Inhibition of Mitogen-induced Changes in Intracellular Fluorescein Fluorescence Polarization of Human Peripheral Blood Lymphocytes by Colchicine, Vinblastine and Cytochalasin B.

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ABSTRACT. We have previously reported that the exposure of human peripheral blood lymphocytes (PBL) to a variety of stimulants caused rapid changes in intracellular fluorescein fluorescence polarization (IFFP) in the activated cells. In the present study we further analyzed possible mechanisms responsible for the changes in IFFP in PBL exposed to phytohaemagglutinin (PHA) and anti-CD3 antibody. By employing several agents which are known to affect the polymerization of the cytoskeleton we showed that both cytochalasin B, which regulates the microfilaments structure, and vinblastine and colchicine, which affect the microtubules, completely abolished the changes induced in IFFP of human PBL by both PHA and anti-CD3. This effect was dose dependent and was noted at concentrations ranging from 10 to 100 μ M of cytochalasin B and 10 μ M of vinblastine and colchicine. The effect of these cytoskeleton modulators occurred within 20 minutes after the initiation of activation with PHA. Our results indicate that activation with PHA and anti-CD3 causes early changes in the microtubules and microfilaments components of the cytoskeleton. The possible application of IFFP measurement in analyzing early changes in the cytoskeleton following cell activation is discussed.

Following stimulation of resting lymphocytes a cascade of biochemical changes occurs within seconds and minutes, which include motile activation, the activation of phospholipase C (1), and phosphoinositol pathway (2). These early metabolic changes then lead to later events such as cell proliferation. One of the mechanisms linking such early and late intracellular events following cell activation involves the cytoskeleton. The cytoskeleton is a complex network composed of microfilaments, microtubules and intermediate filaments which is responsible for the spatial organization of the cytoplasmic matrix. This cellular network plays a role in regulating the activation process which includes gene expression (3), synthesis of RNA and proteins, production of cytokines and cytokine-receptors (3), Ca^{++} levels (4, 5), glucose uptake (6) and cell proliferation (7, 8). Microfil-

aments and microtubules are composed of several cytoplasmic proteins including actin and tubulin, which are also found to be linked to the inner tails of the plasma membrane proteins. Some of the cytoplasmic proteins, such as monomeric G actin, undergo structural changes following exposure of cells to a variety of stimuli (9, 10). The role of the microfilaments and microtubules in cell activation has been demonstrated by employing inhibitors which affect the polymerization of actin. It has been shown that cytochalasins, which are fungal metabolites, inhibit microfilaments polymerization (11, 12, 13) while vinblastine and colchicine disrupt the cytoskeleton polymerization of the microtubule network (12, 13). We have previously shown (14, 15, 16, 17) that the incubation of human PBL with several stimulants induces rapid changes in the IFFP. To analyze the possible involvement of the cytoskeleton in such changes, PBL were activated, by the exposure to PHA and anti-CD3 antibody, in the presence of colchicine, vinblastine and cytochalasin B.

MATERIALS AND METHODS

Lectins and Antibodies. Bottles, containing Phytohaemag-

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IFFP, Intracellular fluorescein fluorescence polarization; PBL, Peripheral blood lymphocytes; PHA, Phytohaemagglutinin; FP, Ficoll Paque; PBS, Phosphate buffer solution; FDA, Fluorescein diacetate; CV, Coefficient of Variables; DFP, Decrease in IFFP; SEM, Standard error of the mean; SD, Standard deviation; CM, Complete medium; DMSO, Dimethyl sulfonic acid.

glutinin A (PHA) (HA15, Wellcome) were reconstituted in 5 ml distilled water and stored at -70°C in aliquots of 100 μl . Mouse anti human anti-CD3 antibody (clone UCH1) was purchased from Dako (M-835).

Inhibitors. Colchicine (Sigma C-9754), Vinblastine (Sigma V-1377), and Cytochalasin B (Sigma C-6762), were brought to 100 μM solution in phosphate buffer solution (PBS), and kept at 4°C until use.

PBL separation. The low density subset of human blood lymphocytes was prepared, with minor modifications, by the regular Lymphrep (LP) or Ficoll paque (FP) technique. Briefly, blood samples (10 ml), were rotated at 25 rev/min with 0.1 gr of iron-carbonyl powder (Genzyme) at 37°C , for 30 minutes, then placed on a magnet for 10 min for the removal of iron absorbing cells. The remaining blood was diluted with saline (1:1) and then separated on a two-layer (100% and 80%) cell density gradient (Ficoll Paque Code No.17-0840-03, Pharmacia, 1.077 g/ml). After spinning at 550 g for 23 minutes at 25°C , cells accumulated at the interphase between the two Ficoll layers, were collected and washed twice in saline, at 280 g for 10 minutes at 25°C . The last wash was performed under identical conditions except that an isotonic Phosphate Buffer Saline (PBS, Biological Industries, ISRAEL) was employed. Cells were then suspended in PBS containing 0.5 mM MgCl_2 (Merck) and 1.3 mM CaCl_2 (Merck) and adjusted to pH 7.4. The cell suspension concentration was then brought to 8×10^6 cells/ml and kept overnight at room temperature.

PBL Stimulation. PBL, kept overnight, were washed and resuspended in PBS containing 1 mM glucose and Ca^{++} and Mg^{++} . Cells underwent incubation for 45 minutes at 37°C prior to stimulation for 40 minutes with PHA or anti-CD3 monoclonal antibody in the presence or absence of selected inhibitors.

IFFP Measurement with the Cellscan.

a. Cell staining: Fifty-five μl of the cell suspension, before or after incubation with reagent, were added to 50 μl of 2 μM fluorescein diacetate (FDA) in PBS+ Ca^{++} and Mg^{++} (FDA, Sigma F7378), and stained for 5 minutes at room temperature. The FDA undergoes fluorochromasia (18) in viable cells. This includes penetration of FDA into the cells, its enzymatic hydrolysis which converts it to fluorescein and the accumulation of the latter in the cell. (The stock solution of FDA was 20 mM in DMSO, final dilution 1:10⁴).

b. Apparatus: The central feature of our "Cellscan" system (described in detail in 19) is a cell carrier incorporating a 100×100 two-dimensional array of holes, each approximately 7 μm in diameter and spaced about 20 μm apart, in which cells are trapped. The carrier is fixed to a holder which allows the introduction and extraction of liquids during which cells are maintained in their individual traps, permitting observation while changes are made in the cellular microenvironment.

A 40 μl aliquot of cell suspension is applied to a carrier; a negative pressure of approximately 1–5 mm H_2O maintained between the upper and lower surfaces of the carrier causes

rapid settling of the cells into traps (19).

Measurements of fluorescence with the Cellscan employ a photon counting technique in which the same number of photons is counted for each cell, regardless of intensity, so that strongly emitting and weakly emitting cells are measured with the same precision. Measurement of background signals due to dark current, stray light, and/or autofluorescence can be made for each cell prior to its fluorescence measurement.

The cell carrier holder is fastened to a scanning table driven by 3 stepping motors; motion in the x-y plane positions any one of the traps in the cell carrier in the center of the epilluminating laser beam and the microscope observation field, while the z axis motor is used to change focus. Under the staining conditions used here, with illumination intensities of 1–5 μW at 441 nm, the scanning time for obtaining counts of 10,000 photons from a single fluorescein-loaded cell varied from 0.05 to 1 second. The coordinates of each cell, its fluorescent intensity (expressed in number of counts per unit time) at two different wavelengths (527 nm and 510 nm), the counting time, and the computed polarization values were recorded, as were the mean, standard deviation and CV.

Calibration and reproducibility of polarization measurements with the Cellscan system were previously discussed in detail (19). Measurements at $22 \pm 1^{\circ}\text{C}$ of cells, glycerin-water mixtures, and beads by the Cellscan and by Perkin-Elmer MPF-44 and SLM-4800 polarization fluorometers consistently yield comparable values. Fading of fluorescence in the Cellscan was insignificant under the measurement conditions used and in any case did not influence the polarization (17, in preparation).

Maintenance of cell position and measurement reproducibility were tested by 25 routine cell scans of a field of 10×10 traps for beads and by 10 scans for cells. Although the particles on the carrier were rinsed between scans, no bead was lost from its position after 25 measurements, and the majority of the cells maintained their position during ten measurements. The CVs for beads never exceeded 3%; some were much lower. For cells, the distribution of polarization values was, understandably, wider, as cells are considerably less homogeneous than beads. However, the average CV for 10 measurements of an individual cell was $< 2.75\%$.

c. IFFP Measurement and Calculation: Cells were applied on the cell carrier (Fig. 1) and the mean IFFP was determined after scanning 400 (20×20) trapped cells. The degree of IFFP was measured at 527 ± 4 nm. At least 3 separate measurements were performed for each sample.

The percentage of decrease in IFFP (DFP) after incubation with a stimulant is given by:

$$DFP = \left[\frac{IFFP_o}{IFFP_s} - 1 \right] 100.$$

where $IFFP_o$ and $IFFP_s$ are the average polarization values of ~ 400 cells after incubation with no stimulant ($\bigcirc$), and with stimulant(s), correspondingly.

Based on multiple experiments, the standard error of the

Fig. 1. Lymphocytes trapped in a hole of a silicon-coated cell carrier ($\times 7,200$ magnification).

mean (SEM) of at least three separate measurements of DFP, of a given sample, was $\leq 2\%$ (19). Thus, any value of DFP greater than 4% ($\sim \times 2$ Standard Deviation (SD)) was considered as a positive indicator for cell activation.

Proliferation assay. PBL at $1 \times 10^5/200 \mu\text{l}$ were seeded in triplicates in a 96-well plate in enriched complete medium (CM) of RPMI containing 10% heat inactivated fetal calf serum, L-glutamine, antibiotics with or without stimulants at different dilutions. Unless otherwise mentioned, $[\text{H}]\text{-thymidine}$ ($1 \mu\text{Ci}/20 \mu\text{l}$) was added for the last 4 of the 72 hours of incubation with the lectin. Cells were then harvested using a cell-harvester device (Packard, USA) and radioactivity determined on a liquid scintillation beta-counter (Packard, USA).

Statistical analysis. Statistical analysis was performed by the Wilcoxon rank-sum test (20). Two sided p-values are presented.

RESULTS

The effect of cytoskeleton modulators on cell proliferation. We have previously shown that incubation of

Fig. 2. Human PBL were incubated for 40 minutes with either PHA and anti-CD3. FDA was then added and cells were applied on the cell carrier and scanned using a polarized laser beam for measuring IFFP. Results were obtained as %DFP (see Materials and Methods). Mean (3 separate measurements) and SEM are presented.

^c $p < 0.05$ compared to unstimulated cells (IFFP₀).

human PBL with several stimulants employed, reduced IFFP (14, 15, 16, 17). To study a possible relation between changes in IFFP and the cytoskeleton in activated cells following stimulation, we employed PHA and anti-CD3 monoclonal antibody, which cause a statistically significant DFP (Fig. 2). For modulating the cytoskeleton integrity, vinblastine, colchicine and cytochalasin B, were employed. Since it has been shown that the cytoskeleton is involved in intracellular events leading to DNA synthesis and cell proliferation (5), we tested the effect on DNA synthesis of the modulators of both microfilaments and microtubules. For this purpose, we incubated PBL for 72 hours with various concentrations of cytochalasin B, a microfilament modulator, or colchicine and vinblastine, which are microtubule modulators. $[\text{H}]\text{-thymidine}$ was added for the last 4 hours of incubation. Representative results are shown in Table I. Both colchicine and vinblastine at $10 \mu\text{M}$ to $0.01 \mu\text{M}$ inhibited DNA synthesis as measured by $[\text{H}]\text{-thymidine}$ incorporation. Cytochalasin B was effective only at $100 \mu\text{M}$ while at $10 \mu\text{M}$ this agent caused a substantial increase in DNA synthesis.

The effect of microtubule modulators on PHA induced changes in IFFP. Since microtubule and microfilament modulators affected DNA synthesis, we tested the effect of these agents on IFFP. For this purpose we incubated PBL for 40 minutes with PHA in the presence or absence of different concentrations of colchicine, vinblastine and cytochalasin B which were found to effectively inhibit DNA synthesis. PHA concentration as well as the incubation period (40 minutes) were selected based on previous results demonstrating that under such conditions a significant reduction in IFFP occurred. Representative results obtained in three sepa-

Table I. EFFECT OF MICROTUBULES AND MICROFILAMENTS REGULATORS ON CELL PROLIFERATION.

Exp.	Inhibitor	μ M	Mean ^a cpm (SEM) ^b			
			CM	PHA	Inhibitor (In)	PHA + In
1	—	—	2036 (318)	27495 (5195)		
	colchicine	10	—	—	565 (27)	9511 (1192) ^c
		1	—	—	264 (165)	6962 (335) ^c
		0.1	—	—	585 (108)	6840 (667) ^c
		0.01	—	—	1618 (121)	12129 (1890) ^c
		0.001	—	—	2632 (722)	20333 (928)
	vinblastine	1	—	—	623 (91)	11492 (868) ^c
		0.1	—	—	1226 (86)	13030 (3732) ^c
		0.01	—	—	1598 (249)	5593 (212) ^c
		0.001	—	—	2720 (343)	24493 (3805)
	cytochalasin B	100	—	—	208 (32)	5054 (1525) ^c
		10	—	—	3247 (526)	61162 (4574) ^c
		1	—	—	2518 (1025)	31601 (5802)
		0.1	—	—	2858 (272)	21852 (3317)
		0.01	—	—	3766 (260)	24411 (2532)
2	—	—	530 (62)	31614 (6152)		
	colchicine	10	—	—		12121 (739) ^c
		0.1	—	—		12447 (819) ^c
		0.001	—	—		30367 (4539)
	vinblastine	10	—	—		10591 (1653) ^c
		0.1	—	—		13479 (1200) ^c
		0.001	—	—		29411 (1717)
	cytochalasin B	100	—	—		17399 (2135) ^c
		1	—	—		27649 (2480)
		0.01	—	—		34594 (2115)

PBL were incubated for 72 hours with PHA and in the presence or absence of colchicine, vinblastine and cytochalasin B at various concentrations. [³H]-thymidine was added for the last 4 hours, before harvesting the cells and determination of radioactivity on a scintillation fluid beta-counter.

^a Mean of three samples.

^b Standard error of the mean.

^c $p < 0.05$ Compared to cells incubated in PHA alone.

Table II. EFFECT OF MICROTUBULES MODULATORS ON IFFP INDUCED IN HUMAN PBL BY PHA.

Exp.	Inhibitor (In)	μ M	Mean ^a IFFP $\pm$ SEM ^b (% DFP)			
			IFFP _o (PBS)	IFFP _s (PHA)	In ^d	IFFP _s (PHA + In ^d)
1	colchicine	—	0.186 $\pm$ 0.005 (0)	0.170 $\pm$ 0.002 ^c	—	—
		10	—	—	0.192 $\pm$ 0.002 (0)	0.187 $\pm$ 0.003 (0)
		1	—	—	—	0.176 $\pm$ 0.003 ^c (6)
2	vinblastine	—	0.181 $\pm$ 0.002 (0)	0.167 $\pm$ 0.003 ^c (8)	—	—
		10	—	—	0.190 $\pm$ 0.002 (0)	0.181 $\pm$ 0.004 (0)
		1	—	—	0.185 $\pm$ 0.003 (0)	0.163 $\pm$ 0.004 ^c (10)
		0.1	—	—	0.184 $\pm$ 0.003 (0)	0.170 $\pm$ 0.003 ^c (7)

PBL were stimulated for 40 minutes with PHA in the presence or absence of colchicine and vinblastine at various concentrations. Cells were then stained with FDA and applied on the cell carrier and scanned using a polarized laser beam for measuring IFFP.

^a Mean of 3 separate measurements.

^b SEM, standard error of the mean.

^c $p < 0.05$ Compared to unstimulated cells (IFFP_o).

^d In, Inhibitor

Table III. EFFECT OF MICROFILAMENTS MODULATOR ON IFFP INDUCED IN HUMAN PBL BY PHA.

Exp.	Cytochalasin B μM	Mean ^a IFFP $\pm$ SEM ^b (% DFP)			
		IFFP _o (PBS)	IFFP _s (PHA)	In ^d	IFFP _s (PHA/anti-CD3) + In ^d
1	—	0.158 $\pm$ 0.002 (0)	0.150 $\pm$ 0.002 ^c (6)	—	—
	100	—	—	0.165 $\pm$ 0.003 (0)	0.164 $\pm$ 0.002 (0)
	10	—	—	—	0.160 $\pm$ 0.002 (0)
	1	—	—	—	0.147 $\pm$ 0.002 ^c (7)
2	—	0.193 $\pm$ 0.001 (0)	0.174 $\pm$ 0.002 ^c (10)	—	—
	10	—	—	0.193 $\pm$ 0.003 (0)	0.191 $\pm$ 0.003 (0)
	1	—	—	—	0.183 $\pm$ 0.001 ^c (6)
	0.1	—	—	—	0.168 $\pm$ 0.003 ^c (13)

PBL were stimulated for 40 minutes with PHA in the presence or absence of cytochalasin B at various concentrations. Cells were then stained with FDA, applied on the cell carrier and scanned using a polarized laser beam for measuring IFFP.

^a Mean of 3 separate measurements.

^b SEM, standard error of the mean.

^c $p < 0.05$ Compared to unstimulated cells (IFFP_o).

^d In, Inhibitor

rate experiments are illustrated in Table II. PHA, as previously described, substantially decreased IFFP when compared to that of cells incubated with no stimulant: In Exp. 1, IFFP_o = 0.186 and IFFP_s = 0.170 respectively, giving DFP = 9. And in Exp. 2, IFFP_o = 0.181 and IFFP_s = 0.167, giving DFP = 8. When colchicine or vinblastine were added to PHA at the beginning of the incubation period, the effect of PHA was completely abolished at 10 μM of both colchicine and vinblastine (IFFP_s from 0.170 to 0.187 and from 0.167 to 0.181 for colchicine and vinblastine, respectively). Incubation of PBL with colchicine alone had no effect on IFFP when compared to unstimulated cells (IFFP_o), whereas vinblastine alone, at 10 μM , increased IFFP_o (0.190) when compared to untreated cells (0.181). Cytochalasin B, a microfilaments modulator, at 10 and 100 μM also abolished the reduction in IFFP_s induced by PHA, from

IFFP of 0.150 to 0.164 and 0.160, respectively (Exp. 1) and from 0.174 to 0.191 in Exp. 2 (Table III). 1 μM of cytochalasin B had no effect on IFFP_s (Table III). Incubation with cytochalasin B alone at both 10 and 100 μM increased IFFP_o from 0.158 in the untreated PBL to 0.165 in Exp. 1 (Table III).

The effect of microtubules and microfilaments modulators on IFFP_s induced by anti-CD3. Since vinblastine and cytochalasin abrogated IFFP_s induced in human PBL by PHA, we tested the effect of these cytoskeleton modulators on IFFP_s induced by a different T lymphocyte polyclonal stimulation. For this purpose, PBL were incubated with 1:5,000 diluted anti-CD3, previously shown to reduce IFFP_o (Fig. 2). As shown in Table IV both vinblastine (10 μM) and cytochalasin B (10 μM) completely abrogated IFFP_s induced in PBL by anti-CD3.

Table IV. EFFECT OF VINBLASTINE AND CYTOCHALASIN ON IFFP INDUCED IN HUMAN PBL BY ANTI-CD3.

anti-CD3	Inhibitor (μM)	Mean ^a IFFP $\pm$ SEM ^b (% DFP)			
		IFFP _o	IFFP _s (anti-CD3)	In ^c	IFFP _s (anti-CD3) + In ^c
—	—	0.277 $\pm$ 0.001 (0)	—	—	—
+	—	—	0.262 $\pm$ 0.001 ^d (6)	—	—
—	Vinblastine (10 μM)	—	—	0.279 $\pm$ 0.001 (0)	—
+	+	—	—	—	0.281 $\pm$ 0.001 (0)
—	Cytochalasin B (10 μM)	—	—	0.300 $\pm$ 0.002 (0)	—
+	+	—	—	—	0.291 $\pm$ 0.001 (0)

PBL were incubated for 40 minutes with 1:5000 diluted anti-CD3 in the presence or absence of vinblastine (10 μM) or cytochalasin B (10 μM). Cells were then stained with FDA, applied on the cell carrier and scanned using a polarized laser beam for measuring IFFP.

^a Mean of 3 separate measurements.

^b SEM, Standard error of the mean.

^c In, Inhibitor.

^d $p < 0.05$ Compared to unstimulated cells (IFFP_o).

Table V. EFFECT OF TIME-EXPOSURE TO COLCHICINE AND VINBLASTINE ON IFFP INDUCED IN PHA ACTIVATED HUMAN PBL.

Exp.	Inhibitor (μ M)	Time (min) ^a	Mean ^b IFFP $\pm$ SEM (% DFP)		
			IFFP _o (PBS)	IFFP _s (PHA)	IFFP _s (PHA) + Inhibitor
1	Colchicine (10 μ M)	—	0.211 $\pm$ 0.002 (0)	0.189 $\pm$ 0.001 ^c (11)	—
		40	—	—	0.214 $\pm$ 0.001 (0)
		20	—	—	0.199 $\pm$ 0.007 (4)
		10	—	—	0.190 $\pm$ 0.003 ^c (9)
		5	—	—	0.193 $\pm$ 0.001 ^c (9)
2	Vinblastine (10 μ M)	—	0.191 $\pm$ 0.002 (0)	0.179 $\pm$ 0.001 ^c (7)	—
		40	—	—	0.185 $\pm$ 0.002 (3)
		20	—	—	0.186 $\pm$ 0.003 (3)
		10	—	—	0.182 $\pm$ 0.002 ^c (5)
		5	—	—	0.174 $\pm$ 0.002 ^c (9)

PBL were stimulated for 40 minutes with PHA. Colchicine and vinblastine (10 μ M) were added together with PHA, washed out at the indicated periods and incubated with PHA to complete 40 minutes of stimulation. PBL were stained with FDA, applied on the cell carrier and scanned using a polarized laser beam for measuring IFFP.

^a minutes of incubation with either colchicine or vinblastine.

^b Mean of 3 separate measurements.

^c $p < 0.05$ Compared to unstimulated cells (IFFP_o).

Kinetics of inhibition induced by cytoskeleton inhibitors. In order to test the kinetics of the effect of vinblastine, colchicine and cytochalasin B on IFFP_s induced by PHA, we performed the following experiment. PBL were stimulated with PHA in the presence or absence of vinblastine and colchicine, at optimal concentrations revealed in previous experiments. These agents were added at various periods after the initiation of stimulation with PHA. As shown in Table V, the addition of PHA at the beginning of culture together with either vinblastine or colchicine, at the optimal concentration (10 μ M), abolished the effect of PHA on IFFP. A similar effect was observed when these agents were added for 20

and 40 minutes following PBL stimulation, whereas no effect was seen when added for only 5 minutes following the entire stimulation period (40 minutes) with PHA (Table VI).

DISCUSSION

Stimulation of human PBL with mitogens has been shown to induce a rapid decrease in IFFP which correlated to later events such as cell proliferation. In the present study we analyzed the biological significance of changes in IFFP. Since it has been shown that the extent of polymerization of the cytoskeleton microtubules and

Table VI. COLCHICINE AND VINBLASTINE INHIBIT THE REDUCTION IN IFFP INDUCED IN HUMAN PBL BY PHA.

Exp.	Inhibitor (μ M)	Time (min) ^a	Mean ^b IFFP $\pm$ SEM (% DFP)		
			IFFP _o (PBS)	IFFP _s (PHA)	IFFP _s (PHA) + Inhibitor
1	Colchicine (10 μ M)	—	0.259 $\pm$ 0.002 (0)	0.235 $\pm$ 0.001 ^c (9)	—
		5	—	—	0.245 $\pm$ 0.001 ^c (5)
		20	—	—	0.281 $\pm$ 0.001 (0)
		40	—	—	0.250 $\pm$ 0.003 (4)
2	Vinblastine	—	0.240 $\pm$ 0.003 (0)	0.219 $\pm$ 0.001 ^c (9)	—
		40	—	—	0.257 $\pm$ 0.002 (0)
3	Cytochalasin B	—	0.192 $\pm$ 0.001 (0)	0.170 $\pm$ 0.001 ^c (15)	—
		40	—	—	0.168 $\pm$ 0.002 ^c (16)

PBL were stimulated with PHA for 40 minutes followed by the addition of either colchicine (10 μ M), vinblastine (10 μ M) or cytochalasin B (100 μ M) for the indicated time (minutes). PBL were stained with FDA, applied on the cell carrier and scanned using a polarized laser beam for measuring IFFP.

^a minutes of incubation with either of the inhibitors.

^b Mean of 3 separate measurements.

^c $p < 0.05$ Compared to unstimulated cells (IFFP_o).

microfilaments is affected during cell activation (10), we tested the effect of agents which modulate the cytoskeleton structure, on IFFP. When adding 10 μ M of colchicine or vinblastine to PBL stimulated with PHA or anti-CD3 monoclonal antibody, we were able to completely reverse the effect of these stimulants on IFFP (Table II). Similarly, cytochalasin B, which affects another component of the cytoskeleton, the microfilaments, also reversed the effect of PHA on IFFP at 10 and 100 μ M (Table III). The effect of these cytoskeleton modulators was achieved at concentrations similar to those obtained by others testing intracellular events, such as target cell lysis, glucose intake, expression of receptors, and synthesis of DNA, RNA and proteins, following specific (11, 21) and non-specific stimulation (22, 23, 24). In most of the studies, the inhibitory effect of cytochalasin, vinblastine and colchicine, was achieved when cells were pre-incubated with the inhibitor before adding the stimulants (5, 25, 26). In our study the inhibitory effect on the cytoskeleton was achieved when the above agents were present during the stimulation period or even after the initiation of stimulation with PHA (Tables II, VI). These results demonstrate that reduction in IFFP may reflect changes in the polymerization of the cytoskeleton. Kinetics analysis revealed that the effect of vinblastine and colchicine occurred when these agents were presented for at least 20 minutes together with PHA (Table V). Furthermore, when these cytoskeleton modulators were added after the termination of PHA activation period (40 minutes), for at least 20–40 minutes, the effect of PHA on IFFP could still be reversed. These results may indicate that changes in the cytoskeleton, which are induced by PHA, exist even after the removal of the stimulant (PHA).

Interestingly, cytochalasin B, when added after the completion of PHA stimulation period (40 minutes) was unable to reverse the effect of PHA on IFFP (Table VI). This phenomenon could be explained by the inability of cytochalasin, at the concentration employed, to reverse the effect of PHA on IFFP. Alternatively, our results point towards the possibility that under identical PHA stimulation conditions, changes induced in the microfilaments might not have similar kinetic characteristics. A similar effect on the cytoskeleton was also noted with anti-CD3 (Table IV), which shows that stimulants which act via different receptors cause similar intracellular effects on the cytoskeleton polymerization. The effect on the cytoskeleton of more specific stimulants, which act via cytokine receptors and T cell receptors, are currently being studied. The mechanism by which PHA affects the cytoskeleton polymerization is still unclear, although, as shown by others, it may involve the conversion of globular (G) to filamentous (F) structure (11). The effect of PHA on the cytoplasmic viscosity correlated with the effect on DNA synthesis as measured by

[3 H]-thymidine incorporation. Colchicine, vinblastine and cytochalasin B inhibited, at similar concentrations, both DNA synthesis and changes in IFFP. Similar results demonstrating the inhibition of DNA synthesis by colchicine were shown by others (7). One exception was, however, noted. While 10 μ M of cytochalasin B reversed the decrease in IFFP (Table III), it substantially increased DNA synthesis at similar concentrations (Table I). This effect of cytochalasin B may be due to differences in the ability to reverse the effect of this modulator at various stages of the signal transduction pathway. The similarity by which the cytoskeleton modulators, which were employed, affected both the cytoskeleton polymerization and cell proliferation, points towards the possible role of the cytoskeleton in signal transduction, as postulated by others (27, 28). Studies to reveal the relation between the cytoskeleton and other early events induced in activated cells by a variety of specific and non-specific stimulants, are currently being studied. Finally, in this study we show that following activation of human PBL with PHA and anti-CD3 monoclonal antibody, rapid changes occur in the both microfilaments and microtubules. The effect of selected stimulants on PBL from both healthy individuals and patients with immunological disorders is being studied.

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