EFFECTS OF COLCHICINE AND VINBLASTINE ON THE PHYTOHAEMAGGLUTININ-INDUCED TRANSFORMATION OF LYMPHOCYTES

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SUMMARY

The effects of 2 microtubular-disruptive drugs, colchicine and vinblastine, on the phytohaemagglutinin (PHA)-induced blast transformation and mitogenic stimulation of human lymphocytes were studied. Both drugs markedly inhibited cell growth and DNA synthesis and lowered the mitotic index. No microtubules were seen with the electron microscope in cells treated with PHA plus colchicine or vinblastine. Moreover, the PHA-induced development of all organelles was partially inhibited by these drugs, especially that of the Golgi complex. As compared to cells treated with PHA alone, the dictyosomes were fewer, not so clearly localized in one area of the cytoplasm, and contained a decreased number of cisternae and an increased number of vacuoles.

These results indicate that cytoplasmic microtubules play an important role in the PHA-induced blast transformation and mitogenic stimulation of lymphocytes. It is suggested that the microtubules function in the structural organization of the cell and particularly the Golgi complex. In the drug-induced absence of microtubules this and other organelle systems do not respond as usual to PHA stimulation, which could largely explain the decreased cell growth. This in turn suggests that lowered mitotic activity is a result of inhibition of cell growth, as a critical amount of G2-associated cell growth is believed to be required for the initiation of DNA synthesis and thus mitosis.

INTRODUCTION

Colchicine and other antimicrotubular agents have been found to inhibit the mitogenic stimulation of lymphocytes by phytohaemagglutinin (PHA) and concanavalin A as measured by presence of blast cells and incorporation of [3H]thymidine (Fitzgerald & Brehaut, 1970; Greene, Parker & Parker, 1976; Gunther, Wang & Edelman, 1976; Medrano, Piras & Mordoh, 1974; Wang, Gunther & Edelman, 1975). The inhibition occurs early after the addition of the lectin and it has been suggested that cytoplasmic microtubules are involved in the lymphocyte stimulation process at a stage preceding the entrance of the cells into the S-phase (Gunther et al. 1976; Wang et al. 1975). The exact function of the microtubules has, however, remained obscure.

In stimulated lymphocytes an increase in general cell metabolism and progressive cell enlargement take place before the start of DNA replication and mitotic activity (for a review, see Wedner & Parker, 1976). A detailed fine-structural characterization

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of this blast transformation has been performed by Biberfeld (1971) and morphometric data have been given by Konwiriski & Kozlowski (1972). Among the cytoplasmic organelles the Golgi complex, located in the centrosphere region, shows a marked increase both in number and size of the dictyosomes (stacks of cisternae). Microtubules increase in number in direct proportion to cell size. They radiate out from the pericentriolar region and display a close spatial connexion to the Golgi complex (Biberfeld, 1971). Similar observations on the relationship between these organelle systems have been made in other cell types and, by using antimicrotubular agents like colchicine and vinblastine (Wilson & Bryan, 1974), it has been demonstrated that the normal organization of the Golgi complex is dependent on intact cytoplasmic microtubules (Hinek, Thyberg & Friberg, 1977; Moskalewski, Thyberg & Friberg, 1976; Moskalewski, Thyberg, Lomander & Friberg, 1975; Thyberg & Hinek, 1977; Thyberg, Moskalewski & Nilsson, 1976). The present report deals with the effects of colchicine and vinblastine on PHA-stimulated lymphocytes and thus with the role of microtubules in lymphocyte mitogenesis. Special attention is paid to fine structural modifications of the cytoplasmic microtubular system, the Golgi complex, and their relationship. A preliminary report of part of this work has been published (Thyberg, Moskalewski & Friberg, 1976).

MATERIALS AND METHODS

Cell culture

For all experiments, lymphocytes were isolated from peripheral blood of one and the same healthy human donor. Heparinized blood (50 units/ml) was allowed to sediment for 3 h at 37 °C. The supernatant was then centrifuged for 15 min at 100 g and the cell pellet resuspended. As judged by light and electron microscopy, more than 95% of the nucleated cells isolated in this way were small lymphocytes. In addition, a few monocytes but essentially no granulocytes were noted. A variable proportion of erythrocytes was also present.

Cultures were set up at a density of $2 \times 10^8$ nucleated cells/ml in medium 199 (Gibco Bio-Cult, Paisley, Scotland) supplemented with 20% autologous, defibrinated plasma, 150 units/ml of penicillin and 150 μg/ml of streptomycin. The experiments were performed in 10-ml Erlenmeyer flasks using a volume of 5 ml. PHA (Bacto, type M; Difco Labs., Detroit, Mich.) was added to a concentration of $0.01$ ml/ml of medium. Colchicine (E. Merck AG, Darmstadt, Germany) and vinblastine sulphate (Sigma Chemical Co., St Louis, Mo.) were both added to a final concentration of $1 \times 10^{-5}$ M, either at the start of culture or after 68 h. For studies of the lysosomal system, $0.02$ ml/ml of medium of Thorotrast (25% stabilized colloidal thorium dioxide by volume; Testagar, Detroit, Mich.) was added after 48 h of culture. All cultures were kept at 37 °C in an atmosphere of 5% CO₂ in air and terminated after 72 h.

Assay of DNA synthesis

DNA synthesis was assayed in triplicate cultures set up in 10 x 80 mm tubes in a volume of 10 ml. The cells were exposed to 1 μCi/culture of [³H]thymidine (20 Ci/mmol; The Radiochemical Centre, Amersham, England) during the last 24 h of culture. Processing of the cells for liquid scintillation counting was performed according to Bach & Voynow (1966).

Light and electron microscopy

Cells were suspended in a small amount of medium and fixed by addition of an equal volume of 4% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3. Further preparation was made in
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delletized form. After postfixation in 1% veronal acetate-buffered osmium tetroxide (Sjöstrand, 1967) the cells were dehydrated in ethanol (70–100%) and embedded in low-viscosity medium (Spurr, 1969). Thin sections were cut with glass knives on an LKB Ultrrotome I and stained with uranyl acetate followed by lead citrate (Reynolds, 1963). They were examined in a Philips EM 300 electron microscope.

Estimations of cell and nuclear sizes were done at the light microscopic level. For this purpose, the cell pellets were all trimmed down to the same level in the Ultrotome. Sections about 1 μm thick were cut on the Ultrotome and photographed in a Leitz photomicroscope. Cross-sectional areas of cells and nuclei were measured on micrographs with a final magnification of 2700 times, using a polar compensating planimeter (Ingut, Stockholm, Sweden). In mitotic cells the nuclear area was recorded as zero. For each experimental group, cells from at least 8 sections (one section per cell pellet) were measured. The statistical significance of the differences of the means between the various groups was analysed using the Mann–Whitney test (Keeping, 1962).

Table 1. Effects of colchicine and vinblastine on cell and nuclear sizes of lymphocytes stimulated with PHA for 72 h

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell sectional area, μm²</th>
<th>Nuclear sectional area, μm²</th>
<th>Statistical significance of differences of means*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>S.D.</td>
<td>Mean</td>
</tr>
<tr>
<td>Control (1; n = 635)</td>
<td>25.0</td>
<td>6.4</td>
<td>17.4</td>
</tr>
<tr>
<td>PHA (2; n = 282)</td>
<td>91.6</td>
<td>38.4</td>
<td>36.7</td>
</tr>
<tr>
<td>PHA + colchicine (3; n = 566)</td>
<td>48.3</td>
<td>25.5</td>
<td>24.1</td>
</tr>
<tr>
<td>PHA + vinblastine (4; n = 457)</td>
<td>48.3</td>
<td>23.6</td>
<td>23.8</td>
</tr>
</tbody>
</table>

* The values given are valid for both parameters. They give the probabilities that the observed difference could arise purely by chance.

RESULTS

Inhibition of [³H]thymidine incorporation

Colchicine inhibited approximately 71% and vinblastine approximately 80% of [³H]thymidine incorporation in PHA-stimulated lymphocytes between 48 and 72 h after the start of culture. In unstimulated cultures the incorporation was about 0.7% of that in cultures exposed to PHA.

Inhibition of cell growth

The effects of colchicine and vinblastine on the stimulation of lymphocytes by PHA were apparent not only in a lower level of [³H]thymidine incorporation but also by a decrease in cell growth (Table 1 and Figs. 1–5). Thus, approximately 65% of the increase in nuclear and total cell sectional areas caused by treatment with PHA for 72 h was inhibited by both drugs. There were, however, large variations in all groups, particularly with regard to total cell sectional area (Fig. 1).
Mitotic indexes were significantly lower in cultures treated with PHA plus colchicine or vinblastine than in cultures treated with PHA alone (Table 2). In each of these cell groups the mean sectional area of the mitotic cells was distinctly larger than that of the whole cell population (Table 3). Moreover, in cultures treated with PHA alone the mitotic cells were, on the average, larger than in cultures treated with PHA plus colchicine or vinblastine (Table 3).

![Graph showing distributions of nuclear and total cell sectional areas of lymphocytes cultured for 72 h](image)

**Effects of colchicine and vinblastine on cell fine structure**

Fresh lymphocytes and lymphocytes cultured for 72 h without PHA (controls) were similar. The Golgi complex consisted of one or sometimes a few dictyosomes per cell section, usually located close to the centrioles (Fig. 6). PHA stimulation for 72 h resulted in a prominent growth in cytoplasmic volume with development of all organelles. Lymphoblast structure was as described by Biberfeld (1971). For example,
Figs. 2–5. Light micrographs of lymphocytes cultured for 72 h without PHA (Fig. 2), with PHA (Fig. 3), with PHA plus colchicine (Fig. 4), and with PHA plus vinblastine (Fig. 5). Distinct differences in cell size and mitotic activity between the groups are demonstrated. × 1600.
Table 2. Effects of colchicine and vinblastine on mitotic index of lymphocytes stimulated with PHA for 72 h

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mitotic index, %</th>
<th>Statistical significance of differences*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (1; n = 635)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>PHA (2; n = 282)</td>
<td>13.1</td>
<td>2-1  $P &lt; 0.001$</td>
</tr>
<tr>
<td>PHA + colchicine (3; n = 566)</td>
<td>4.2</td>
<td>3-4  $P &lt; 0.005$</td>
</tr>
<tr>
<td>PHA + vinblastine (4; n = 457)</td>
<td>7.7</td>
<td></td>
</tr>
</tbody>
</table>

* Analysed by $\chi^2$ test. The values given are the probabilities that the observed difference could arise purely by chance.

Table 3. Effects of colchicine and vinblastine on mitotic cell size of lymphocytes stimulated with PHA for 72 h

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell sectional area, $\mu m^2$</th>
<th>Statistical significance of means*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA (n = 37)</td>
<td>Mean: 122.7, S.D. 29.4</td>
<td>$P &lt; 0.001$†</td>
</tr>
<tr>
<td>PHA + colchicine (n = 24)</td>
<td>78.4, 41.6</td>
<td>$P &lt; 0.001$†</td>
</tr>
<tr>
<td>PHA + vinblastine (n = 35)</td>
<td>81.1, 34.6</td>
<td>$P &lt; 0.001$†</td>
</tr>
<tr>
<td>PHA mitosis</td>
<td>—</td>
<td>$P &lt; 0.001$</td>
</tr>
<tr>
<td>PHA colchicine mitosis</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>PHA mitosis</td>
<td>—</td>
<td>$P &lt; 0.001$</td>
</tr>
<tr>
<td>PHA vinblastine mitosis</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>PHA colchicine mitosis</td>
<td>—</td>
<td>$P &gt; 0.1$</td>
</tr>
<tr>
<td>PHA vinblastine mitosis</td>
<td>—</td>
<td>$P &gt; 0.1$</td>
</tr>
<tr>
<td>PHA —</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>PHA colchicine mitosis</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>PHA vinblastine mitosis</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

* The values given are the probabilities that the observed difference could arise purely by chance.
† Compared to the whole cell population of the group.

Fig. 6. Juxtanuclear area in a freshly isolated lymphocyte showing 2 centrioles associated with microtubules (arrows) and 2 dictyosomes (d). The surrounding cytoplasm contains a few mitochondria (m) and numerous free ribosomes. x 50000.

Fig. 7. See p. 191 for legend.
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Lymphocyte transformation

there was a marked increase in the size of the Golgi complex. Five or more dictyosomes were seen per cell section. They were organized in the area around the centrioles and associated with varying numbers of dense and multivesicular bodies (Figs. 7–9). The phagosomal/lysosomal nature of these bodies was demonstrated by their ability to accumulate exogenous thorium dioxide particles (Figs. 8, 9). Microtubules were particularly numerous within the Golgi area and appeared to be radiating out from the pericentriolar region (Figs. 7, 8).

Cells exposed to colchicine or vinblastine, either throughout the culture period or just for the last 4 h, lacked cytoplasmic microtubules, but contained microfilaments in increased numbers (cf. De Brabander, Aerts, Van der Veire & Borgers, 1975; Thyberg & Hinek, 1977). Paracrystals and occasional macrotubules associated with ribosomal aggregates were found in vinblastine-treated cells (Figs. 12–14). Such structures also occur in other cell types exposed to vinblastine and they are believed to contain the microtubular protein tubulin (e.g. Bensch & Malawista, 1969; Bryan, 1972).

In addition to the modification of the microtubular and microfilamentous systems colchicine and vinblastine induced other characteristic changes in lymphocyte structure. As mentioned above (see also Table 1 and Fig. 1), cells treated with either of these drugs throughout the culture period were, on average, markedly smaller than cells treated with PHA alone but, nevertheless, larger than lymphocytes cultured without PHA. Likewise, the different cytoplasmic organelles were poorly developed compared to cells treated with PHA alone but, nevertheless, distinctly more prominent than in lymphocytes cultured without PHA. For example, the dictyosomes of the Golgi complex were fewer (seldom more than 3 per cell section) and structurally abnormal, with a decreased number of cisternae partially transformed into or replaced by empty-looking vacuoles. These latter changes were also evident in cells exposed to the antimicrotubular agents only for the last 4 h of culture (Figs. 11, 13, 14). Moreover, the dictyosomes were not organized in one distinct area of the cytoplasm but were dispersed in a haphazard way. This was particularly clear in the 4-h treated cells which had a higher number of dictyosomes. In vinblastine-treated cells the dictyosomes were often closely related to the paracrystals (Figs. 12, 13).

Phagosomes/phagolysosomes were frequently found in small clusters in cells exposed to the antimicrotubular drugs (Figs. 12–14). Whereas the number of lysosomes was low in colchicine-treated cells, there appeared to be a relative increase in their

Figs. 7–9. Juxtanuclear areas of lymphocytes stimulated with PHA for 72 h.

Figs. 7 and 8 show dictyosomes (d) arranged in a ring-like pattern. The individual dictyosomes are composed of 3–5 cisternae with associated vesicles. Microtubules (arrows) are seen in close connexion to the dictyosomes and appear to be radiating out from the pericentriolar region located within the Golgi area (c, centriole; pb, pericentriolar body). A few lysosomes are also seen within this area; the cell in Fig. 8 has been exposed to thorium dioxide particles which after ingestion have accumulated in the lysosomes.

Fig. 9 shows a few dictyosomes and surrounding thorium dioxide-labelled lysosomes. Except for the above mentioned organelles, mitochondria and numerous free polyribosomes are found in the cells. A few cisternae of granular endoplasmic reticulum (arrowheads) are also seen. Fig. 7, x 30000; Fig. 8, x 30000; Fig. 9, x 35000.
number in vinblastine-treated cells. As in cells stimulated with PHA alone, the number of lysosomes was both in colchicine- and vinblastine-treated lymphocytes highest after exposure to exogenous thorium dioxide particles. The ingestion of these particles was not prevented by the antimicrotubular drugs (Fig. 12).

**DISCUSSION**

The present results confirm previous observations of an inhibitory effect of microtubular-disruptive drugs on DNA synthesis by lectin-stimulated lymphocytes (Fitzgerald & Brehaut, 1970; Greene et al. 1976; Gunther et al. 1976; Medrano et al. 1974; Wang et al. 1975): this effect has been shown not to be due to blockage of thymidine transport or cell death (Greene et al. 1976; Medrano et al. 1974; Wang et al. 1975). Mitotic indexes were also markedly lower in cultures treated with PHA plus colchicine or vinblastine than in cultures treated with PHA alone (Table 2), again indicating that these drugs interfere with the commitment of lymphocytes to undergo mitosis (cf. Fitzgerald & Brehaut, 1970; Gunther et al. 1976; Wang et al. 1975). Furthermore, the PHA-induced blast transformation, as measured by increase in cell size, was strongly inhibited by colchicine and vinblastine. Thus, the sectional area of cells treated with PHA plus either of these drugs for 72 h was, on the average, about 50% lower than that of cells treated with PHA alone for the same time, but larger than that of lymphocytes cultured without PHA.

This inhibition of cell growth may be an important factor in preventing the cells from entering mitosis. Thus, in all groups the size of the mitotic cells was, on average, distinctly larger than that of the whole cell population. Hence, the decrease in thymidine incorporation and mitotic index of cultures treated with PHA plus colchicine or vinblastine could conceivably be due to fewer cells reaching a size necessary for entrance into S-phase and mitosis. This suggestion is in agreement with findings on other cell types indicating that a critical amount of G1-associated cell growth is required for initiation of DNA synthesis and mitosis (Killander & Zetterberg, 1965a, b; Yen et al. 1975a, b).

No microtubules were found in cells exposed to PHA plus colchicine or vinblastine. Otherwise, excepting the occurrence of paracrystals and macrotubular arrays after vinblastine treatment (see the Results section), the most prominent fine-structural changes caused by these drugs concerned the Golgi complex. In cells treated with the microtubular-disruptive drugs from the start of PHA stimulation this organelle...
System was notably less developed than in cells treated with PHA alone but, nevertheless, larger than in cells cultured without PHA. Moreover, the individual dictyosomes were structurally altered and showed a reduced number of narrow cisternae associated with a group of empty-looking vacuoles. These latter changes were also evident in cells treated with colchicine or vinblastine only for the last 4 h of PHA stimulation. In both cases, the spatial distribution of the dictyosomes within the cell was also disturbed. Similar alterations in Golgi structure have been found in other cell types exposed to microtubular-disruptive drugs (Moskalewski et al. 1975, 1976; Thyberg & Hinek, 1977; Thyberg et al. 1976b). Together with the observations of a

Fig. 14. As Fig. 13. × 24000.

Fig. 12. Lymphocyte treated with PHA plus vinblastine for 72 h. Furthermore, the cell was exposed to colloidal thorium dioxide for the last 24 h of culture. Two dictyosomes (d) largely consisting of swollen cisternae and/or vacuoles are shown. A few lysosomes with ingested thorium dioxide particles are seen in close proximity to the dictyosomes. pc, vinblastine-induced paracrystals; ld, lipid droplets. × 37000.

Fig. 13. Lymphocytes treated with PHA for 72 h with addition of vinblastine for the last 4 h. The dictyosomes (d), largely consisting of swollen cisternae and/or vacuoles, are widely separated in the cytoplasm. Paracrystals (pc) are found close to some of the dictyosomes. The lysosomes (l) partly occur in small clusters. ap, autophagosome; mf, microfilaments. × 32000.
close morphological relationship between microtubules and the Golgi complex in various cell types (Hinek et al. 1977; Kern, 1975; Mollenhauer, 1974; Moskalewski et al. 1975, 1976; Warchol, Herbert, Williams & Rennels, 1975) including PHA-stimulated lymphocytes (cf. Biberfeld, 1971), these results suggest an integral role of the cytoplasmic microtubular system in the organization and function of the Golgi complex. Such a role for microtubules could be ascribed to a general cytoskeletal action but it is also possible that they may be more directly involved in the transport of membrane vesicles into and out of the Golgi complex (cf. Mollenhauer, 1974; Thyberg & Hinek, 1977).

Conceivably, defective development and function of the Golgi complex could be partly responsible for the inhibition of cell growth caused by the microtubular-disruptive drugs. This organelle system has important functions in the synthetic and secretory activities of the cell (Dauwalder, Whaley & Kephart, 1972; Flickinger, 1975; Hodson & Brenchley, 1976; Michaels & Leblond, 1976) and endoplasmic reticulum membrane material (Autori, Svensson & Dallner, 1975; Elhammer, Svensson, Autuori & Dallner, 1975). Because of the close functional relationship between the Golgi complex and the granular endoplasmic reticulum (Dauwalder et al. 1972; Palade, 1975) it seems possible that a disturbed function in the former organelle may lead to derangements in the activities of the latter (e.g. feedback inhibition). In this context it is interesting to note that membrane-bound ribosomes have been found to be of particular importance in protein synthesis in lectin-stimulated lymphocytes (Wettenhall & Slobbe, 1976).

Summing up the above discussion, it is concluded that cytoplasmic microtubules play an important role in the blast transformation and mitogenic stimulation of lymphocytes produced by PHA and other lectins. This could occur either by direct or indirect interaction between lectin receptors at the cell surface and microtubules (Edelman, Yahara & Wang, 1973). In this context it is interesting to note that cyclic nucleotides appear to be involved in the lectin-induced lymphocyte proliferation (Hadden, Hadden, Sadlik & Coffey, 1976; Wedner & Parker, 1976) and that much attention also is paid to their possible role in the regulation of microtubule assembly/disassembly (Sandoval & Cuatrecasas, 1976; Snyder & McIntosh, 1976).

It is furthermore suggested that the functional role of microtubules is in the structural organization of the cell and particularly the Golgi complex. In the colchicine- and vincristine-induced absence of microtubules, this and other organelle systems do not develop normally, leading to an inhibition of lymphocyte growth and a decreased rate of entrance into mitosis. It should again be stressed, however, that lectin-stimulated lymphocyte growth and mitotic activity was only partially inhibited by the microtubular-disruptive drugs. At the fine-structural level the different organelles, including the dictyosomes, were more prominent and more numerous in cells treated with PHA plus colchicine or vincristine than in cells cultured without PHA. It therefore seems probable that cytoplasmic microtubules are not an absolute requirement for the blast transformation of lymphocytes but enable this process to proceed in a more effective way. This concept conforms to the findings of Wang et al. (1975) of a late rise of DNA synthesis and blast transformation in cultures containing colchicine,
indicating that the effect of the drug may be to slow the passage of the cells through the $G_1$ phase of the cell cycle.

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