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Stimulation of Cell Cycle Traverse: Synergism between
Anti-Microtubule Agents and Growth Stimulants.

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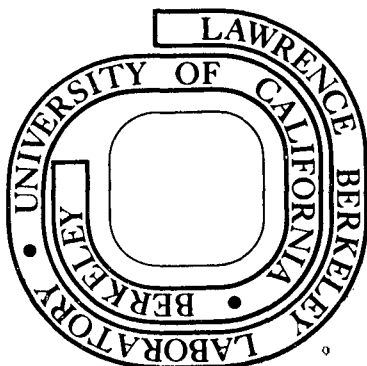
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STIMULATION OF CELL CYCLE TRAVERSE: SYNERGISM BETWEEN

ANTI-MICROTUBULE AGENTS AND GROWTH STIMULANTS

For years colchicine and other mitotic inhibitors have been used in cell cycle studies to produce synchronous cell populations[1]. This application is based on the ability of these drugs to inhibit mitosis by binding to tubulin and preventing microtubule (mitotic spindle) formation [2]. In addition to their inhibition of mitosis, these drugs also modify morphology and functions of cells during interphase. For example, it has been found that colchicine inhibits the formation of cilia[3]; affects phagocytosis, the normal distribution of lysosomes[4], the synthesis and secretion of a variety of proteins[5-10] and the transport of certain nucleosides [11].

At very high concentrations (2-35 x 10^{-3}M) colchicine and colcemid have been reported to retard the initiation of DNA synthesis and to prolong the period of DNA synthesis [12,13]. In this report we describe the synergistic effects of this and other anti-microtubule agents at lower concentrations (1-2 x 10^{-7}M) on the insulin- or serum-induced increase in cells moving from G_1 to S phase. These results suggest that microtubles play an important role in the regulation of DNA synthesis by growth factors.

We have previously demonstrated that high density chick cells starved of serum could be stimulated synchronously by insulin to traverse at least one cell cycle[14]. This stimulation was detected as an increase in the proportion of cells in S phase with 8-10 hr lag when the increase in DNA per cell was detected by flow microfluorometry (FMF). When similar experiments were performed in the presence of 2 x 10^{-7}M colchicine, it was evident that colchicine significantly increased the population in the S phase of the cell cycle (Fig. 1). The stimulated cells progressed through S phase

more slowly than in the absence of colchicine, but the degree of synchrony was as good as the stimulated population when insulin was used alone.

Two other anti-microtubule agents, colcemid and vinblastine[15], were compared to cholchicine in their ability to affect the proportion of cells stimulated to make DNA in insulin-treated chick cell cultures. The results shown in Table 1 indicate that all of these compounds increased the cell population moving out of G_1 phase in the presence of insulin. Colchicine and vinblastine were more effective than colcemid at lower concentrations. Furthermore, the continuous presence of anti-microtubule agents was not necessary, for their removal from media after 6 hours had the same effect (data not shown). These observations suggest that the primary effect of anti-microtubule agents at low concentrations is on the early events in the stimulation of DNA synthesis. At higher concentrations (above $5 \times 10^{-7}M$), all of these compounds decreased the number of cells engaged in DNA synthesis. Thus the mode of action of these drugs is different at low and high concentrations.

In order to see whether or not this stimulatory effect also applied to cell lines, the effect of anti-microtubule agents on Balb 3T3 cells was investigated along with chick fibroblasts. In these experiments cultures at their saturation density were stimulated with fresh medium containing either insulin or serum. The proportion of cells in the $S+G_2+M$ phases with or without added growth stimulants are listed in Table 2. As has been reported previously [14], we found that insulin had little or no stimulatory activity on DNA synthesis in Balb 3T3 A31 cells. This was true even when the insulin concentration was raised to 128 munits/ml. Similar results were obtained with Don hamster cells at high density (results not shown). Furthermore, only chick cells could be stimulated to synthesize DNA by

fresh medium. It is important to note that colchicine was effective in stimulating cell cycle from G_1 to S only when the primary growth stimulant, a mitogen, was able to stimulate some cell traverse. Therefore, colchicine and other anti-microtubule agents act as "synergistic agents" to a "mitogen".

How does the message for DNA synthesis get transferred from the mitogen to the cell? The cell membrane is believed to play an important role in such a process. Some growth stimulants have been partially purified from various sources, and shown to bind to the cell membrane of cultured cells[16-18]. What occurs between the binding of growth stimulants and their relation to the initiation of DNA synthesis is, however, poorly understood.

The dynamics of the membrane receptors and other components seems to be subject to the controls from both sides of the membrane. The evidence for this assumption is the fact that concanavalin A (Con A) and other related plant lectins inhibited the immunoglobulin capping on lymphocytes [19,20]. Furthermore, Con A by itself could form caps on various cell types and the process was either inhibited or stimulated by anti-microtubule or anti-microfilament agents depending on cell types studies[20-25]. This evidence was further supported by a recent report indicating that colchicine must get inside the cell to be effective in the inhibition of Con A capping in Chinese hamster cells[20]. Except in a case for microfilaments[26], the evidence for the involvement of microtubules and microfilaments in growth regulation has been of a "negative" nature so far, i.e. agents affecting these organelles would inhibit the stimulatory action caused by a mitogen[12,13]. Most of these studies were carried out at high concentrations of inhibitors, where possible toxic side effects could have complicated the interpretation of the results. Our evidence of the positive effect of anti-microtubule agents on mitogen-stimulated DNA synthesis directly demonstrates a role

for involvement of microtubules in growth regulation. Furthermore, site-site interaction and redistribution of insulin receptors after the binding of insulin to the membrane of rat liver or human lymphocytes have been reported biochemically and with electron microscopy[27,28]. The presence of insulin receptor on chick fibroblasts was recently demonstrated in detail[29]. The striking synergistic effect of colchicine and insulin on stimulation of DNA synthesis in chick fibroblasts reported here makes it possible to propose the following simple model to explain such synergism (Fig. 2). In this model which is a modification of that proposed by Nicholson[30], as well as that by McGuire and Barber[31], a growth stimulant activates its receptor on the membrane after binding by forming an active stimulant-receptor complex. In order to complete a signal for DNA synthesis, however, a cooperation of this complex with certain membrane components has to take place. If the mobility of membrane receptors and other components is an important factor in the signal transfer, any compound which increases this mobility should facilitate DNA synthesis. Microtubules have been proposed to be an anchorage agent for certain membrane components[19,30]. Our results indicate that this may indeed be the case. A factor which destroys microtubule structure, colchicine in this case, can be a synergistic agent for growth stimulation. Such an agent by itself is incapable of triggering DNA synthesis. It acts only when some DNA synthesis capacity is already present.

It has been reported that Balb 3T3 cells indeed have insulin receptors[32]. However, such binding fails to stimulate DNA synthesis in these cells (14,33, and the results here). In addition, the cell lines of this type and human diploid foreskin or bovine embryonic trachea fibroblasts failed to respond to the growth stimulation by trypsin, an agent which stimulates growth in secondary chick fibroblasts[34]. The most likely possibility is that there is fundamental difference in the nature of membranes of primary cell

cultures and cell lines. The interaction between mitogen-receptor complexes themselves or with other membrane components is blocked in cell lines unless a more complicated growth mixture such as serum is provided to potentiate the cooperation. Alternatively, the activation of certain mitogen receptors, e.g. that of insulin, may not occur after the binding of the mitogen. Therefore, no message is generated and transferred to the second component. There is an additional possibility that the second membrane component for message transfer from particular single growth factor may be altered in cell lines. As a result, the growth control of cell lines in culture should be much more stringent than that of primary cell cultures as discussed previously [14]. The exact nature of the synergistic effects of anti-microtubule agents and growth stimulants is currently under investigation.

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Table 1

Effect of Colchicine, Colcemid, and Vinblastine on the Cell Cycle Traverse of Insulin-treated Chick Embryo Fibroblasts

Concentrations of mitotic inhibitors (M)	Percentage of cells in $S+G_2+M$ phases		
	Colchicine	Colcemid	Vinblastine
0 (insulin only)	25	25	25
5×10^{-8}	47	27	58
1×10^{-7}	60	25	64
2×10^{-7}	69	54	60
5×10^{-7}	64	62	63

Cells were grown and prepared for FMF analysis as in Figure 1.
Colcemid, vinblastine, and colchicine were tested at the concentrations
indicated. Insulin was present in all cultures. Cells were harvested
22 hrs after addition of mitotic inhibitors and insulin. After FMF
analysis, the percent population in $S + G_2 + M$ phases were calculated
with a computer program.

Table 2

Response of Cell Cycle Traverse of Cell Cultures

to Colchicine and Growth Stimulants

Cell Types and Media	Percentage of cells in $S+G_2+M$ Phases	
	-Colchicine	+Colchicine
Secondary chick embryo fibroblasts		
Medium 199 (control)	14	32[*]
+ insulin, 16 m u/ml	29	66
+ chick serum, 3%	33	58
Balb 3T3 A31		
Medium DME (control)	4	4
+ insulin, 128 m u/ml	4	4
+ calf serum, 10%	15	29
+ calf serum, 20%	30	78
+ calf serum, 30%	33	90

The data for secondary chick embryo fibroblasts was obtained as described in Table 1. The numbers presented were the average of two experiments. Cells of Balb 3T3 A31 were grown to confluency in Medium DME containing 10% calf serum for 5 days. Cells were washed with saline and provided with fresh Medium DME containing additional components indicated. After 28 hrs, cells were harvested and prepared for FMF analysis.

*Stimulation by colchicine on cells in Medium 199 (control) varied with each experiment depending on the batch of eggs.

Figure 1. Progress of chick embryo fibroblasts through the cell cycle in the presence of insulin and colchicine.
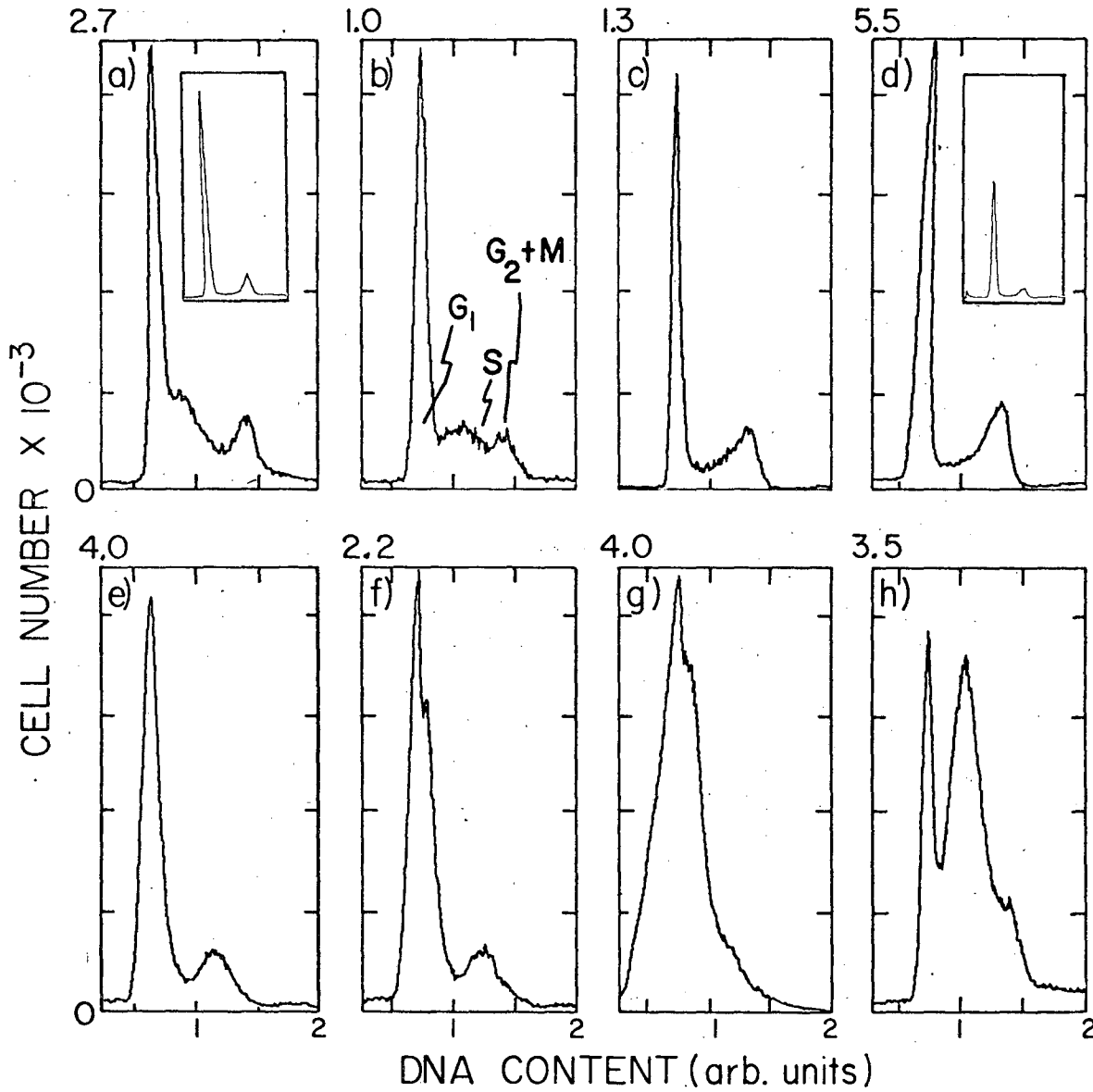
Confluent secondary chick embryo fibroblasts in Medium 199 were prepared and deprived of serum as described previously[14]. After serum starvation, insulin was added at a concentration of 16 m units/ml medium. Parallel cultures were provided with insulin and 2×10^{-7} M colchicine and were incubated in 5% CO_2 at 39°C. Cells were harvested and prepared for FMF analysis, us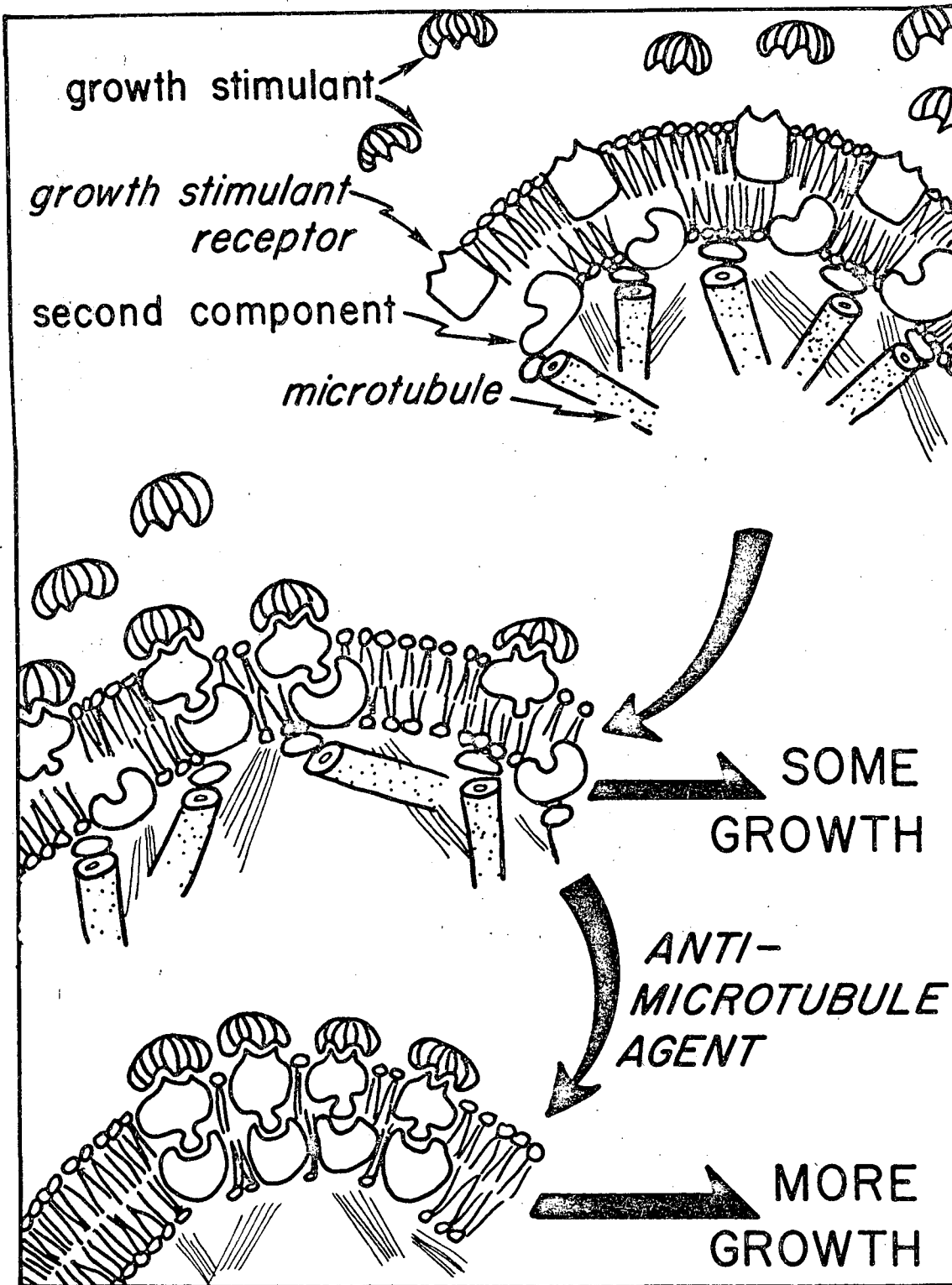ing propidium iodite as the fluorescent stain[35]. FMF patterns of insulin-treated cultures are a) to d) for the time points of 13, 17, 23, and 29 hrs, and insulin plus colchicine are e) to h) for the same time sequence. The inserted histograms in a) and d) are control cultures in Medium 199 at 0 and 29 hrs.

Figure 2.   Schematic representation of message transfer from growth stimulant
through membrane and the influence by anti-microtubule agents.

In this model, the inactive receptor of "growth stimulant (mitogen)"
and "second component" for carrying on message transfer are
distributed randomly on the membrane, and their mobilities
are controlled by membrane fluidity which, in return, is influenced
by the cytoplasmic microtubules along the inner side of the
membrane. Binding of growth stimulant activates the receptor.
Within the limit of membrane fluidity, the message from stimulant-
receptor complex is transferred from the complex to the second
components through the interaction between those two compartments.
From there, the message for growth is transferred further through
cytoplasm into nuclei. Growth will occur to some cells on
which the message transfer is completed through those various
interactions. When anti-microtubule agents are present, the
cytoplasmic microtubules are depolymerized and the mobilities
of membrane components increase. The aggregation of active
growth stimulant-receptor complex themselves results in the
generation of stronger message and the increased probability
of cell cycle traverse. In addition, the increased membrane
fluidity facilitates the interaction of second components and
active complexes. Message is then transferred much more efficiently.
As a result, the probability of cell cycle traverse[36] increases
and more cells will engage in DNA synthesis.

Figure 1

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XBL 771-4129

growth stimulant

growth stimulant receptor

second component

microtubule

SOME GROWTH

ANTI- MICROTUBULE AGENT

MORE GROWTH

XBL 772-4184

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