector cells were pretreated with 1.0 μM taxol for 4 h, exposed to 42^{0}C for 30 min, and then conjugates were formed and prepared for immunofluorescence microscopy with antibody to tubulin. The histogram shows the effect of hyperthermia treatment on the ability of taxol-treated CTLs to orient their MTOC towards a TC. As previously demonstrated taxol treatment alone has no effect on the ability to orient (X=0.0112, p=0.9159). Hyperthermia treatment, however, reduces the number of taxol treated CTLs oriented towards the TC (X=9.3711, p=0.0022). Duplicate counts of ≥25 cells were made of each sample and the values shown are the average of the mean of 2 experiments ±SE.

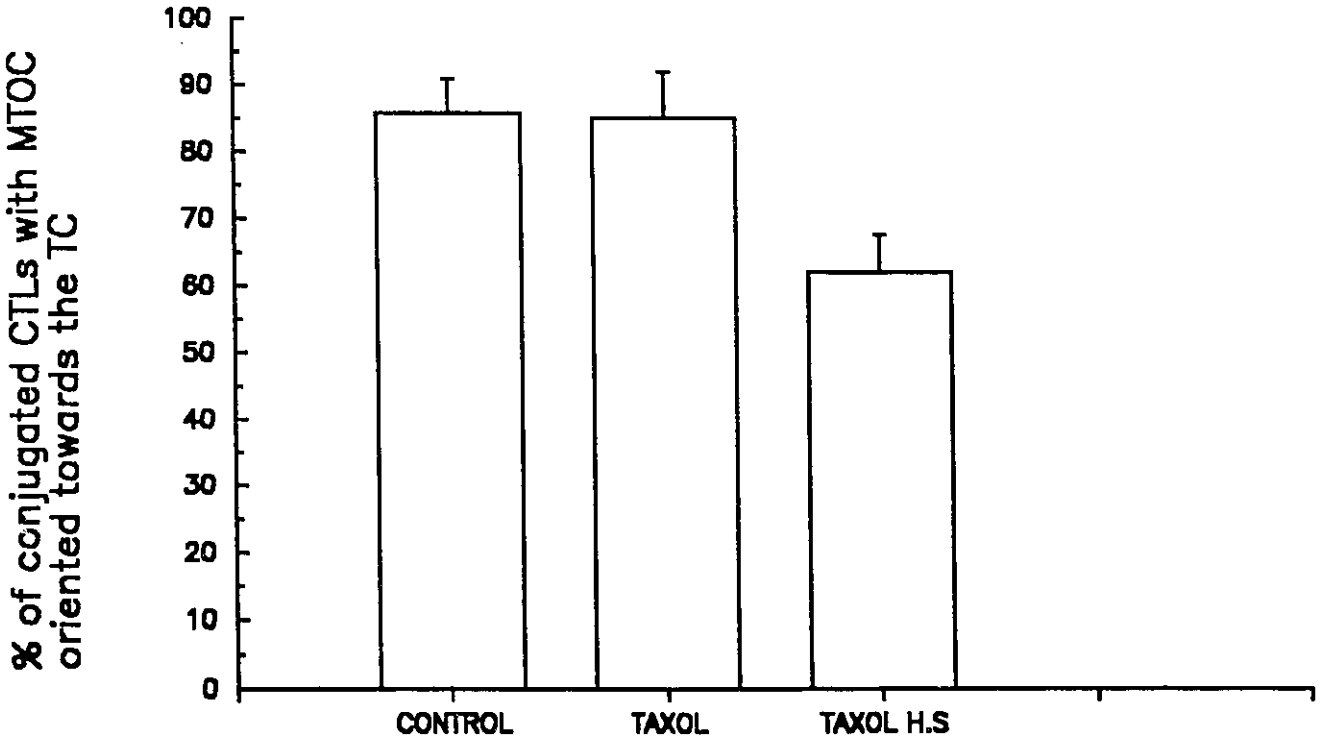
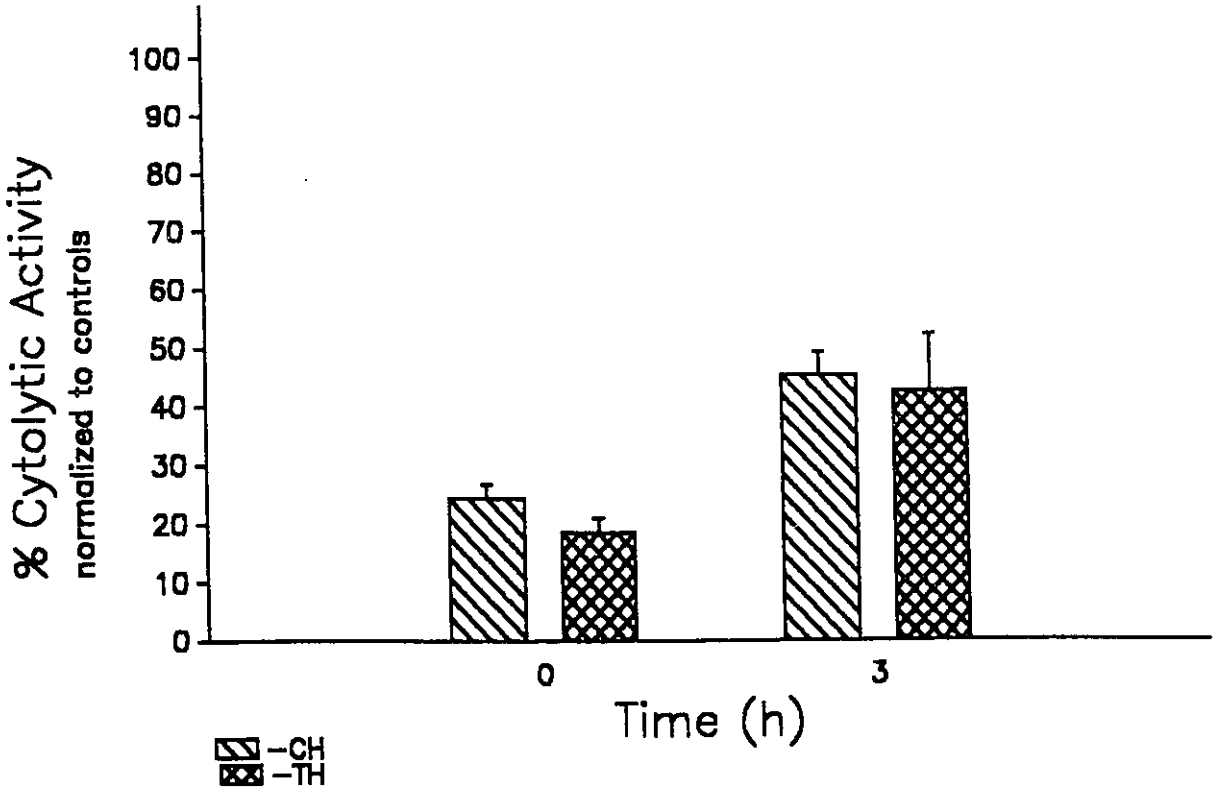

% of conjugated CTLs with MTOC oriented towards the TC
100
90
80
70
60
50
40
30
20
10
0
CONTROL
TAXOL
TAXOL H.S

Figure 3.8. Effect of Hyperthermia on the Cytolytic Ability of Taxol-Treated CTLs. Effector cells were treated with 1.0 μM taxol or incubated in control medium for 4 h before being exposed to 42^0C for 30 min. Immediately following the hyperthermia treatment a cytolytic assay was performed. No significant difference was observed in the cytolytic ability of the taxol-treated (TH) and control (CH) cells ($F=0.6596$, $DF_1=1$, $DF_2=15$, $p=0.4294$). In addition, the continued association of the microtubule bundles with the centrosome did not significantly affect the extent of cytolytic activity when measured after 3 h of recovery ($F=0.1366$, $DF_1=1$, $DF_2=14$, $p=0.7181$). All samples were done in triplicate and the values shown are the average of the mean of 3 experiments ±SE.


% Cytolytic Activity
normalized to controls
100
90
80
70
60
50
40
30
20
10
0
0
3
Time (h)
-CH
-TH

DISCUSSION

MTOC orientation and cytolytic activity were shown to be microtubule dependent by Kupfer et al. (1983). In these studies the MTOCs of CTLs, whose microtubules had been disassembled by drug treatments prior to conjugation with TCs, were demonstrated to be randomly oriented with respect to the TCs. The incubation of these treated cells with ^{51}Cr labelled TCs produced little TC lysis. When the drugs were subsequently removed to permit the reassembly of the microtubules the ability of the cell to orient towards and to lyse TCs was shown to recover. Other experiments in which the orientation of the MTOC was reversibly inhibited by removal of Ca^{++} from the medium (Kupfer et al., 1985), and the observation that when CTLs are conjugated to several targets the TCs are not lysed simultaneously but rather sequentially (Rothstein et al., 1978; Zagury et al., 1979) provided additional circumstantial evidence in support of the hypothesis that MTOC reorientation is a prerequisite for TC lysis.

In Chapter 2 of this study it was established that hyperthermia treatment results in a disorganization of the MTOC-microtubule system and prevents the immunodetection of the centrioles by the rabbit autoimmune serum (Figs. 2.6 and 2.7). This made it impossible to determine the effect of hyperthermia on the orientation of the MTOC by immunofluorescence. Therefore, while the close correlation observed between the loss and recovery of MTOC-microtubule organization and the loss and recovery of

cytolytic activity suggested that the initial inhibitory effect of hyperthermia was due to the disruption of MTOC-microtubule organization, whether or not this treatment inhibited MTOC orientation was unclear.

In this chapter taxol was used to test the relationship between the hyperthermia-induced disruption of the MTOC-microtubule array and its effect on cytolytic activity. As previously observed (Fig. 1.16) treatment of effector cells with 10 μM taxol for 4 h leads to an inhibition of MTOC orientation. The surprising result was that treating cells with 1 μM taxol for 4 h, a dose that results in the same amount of microtubule bundling, does not inhibit MTOC orientation. That treatment of cells with 1 μM taxol for 4 h has no effect on the ability of CTLs to orient their MTOC towards the TC, would seem to suggest that a dynamic, radially arranged microtubule array is not required for MTOC orientation. Why the higher taxol dose inhibits MTOC orientation is unclear. In the only other study I am aware of where the effect of taxol on MTOC orientation was determined, prolonged (24-48 h) exposures to very low doses (1 ng/mL) of taxol that did not alter microtubule organization slowed, but did not prevent, the orientation of the MTOCs of porcine aortic endothelial cells to face a wound edge (Coomber and Gotlieb, 1990).

Neither of the taxol doses discussed above had an effect on cytolytic activity. In this instance the surprise is that the 10 μM for 4 h treatment, which led to a significant inhibition of MTOC orientation, did not result in a decrease in cytolytic activity.

This result appears to run contrary to all the other reports in the literature that suggest that MTOC orientation is a prerequisite for lysis; however, the taxol treatment results in only a partial inhibition of orientation and the high ratio of effector cells to TCs used in the cytolytic assays (≥25:1) makes it possible that many of the TCs will be bound by more than one CTL, at least one of which will be able to orient towards it.

The taxol-induced microtubule bundles are not disorganized and remain associated with the centrosome following a 42^{0}C for 30 min hyperthermia treatment. Nevertheless, the taxol treatment does not prevent the loss of immunodetection of the centrioles by the rabbit autoimmune serum, the aggregation of the PCM about the centrioles, or an inhibition of MTOC orientation caused by hyperthermia. These results demonstrate that the preservation of a centrosome-associated microtubule array does not prevent hyperthermia from inhibiting both MTOC orientation and cytolytic activity. Therefore, while MTOC orientation and TC lysis may be dependent upon a centrosome associated microtubule array these results suggest that the initial inhibition of hyperthermia on CTL function is probably not due to the disruption of microtubule organization.

Cold, like heat, has been shown to produce changes in both the microtubule system and centrosome organization (Schweitzer and Brown, 1984). The results of this earlier study and the hyperthermia results presented here demonstrate that following either physical treatment the recovery of microtubule and centrosome organization occur concomitantly. This indicates that

the organization of the microtubules and the centrosome are linked, but it is impossible to infer from these results whether one is imposing organization on the other.

Microtubule specific drug treatments, which result in either the disassembly of most microtubules (Schweitzer and Brown, 1984) or promote microtubule assembly and cause an extensive reorganization of the microtubule system (Brown et al., 1985), do so without having an effect on the organization of the PCM. If maintenance of microtubule organization was one of the functions of the PCM it can be argued that the microtubule specific drugs that affect microtubule organization without visibly affecting the PCM are simply able to circumvent the influence of the PCM. If, on the other hand, microtubules are responsible for the organization of the PCM it is possible, though perhaps less likely, that the residual microtubules or the extensively rearranged microtubules resulting from the drug treatments are still able to perform this function. In general these observations show that treatments which affect the microtubule system do not necessarily affect the organization of the PCM while treatments which affect the organization of the PCM also affect the organization of the microtubule system.

The observation that the maintenance of a centrosome-associated microtubule array by taxol does not prevent the hyperthermia-induced aggregation of the PCM supports the suggestion that microtubules are unable to impose order on the PCM. This observation also suggests that an alteration in centrosome

organization may be the key lesion caused by the hyperthermia treatment and that the disruption of the radial microtubule array is a consequence of the aggregation of the PCM.

The evidence in the literature and presented in this study suggests that MTOC orientation is a prerequisite for efficient TC lysis. The taxol results presented in this chapter demonstrate that hyperthermia blocks MTOC orientation and therefore suggest that the initial inhibition of cytolytic activity caused by hyperthermia treatment is a consequence of the CTLs losing their ability to become polarized towards their bound TCs. The loss and recovery of PCM organization is coincident with the loss and the recovery of the ability of the MTOC of CTLs to become oriented towards their bound TCs and with the loss and recovery of cytolytic activity. This suggests that a component of the PCM is involved in MTOC orientation and that the initial inhibition of cytolytic activity caused by hyperthermia is due to a heat-induced effect on this component; however, the possibility that the signal to orient is blocked can not be excluded (see pp. 116-117 in Chapter 2 for discussion). The hyperthermia treatment may either be acting on a component of the PCM directly, causing a deleterious alteration in its structure, or the heat-induced aggregation of the PCM may frustrate its function by physically preventing the interaction of the centrosome-associated component with another cytoplasmic or membrane-associated component.

CHAPTER 4

INTRODUCTION

The results of Chapter 3 suggested that hyperthermia prevents the polarization of CTLs towards a bound TC by interfering with the function of a component of the PCM. The loss and recovery of the ability of the CTLs to orient their MTOCs towards the TC is coincident with the loss and recovery of cytolytic activity indicating that this heat-induced effect was responsible for the initial loss of cytolytic activity observed. Although the MTOC-microtubule organization, and the ability of the CTLs to orient their MTOCs, recover completely from the effects of the hyperthermia treatment within 6 h the recovery of cytolytic activity is incomplete and transient. This indicates that hyperthermia is having a long term effect on a process which occurs after the initial steps of the lytic process; TC recognition and binding, and polarization of the CTL towards the TC. The goal of the experiments of this chapter was to provide an explanation for the partial and transient recovery of cytolytic activity.

In Chapter 2 it was suggested that the heat-induced alteration in protein synthesis resulted in the depletion of a preformed pool of a component required for cytolytic activity and was responsible for the limited recovery and long term loss of cytolytic activity. To test this hypothesis the effect of the reversible protein synthesis inhibitor cycloheximide on cytolytic activity and

recovery from hyperthermia as well as the duration of the heat-induced alterations in protein synthesis have been ascertained.

A discussion of the mechanism of CTL-mediated cytotoxicity is required to provide an explanation for the effects of hyperthermia on cytolytic activity. Differences in the dependence of different populations of CTLs on extracellular Ca^{++} and protein synthesis for efficient TC lysis (Ostergaard and Clark, 1989), as well as differences in the morphology of TC being lysed by CTLs (Duke et al, 1983) have led to the suggestion that CTLs use several different lytic pathways (for reviews see Clark et al., 1988; Martz and Howell, 1989). It was not the purpose of this study to rigorously determine the pathway or pathways utilized by primary cultures of CTLs, but based on the Ca^{++} requirement for cytolytic activity (see Fig. 1.10) and results presented in this chapter it is suggested that the TC lysis observed in this study occurs as a result of both the directed secretion of cytolytic proteins and CTL-induced apoptosis. Using this as the model for cytolytic activity the effect of hyperthermia on cytolytic activity is discussed.

RESULTS

Effect of cycloheximide on TC recognition and binding. Cycloheximide is a reversible protein synthesis inhibitor that acts by inhibiting the transfer reaction in protein synthesis (Grollman, 1966). Centrifugation and resuspension of cells in fresh medium after a 30 min incubation with 100 μM cycloheximide permitted some protein synthesis (Fig. 1E). In the following studies cells that were 'cycloheximide-treated' were exposed to a given concentration of the drug 30 min prior to any subsequent treatment or assay and this concentration was maintained for the duration of the experiment. The treatment of effector cell populations with 100 μM cycloheximide inhibits most ^{35}S-methionine incorporation (Fig. 1F). Random fields of conjugates that had been stained with anti-Lyt 2.2 antibody were examined by epifluorescence and then by phase contrast microscopy and the percentage of Lyt 2.2$^+$ cells bound to a TC determined. Figure 2 shows that the treatment of effector cells with 100 μM cycloheximide prior to hyperthermia treatment has no effect on their ability to form conjugates.

Effect of cycloheximide on microtubule organization and MTOC orientation. Immunofluorescence microscopy, with a monoclonal antibody to tubulin, was used to determine if the hyperthermia treatments used in this study had the same effect on the microtubule system of cycloheximide treated cells as it did on

Figure 4.1. Effect of Cycloheximide on ^{35}S-Methionine Incorporation. Effector cells were pretreated with 100 μM cycloheximide and then centrifuged once and resuspended in fresh medium either without (B) or with (C) cycloheximide. 30 μCi/mL of ^{35}S-methionine was then added to the two above samples and an untreated control sample (A). After a 6 h incubation at 37^{0}C equal amounts of total cell protein were run on a 9.0% polyacrylamide gel and then stained with Coomassie blue. The corresponding autoradiograph shows the pattern of ^{35}S incorporation in the control (D), the washed (E), and the cycloheximide treated (F) cells.

A B C D E F

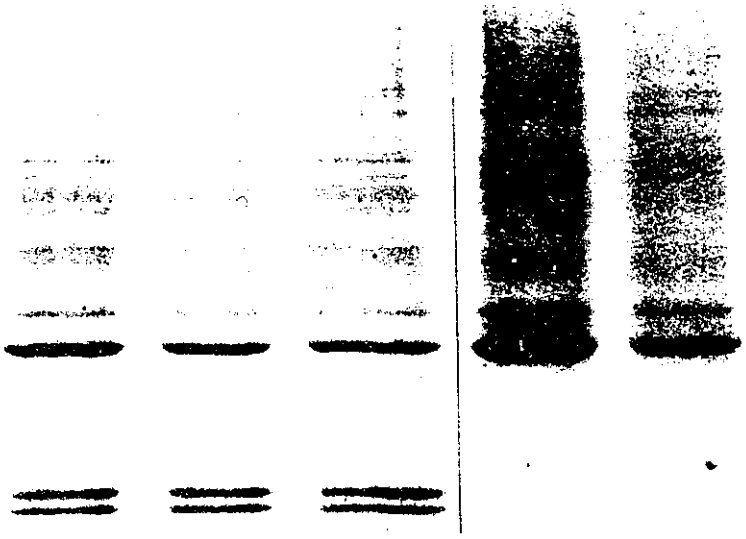
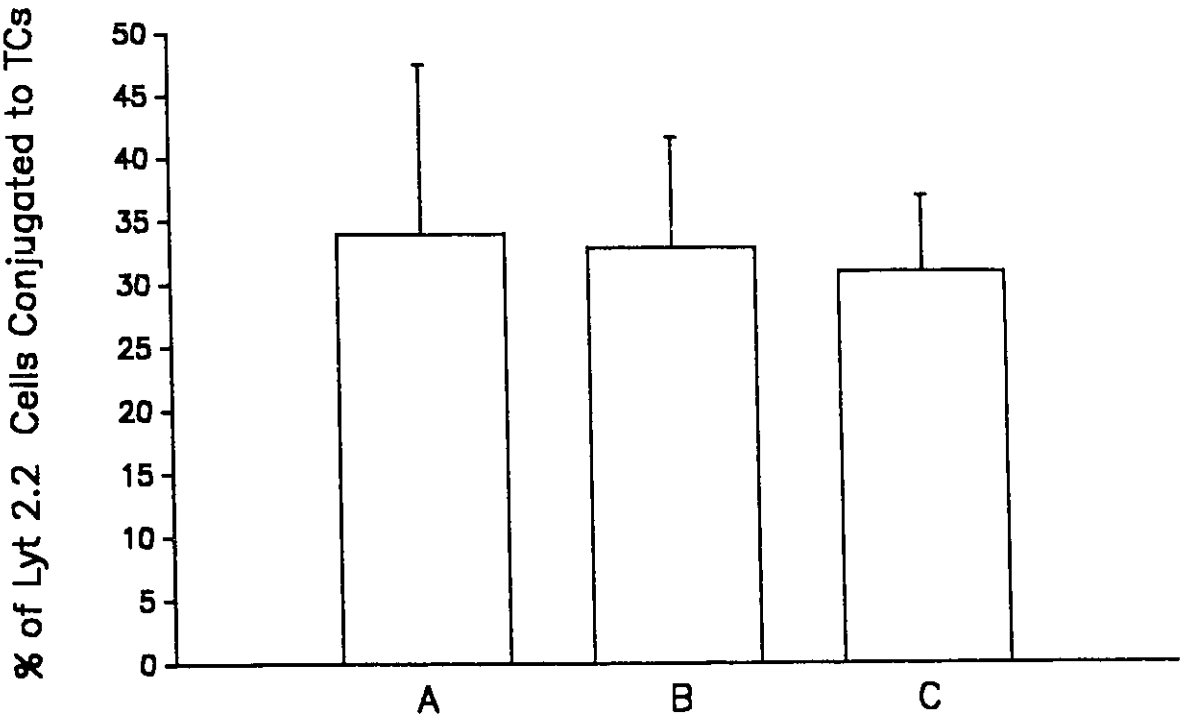


Figure 4.2. Effect of Cycloheximide and Hyperthermia on TC Recognition and Binding. Two aliquots of effector cells were suspended in medium containing 100 μM cycloheximide. After a 30 min incubation at 37^0C one aliquot was left at 37^0C and the other exposed to 42^0C for 30 min. Immediately following these treatments CTL/TC conjugates were formed using control (A), cycloheximide-treated (B), or cycloheximide and hyperthermia-treated (C) cells. The percentage of Lyt 2.2^+ cells bound to a TC is plotted. The histogram shows that there was no significant difference in the ability of these cells to bind TCs ($X=0.7566$, $p=0.6850$). Triplicate counts of ≥ 25 cells were made and the values presented are the average of the mean of two experiments $\pm$SE.


% of Lyt 2.2 Cells Conjugated to TCs
50
45
40
35
30
25
20
15
10
5
0
A
B
C

control cells. Figure 3 demonstrates that cycloheximide treatment does not alter the effect of hyperthermia on the microtubule network of effector cells. Interestingly, cycloheximide also had no effect on the recovery of microtubule organization following hyperthermia treatment. The recovery of microtubule organization in virtually all cycloheximide treated cells occurs within 6 h of the hyperthermia treatment. This allowed the ability of these cells to orient their MTOC towards a bound TC to be determined. As shown in Figure 4, the exposure of cells to cycloheximide and hyperthermia or cycloheximide alone has no affect on the ability of bound CTLs to orient their MTOC towards the TC after a 6 h incubation at 37^0C.

Effect of cycloheximide on cytolytic activity. Since cycloheximide does not affect the initial steps of the lytic pathway (TC recognition, conjugation, and MTOC orientation) in control or hyperthermia-treated CTLs, any effect of this drug on cytolytic activity must involve one or more subsequent lytic events. Cycloheximide treatment alone causes the level of cytolytic activity to drop suddenly to approximately half the level seen in control cells (Fig. 5A). No further inhibition of cytolytic activity by this treatment is seen in assays started 3 h after exposure to the drug but a further drop in activity is evident in assays started 6 h after the inhibition of protein synthesis. The inhibition of protein synthesis by cycloheximide in CTLs exposed to 42^0C for 30 min results in the complete abrogation

Figure 4.3. Effect of Cycloheximide and Hyperthermia on Microtubule Organization. Control and cycloheximide-treated effector cells were exposed to 42^{0}C for 30 min. Following the hyperthermia, samples of control (A), cycloheximide-treated (B), control hyperthermia-treated (C), and cycloheximide and hyperthermia-treated (D) cells were prepared for immunofluorescence microscopy with antibody to tubulin. The remaining cells of the four treatments were incubated 6 h at 37^{0}C and then prepared for immunofluorescence microscopy. The histogram shows that pretreatment with cycloheximide did not alter the effect of hyperthermia treatment on microtubule organization ($X=2.0261$, $p=0.1546$) and after a 6 h recovery no significant difference in the microtubule organization of the 4 samples was observed ($X=3.4524$, $p=0.3270$). Duplicate counts of ≥25 cells were made and the values presented are the means of the counts obtained from two experiments ±SD.

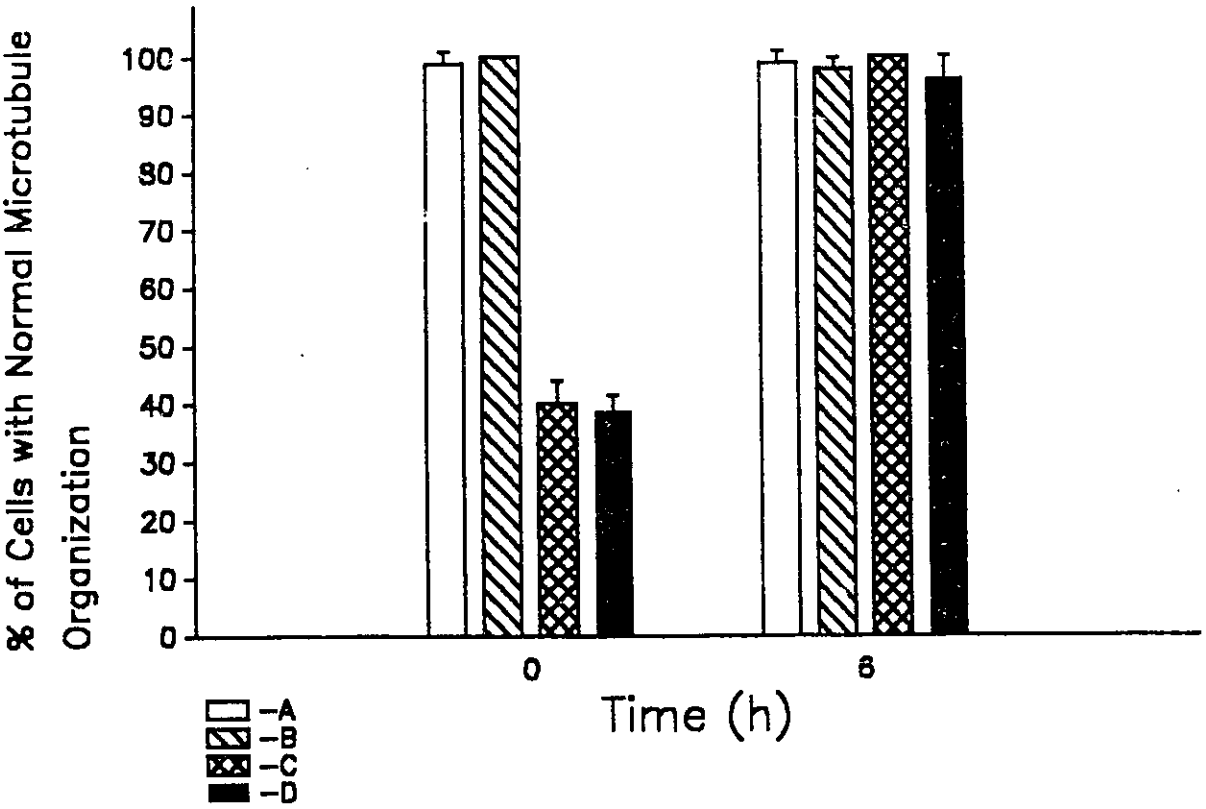
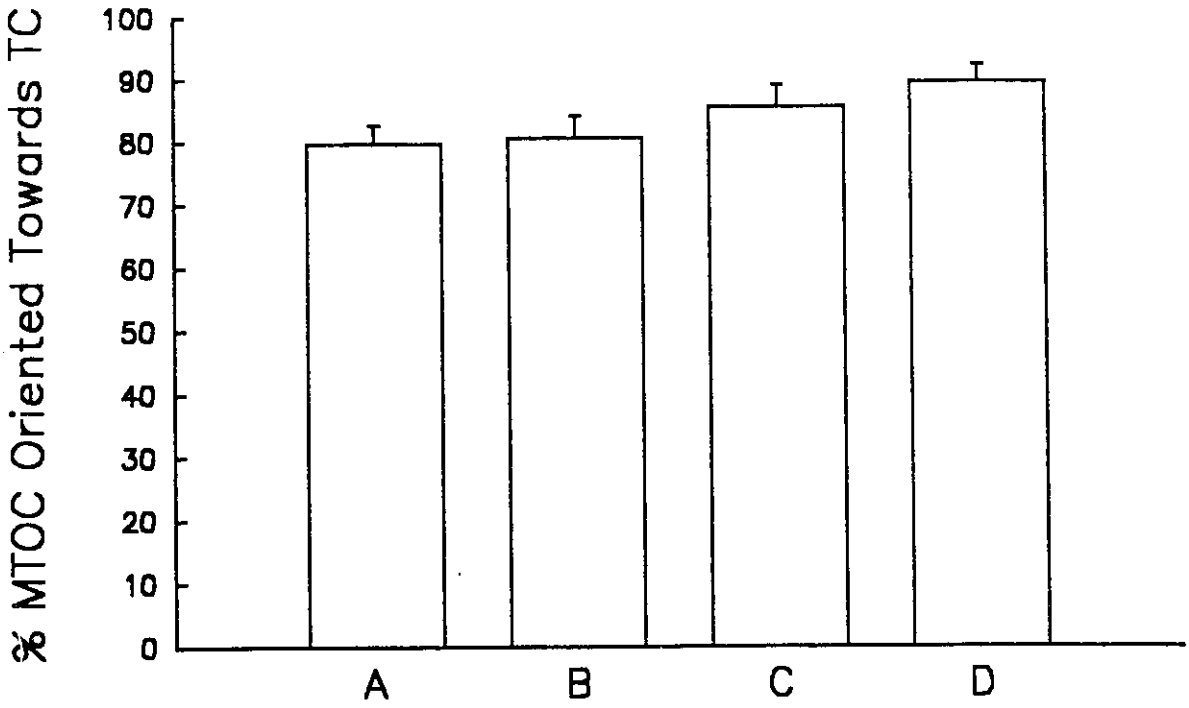

% of Cells with Normal Microtubule Organization
100
90
80
70
60
50
40
30
20
10
0
0
6
Time (h)
A
B
C
D

Figure 4.4. Effect of Cycloheximide and Hyperthermia on MTOC Orientation. Control and cycloheximide-treated effector cells were exposed to $42^{0}C$ for 30 min before being returned to $37^{0}C$. After a 6 h incubation CTL/TC conjugates were formed using control (A), control hyperthermia treated cells (B), cycloheximide treated cells (C), or cycloheximide and hyperthermia treated cells (D) and then stained with a monoclonal anti-tubulin antibody. The histogram shows that there was no significant difference in the ability of these cells to orient their MTOCs towards a bound TC ($X=2.1760$, $p=0.5367$). Duplicate count of ≥ 25 cells were made and the values presented are the means of the count of two experiments ±SD.


% MTOC Oriented Towards TC
100
90
80
70
60
50
40
30
20
10
0
A
B
C
D

(Fig. 5C) of the partial recovery cytolytic activity that normally coincides with the recovery microtubule reorganization. The difference in the cytolytic activity of control and cycloheximide treated cells in cytolytic assays started immediately after the hyperthermia treatment represents the amount of recovery of lytic activity occurring in the control cells during the course of the assay (Fig. 5B).

Duration of the heat-induced alteration in protein synthesis. An inspection of the autoradiograph shown in Figure 6 clearly illustrates, as has been previously established, that hyperthermia treatment results in a reduction in the total amount of protein synthesis. The major protein products still synthesized have molecular weights corresponding to those of the major heat shock proteins in lymphocytes (Fig. 6F). After having been allowed to recover from hyperthermia treatment for 24 h at 37^0C a substantial recovery of the amount and pattern of protein synthesis was observed (Fig. 6G). This recovery of protein synthesis is in contrast to the results of earlier studies of human leukocytes exposed to 42.5^0C for 2 h where protein synthesis remained thoroughly depressed up to 5 days following exposure (Roberts et al., 1985). In the experiments performed in this study the pattern of protein synthesis appeared unchanged at 48 h after hyperthermia treatment; however, a distinct reduction in the total amount of protein synthesis was observed (Fig. 6H).

Figure 4.5. Effect of Cycloheximide and Hyperthermia on Cytolytic Activity. This histogram represents the results of two different experiments. In one (A), effector cells were pretreated with 100 μM cycloheximide for 30 min, 3 h, or 6 h, mixed, in the continued presence of 100 μM cycloheximide with ^{51}Cr loaded TCs, and their cytolytic activity relative to untreated controls assessed. Incubation of the effector cells for either 30 min or 3 h inhibited cytolytic activity to the same extent ($F=0.0050$, $DF_1=1$, $DF_2=16$, $p=0.9445$). Prolonged treatments (6 h) prior to the start of the cytolytic assay further reduced cytolytic activity ($F=21.5429$, $DF_1=2$, $DF_2=24$, $p=4.4\times10^{-6}$). In the second experiment the cytolytic activity of control (B) and cycloheximide-treated (C) effector cells exposed to the same 42^0C for 30 min hyperthermia treatment was determined. The inhibition of the recovery of cytolytic activity caused by cycloheximide is evident in the cytolytic assay started immediately after the hyperthermia treatment ($F=3050.83$, $DF_1=1$, $DF_2=10$, $p=< 10^{-6}$). All samples were run in triplicate and the values shown are the mean of the counts three (A) and two (B and C) experiments respectively ±SD.

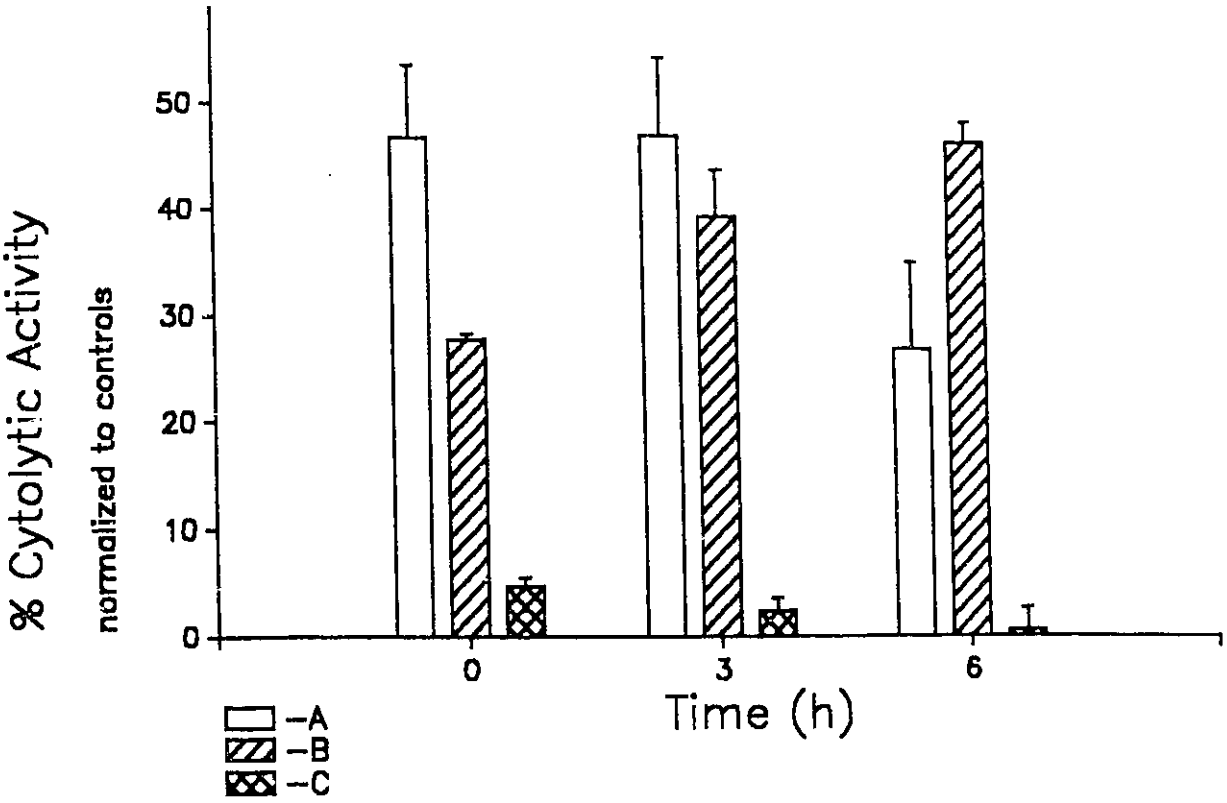
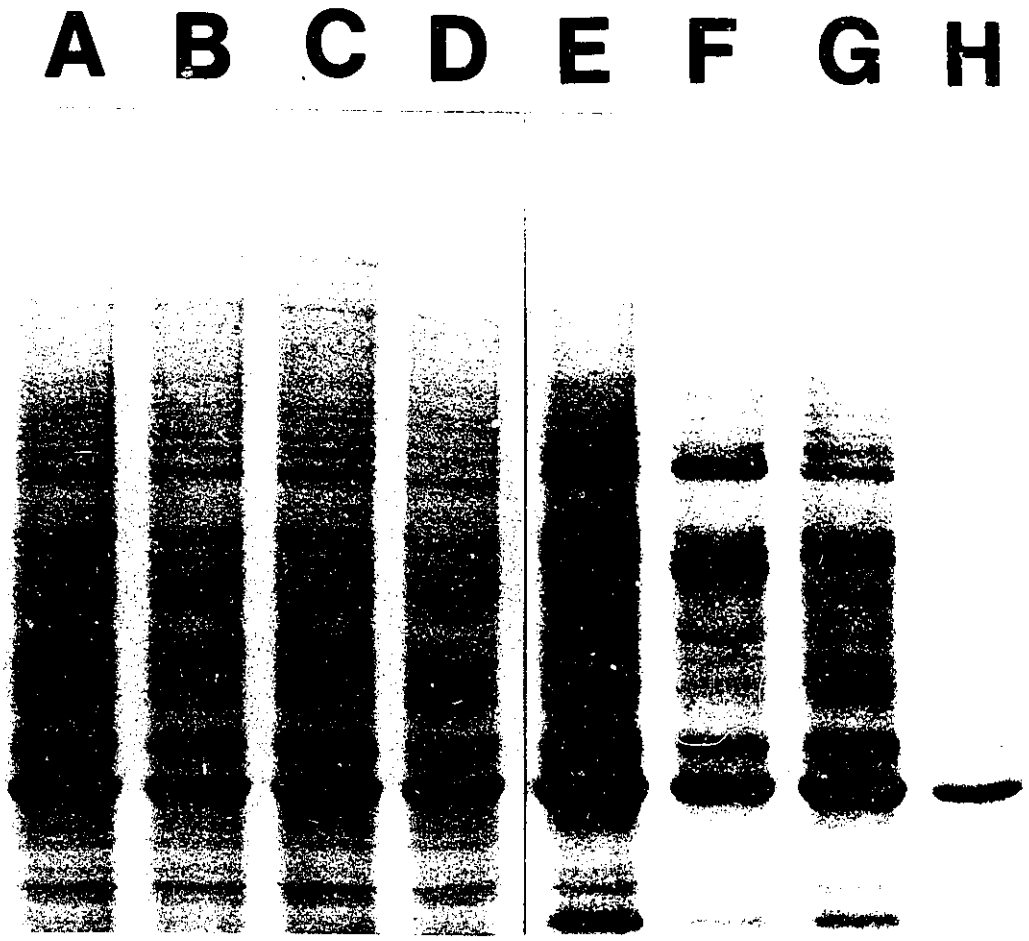

% Cytolytic Activity
normalized to controls
50
40
30
20
10
0
0
3
6
Time (h)
–A
–B
–C

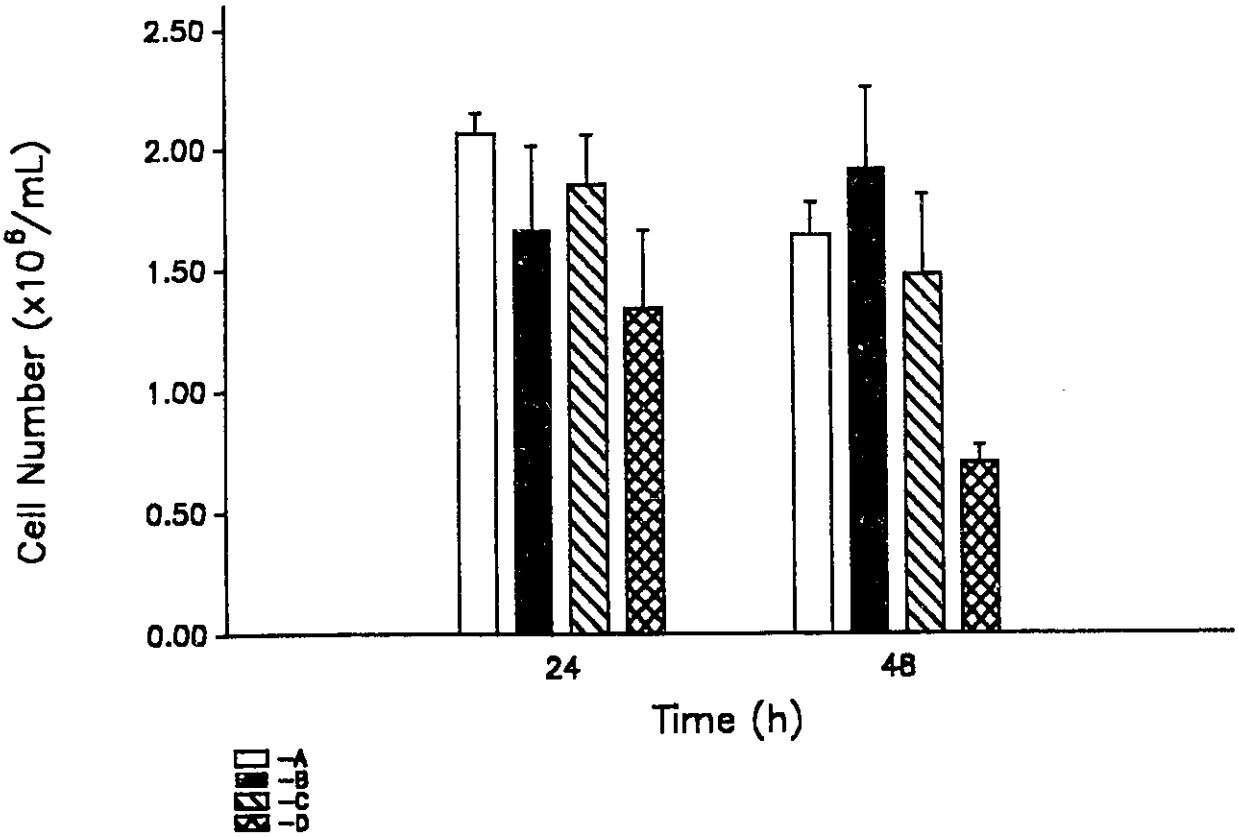
Figure 4.6. Effect of Hyperthermia on Protein Synthesis. Control cells (A) and cells given a 42^{0}C for 30 min hyperthermia treatment immediately (B), 24 h (C), or 48 h (D) prior to being incubated for 6 h in medium containing 30 μCi/mL of ^{35}S-methionine. Equal amounts of total cell protein obtained from these samples were run on a 11% polyacrylamide gel and then stained with Coomassie blue. The corresponding autoradiograph shows the pattern of ^{35}S incorporation in the control cells (E), and the cells heated immediately (F), 24 h (G), and 48 h (H) prior to the addition of the ^{35}S-methionine.


A B C D E F G H

Effect of hyperthermia on the response of effector cell populations to mitogenic stimulation. The ability to exclude dyes is a commonly used and accepted assay of leucocyte viability, but its exact relationship to viability is uncertain (Roberts et al., 1985). The long term loss of cytolytic activity and macromolecular protein synthesis after the transient recovery of both these functions suggested that the continued ability of these cells to exclude trypan blue was perhaps not a true indication of their viability.

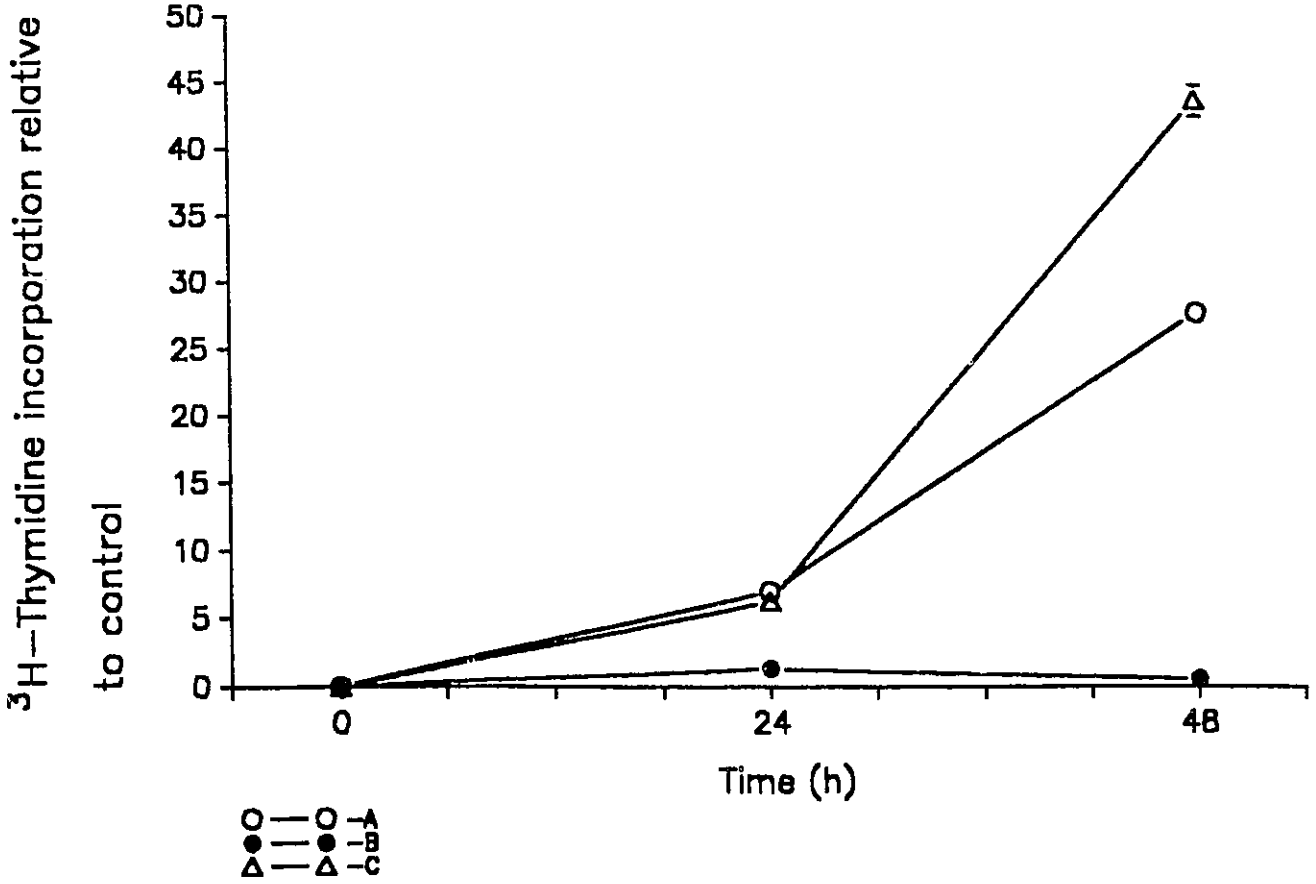
Vidair and Dewey (1988) identified two modes of cell death following hyperthermia: 1) a rapid mode occurring prior to cell division, and 2) a slow mode of death affecting cell division. It was demonstrated in Chapter 1 that the effector cells isolated 10 days after the intraperitoneal injection of EL4 cells are non-dividing cells. This suggested that the arrest of the effector cells in G_0-G_1 might be sustaining their ability to exclude trypan blue. To test this hypothesis the effect of hyperthermia on the ability of effector cell populations to respond to mitogen was determined. The binding of mitogenic lectins to cell surface receptors of lymphocytes in G_0-G_1 leads to rapid entry into the cell cycle and ultimately cell division (Hungerford et al., 1959). Figure 7 shows that the addition of the mitogen con A to hyperthermia-treated effector cells leads to a detectable decrease in cell number by 24 h and a clear reduction in cell number in cell populations stimulated for 48 h. The addition of con A or hyperthermia-treatment of control cells does not cause their cell

Figure 4.7. Effect of Mitogenic Stimulation on Effector Cell Viability. Hyperthermia-treated (42^{0}C, 30 min) and control cells were stimulated with 4 μg/mL of con A. At the times indicated the number of trypan blue impermeable cells in each sample was determined. At either time point no significant difference in the number of trypan blue impermeable cells was observed in the control (A), control con A stimulated (B), and the hyperthermia-treated (C) samples (T=24 h., F=2.8341, DF_1=2, DF_2=9, p=0.1110; T=48 h., F=2.4411, DF_1=2, DF_2=9, p=0.1422). Con A stimulation caused a significant reduction in the viability of the hyperthermia-treated cells (D) relative to the control at both time points (T=24 h., F=18.8989, DF_1=1, DF_2=6, p=0.0048; T=48 h., F=146.7391, DF_1=1, DF_2=6, $p=1.93\times10^{-5}$). Quadruplicate counts of each sample were made and the values shown are the means of the counts of a representative experiment ±SD.


2.50
2.00
1.50
1.00
0.50
0.00
Cell Number (x10^6/mL)
24
48
Time (h)

number to differ significantly from the unstimulated control population at either time point. When the decrease in cell number is taken into account the incorporation of ^{3}H-thymidine shown in Figure 8 indicates that there are cells within the hyperthermia-treated population that are able to respond effectively to the mitogen.

Figure 4.8. Effect of Hyperthermia on DNA Synthesis. Control and hyperthermia-treated (42^{0}C, 30 min) effector cells were divided into 2 groups one of which received 4 μg/mL of con A. At the times indicated 4 μCi/mL of ^{3}H-thymidine was added to an aliquot of each sample that were then incubated at 37^{0}C for 1 h. After the incubation the cells were lysed and the DNA harvested on Whatman glass fibre filters. The amount of ^{3}H incorporation was then determined and recorded as DPM/1x10^6 trypan blue impermeable cells (see Figure 7). The graph shows the amount of ^{3}H incorporation in control con A-stimulated (A), hyperthermia-treated (B), and hyperthermia-treated and con A-stimulated (C) cells relative to that of unstimulated control cells.


^{3}H–Thymidine incorporation relative to control
50
45
40
35
30
25
20
15
10
5
0
0
24
48
Time (h)
A
B
C

DISCUSSION

The proposed function of the MTOC orientation is to direct the secretion of lytic proteins into the intercellular junction formed between the CTL and the TC. A Ca^{++}-dependent degranulation after appropriate stimulation by the TC is the dominant model for CTL-mediated TC lysis; however, several other reports using CTL clones (Ostergaard et al., 1987; Trenn et al., 1987; Ostergaard and Clark, 1987) or primary CTLs and CTL clones conjugated to con A-coated TCs (Ostergaard and Clark, 1989) have documented significant cytolytic activity in the absence of extracellular Ca^{++}. These results indicate that there are at least two pathways for CTL killing: a Ca^{++}-dependent pathway and a Ca^{++}-independent pathway that does not involve protein secretion in any form (Clark et al., 1988; Ostergaard and Clark, 1989).

At present, the nature of the Ca^{++}-independent pathway is unknown; however, it is most characteristic of CTL lines maintained on high levels of IL-2 without periodic antigen stimulation. Many of these cell lines begin to lyse target cells promiscuously, and most fail to degranulate during TC lysis even in the presence of Ca^{++} (Clark et al., 1988). Based on this evidence it seems likely that this Ca^{++}-independent pathway is used only under conditions of unusual immunological stress that lead to the production of high local concentrations of IL-2. As primary CTLs require Ca^{++} to lyse most TCs (Clark et al., 1988), and Ca^{++} is required for efficient

TC lysis under the conditions used in this study, the Ca^{++}-independent pathway will not be further discussed.

Ironically, the putative lytic proteins thought to be employed by the Ca^{++}-dependent pathway have for the most part been isolated from the cytotoxic granules of CTL clones cultured in the continuous presence of IL-2. These proteins include a pore forming protein called either perforin, cytolysin, or pore-forming protein (Henkart et al., 1984; Podack et al., 1985), a Ca^{++}-independent tumour necrosis-like molecule (Lui et al., 1987), and a number of serine esterases (Masson and Tschopp, 1987; Young et al., 1986). Attempts to demonstrate the presence of these granules and/or the lytic proteins they contain in primary CTLs have failed (Berke and Rosen, 1987; Dennert et al., 1987; for an exception see Munger et al., 1987). The failure to detect these molecules in primary CTLs however, does not mean that they are not there. It is possible that small undetectable amounts of these proteins are sufficient for effective TC lysis and that the large granules of cytolytic molecules found in cloned CTL lines are an aberrant effect of the continuous stimulation with IL-2. Another possibility is that instead of being constitutive components of CTLs these molecules are only synthesized after TC binding.

Many TCs attacked by CTLs undergo violent blebbing followed by a fragmentation of the nucleus and cytoplasm into initially sealed vesicles (Martz and Howell, 1989). This morphology is termed apoptosis and is characteristic of cells undergoing programmed cell death during embryonic development (Duvall and Wyllie, 1986) and

lymphocyte death occurring spontaneously in the thymus (Wyllie, 1980). The DNA of apoptotic cells is cleaved by a Ca^{++}, Mg^{++}-dependent endogenous endonuclease into discrete fragments that are all integer multiples of approximately 190 base pairs (Bursch et al., 1990). In contrast, complement, or other chemical or physical means of cytolysis, induces little or no DNA fragmentation (Duke et al., 1983). CTL-induced DNA fragmentation prior to TC lysis has been observed in several studies (Martz and Howell, 1989). In most systems apoptosis requires specific gene transcription and protein synthesis in the affected cell, whereas the CTL-induced apoptosis is apparently independent of new protein synthesis in the TC and the CTL (Duke et al., 1983; Martz and Howell, 1989). It is unclear how the CTLs induce apoptosis though tumour necrosis factor (despite its name this protein does not induce 'necrotic-like' cell death) is thought to induce apoptosis in some cell lines (Bursch et al., 1990) and a membrane bound tumour necrosis-like molecule has been demonstrated in CTLs (Young and Lui, 1988). Thus, the literature suggests that the cytolytic activity of primary CTLs is usually due to the secretion of lytic proteins that are present in only small amounts or synthesized after conjugation and/or protein synthesis-independent CTL-induced apoptosis.

The inhibition of protein synthesis immediately prior to the start of a cytolytic assay should abrogate cytolytic activity if a component or components required for the delivery of the lethal hit are synthesized after the signal to kill is received. Earlier studies that have examined the effect of the inhibition of protein

synthesis on cytolytic activity have not produced a clear cut result. Three reports where an inhibition of cytolytic activity by 1/3 or more was observed have been published. The authors of the original study (Brunner et al., 1968) concluded that this result was consistent with the concept that cell-mediated cytolytic activity is dependent upon protein synthesis and suggested that the partial resistance to the drug could be explained by the presence of preformed pools of the required components in sensitized cells. The authors of two subsequent reports containing similar results (Thorn and Henney, 1976; Landon et al., 1990) concluded correctly that protein synthesis was not required for CTL-mediated cytolysis, but dismissed the significant inhibition observed.

In the present study the inhibition of protein synthesis resulted in the loss of approximately 50% of the cytolytic activity. As was shown by Landon et al. (1990) the treatment was not observed to have an effect on TC binding and, in addition, it was demonstrated that even after a preincubation of 6 h cycloheximide had no effect on MTOC orientation. This indicates that the lytic step or steps dependent upon protein synthesis, like those affected by the hyperthermia treatment, occur subsequent to these two initial events. The observed resistance of the remaining cytolytic activity to protein synthesis inhibition for several hours is similar to that reported by Thorn and Henney (1976); however, when the preincubation time with cycloheximide is extended to 6 h a further decline in cytolytic activity relative to controls is observed.

The observation that 50% of cytolytic activity is affected immediately by protein synthesis inhibition is consistent with the hypothesis that a cytolytic protein is produced and secreted subsequent to TC binding. The resistance of the remaining cytolytic activity suggests that it may be occurring via the protein synthesis-independent, apoptotic pathway described in the literature (Duke et al., 1983; Martz and Howell, 1989). Differences in the amount of cytolytic activity lost as a result of the inhibition of protein synthesis may represent the relative reliance of the cells used in the assay upon the protein synthesis dependent pathway. The preference of CTLs for one pathway over another may be influenced by the same factors that have been shown to influence the reliance of CTLs upon the Ca^{++}-dependent or Ca^{++}-independent pathways: the identity of the TC, the culture conditions of the assay, and the fashion in which the CTLs were generated.

It is immediately clear that hyperthermia treatment, which reduces cytolytic activity to approximately 25% of that observed in control cells, is initially having at least a partial effect on both the secretory and apoptotic cytolytic pathways. One possible explanation for the partial recovery of cytolytic activity is that one of the two cytolytic pathways is irreversibly inactivated by the hyperthermia treatment while the other is only partially affected and recovers completely. The ability of cycloheximide to negate the secretory lytic pathway suggested that it might provide a means to elucidate that effect of hyperthermia on each of the

lytic pathways.

The dual treatment was found not to affect TC recognition and binding, which is not surprising considering that neither treatment alone affected this event. More interestingly, cycloheximide treatment had no effect on the hyperthermia-induced disorganization of the MTOC-microtubule array or on its ensuing reorganization and the concomitant recovery of the ability of the MTOC to orient towards the TC. The addition of cycloheximide did however, result in a complete abrogation of the recovery of cytolytic activity ordinarily observed following hyperthermia treatment. This indicates that the recovery of cytolytic activity following hyperthermia treatment is dependent upon protein synthesis for either the replacement or the repair of a component or components involved in a lytic event that occurs subsequent to MTOC orientation. This result does not, however, indicate which of the two pathways is normally responsible for the recovery of cytolytic activity. It is also possible that both the pathways recover to a limited extent.

The decline in cytolytic activity observed after the partial recovery of cytolytic activity may be explained by the decline in protein synthesis observed 24 and 48 h after the hyperthermia treatment. These results suggest that the $42^{0}C$ for 30 min hyperthermia treatment is lethal despite the fact that these cells remain impermeable to trypan blue (see Fig. 2.2). When hyperthermia treated cells are stimulated to divide by the addition of con A the lysis of many of the cells ensues which confirms that

these cells are undergoing the slow mode of hyperthermia-induced death described by Vidair and Dewey (1988).

A MODEL FOR MTOC ORIENTATION

As stated in the introduction one of the origianl goals of this study was to determine the mechanism of MTOC orientation. While the results presented suggest that the selective stabilization of a subset of microtubules is not responsable for MTOC movement no alternative hypothesis was proposed. Therefore, in this section I shall develope an alternative hypothesis and propose some experiments that could be used to test it.

The inability of colchicine treated cells, whose PCM organization appears normal, to orient towards the TC suggests that whatever is applying the motive force to the MTOC probably does so indirectly through the microtubules. It is difficult to imagine the large taxol-induced bundles of microtubules being dragged through the dense cytoplasm of CTLs. As the 1 μM taxol treatment does not impair the rapid orientation of the MTOC to face the TC (see Fig. 3.3) an attractive hypothesis is that the entire contents of the cell move coordinately at an interface immediately beneath the plasma membrane. This hypothesis could be tested by live observations of cells in which one or more organelles had been stained with a vital dye.

Should the interface prove to be proximal to the plasma membrane a likely candidate for applying the motive force is the dense network of actin filaments situated there. Immunolocalization of actin in the region of cell-cell contact and pharmacological data have implicated actin in TC binding. My own immunofluorescence observations (data not shown) suggest that actin

the CTL is firmly attached to the TC the actin polarized in the region of cell-cell contact during conjugate formation evenly redistributes itself beneath the plasma membrane. This observation suggests that treatment of conjugates at this point with cytochalasin D may have no effect on the association of the two cells. If this is the case it would be possible to test the involvement of the microfilaments in MTOC orientation. Conjugates would be formed in the absence of Ca^{++}. Cytochalsin D would then be applied and subsequent to this application Ca^{++} would be added to the medium. Inhibition of MTOC orientation by this procedure would implicate the microfilaments as the source of the motive force applied on the microtubules.

While this model suggests a means by which the MTOC may be moved about beneath the plasma membrane it does not provide an explanation for how the MTOC becomes stably oriented towards the TC. In published studies where they demonstrated that microtubules are essential for MTOC orientation (see Chapter 3, p. 140) Kupfer et al. (1983) speculated about how the signal to orient could persist so long after the transient flux of Ca^{++} in the CTLs. Their hypothesis was that the MTOC was not the primary target of the Ca^{++} signal. Instead they suggested that the transient rise in Ca^{++} instead produced a conformational change in some structure associated with the membrane in the area of cell-cell contact that then resulted in the orientation of the MTOC by an unpostulated mechanism. It has subsequently been shown that the actin-binding protein talin becomes localized to this area of cell-cell contact

between CTLs and their specific target and that this protein is able to interact with the cytoplasmic region of at least one of the membrane bound receptors that become clustered there. These observations support the suggestion that the plasma membrane of the CTLs in the region of cell-cell contact becomes structurally as well as functionally distinct from the rest of the cell membrane and is able to interact with the cytoskeleton.

The results of this study suggest the following model: Contact with another cell causes the polarization of actin which increases the surface area between the two cells and results in the non-specific adhesion of the two cells. The specific interaction of the T cell receptor and antigen/MHC-1 complexes results in the generation of a signal or signals that precipitate the polarization of talin, and the increase in LFA-1 avidity as well as its migration to the region of cell-cell contact. As the CTL begins to adhere more firmly to the TC more receptors become engaged which results in a transient rise in the intracellular Ca^{++}. The Ca^{++} flux increases the mobility of the MTOC, perhaps by promoting the disassociation of the microtubules from the intermediate filaments and the application of force on the microtubules by the microfilaments. The transient rise of Ca^{++} may also result in a change in the conformation of a component of the PCM. The increased mobility of the MTOC increases the frequency with which it will become oriented towards the TC and when it does now a component of the PCM will interact with a component of the structurally distinct membrane and stably 'anchor' the MTOC in that

orientation.

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APPENDIX

	CPM				
CONTROLS	1	2	3	AVG	STD
100%	2845.0	2798.0	2557.0	2733.3	154.5
0%	507.0	570.0	556.0	544.3	33.1
TEMPERATURE (°C)					
37	1510.0	1477.0	1055.0	1347.3	253.7
41	991.0	1101.0	1226.0	1106.0	117.6
42	637.0	603.0	667.0	604.7	38.9
43	574.0	597.0	618.0	650.3	112.9

From the values shown in the table above the % cytotoxicity is calculated. For example the % cytotoxicity of the first sample at 37°C would be calculated as shown below.

% cytoxicity = 100 x (1510.0 - 544.3)/(2733.3 - 544.3)
= 44.1

	% cytotoxicity				
TEMPERATURE (°C)	1	2	3	AVG	STD
37	44.1	42.6	23.3	36.7	11.6
41	20.4	25.4	31.1	25.7	14.7
42	4.2	2.7	5.6	4.2	1.5
43	1.4	2.4	3.4	2.4	1.0

From the values shown in the above table the relative cytotoxicity of the hyperthermia-treated samples is calculated. For example, the % cytotoxicity relative to the control of the first sample at 41°C would be calculated as shown below.

% relative cytotoxicity = 100 x (20.4/36.7)
= 55.6

	% relative cytotoxicity				
TEMPERATURE (°C)	1	2	3	AVG	STD
41	55.6	69.3	84.9	70.0	14.7
42	11.4	7.4	15.3	11.4	4.0
43	3.8	6.5	9.2	6.5	2.7