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COLCHICINE, VINBLASTINE AND GRISEOFULVIN

PHARMACOLOGICAL STUDIES WITH HUMAN LEUKOCYTES*

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Abstract—Some aspects of the interactions of the vinca alkaloids, colchicine and griseofulvin with human leukocytes in suspension have been explored. Tritiated colchicine, vinblastine and griseofulvin enter the cells where significant binding by a supernatant protein takes place. Glutamate competitively inhibits the entry of vinblastine into the leukocytes when present in large excess. Inhibition of the incorporation of valine-^{14}C into leukocyte protein occurred in the presence of vinblastine and vincristine at concentrations down to 6×10^{-6} M; colchicine and griseofulvin did not affect this incorporation until levels of 3×10^{-4} M were attained. Concentrations of these agents as high as 1×10^{-4} M did not inhibit the incorporation of uridine-^{3}H into RNA or of acetate-^{14}C into lipid. The data were considered to support the concept that the anti-mitotic and anti-inflammatory effects of vinca alkaloids, colchicine and griseofulvin result from binding to precursor units of the microtubules.

AMONG the agents that induce metaphase arrest in dividing cells, several, including colchicine, vinblastine and griseofulvin, have an anti-inflammatory effect in acute gouty arthritis.[1–3] Although these two biological activities are seemingly unrelated recent work on the pharmacological effects of these compounds[4–10] has provided evidence to support an earlier suggestion of a common underlying mechanism of action,[11] one that involves interactions between the drugs and subunits of the microtubules,[1,9,10,12] which are involved both as components of the mitotic spindle and in other situations where movement occurs, both of whole cells and of intracellular structures. On the other hand, these agents also inhibit significantly the synthesis of nucleic acids, proteins and lipids in a wide range of cell types; these biochemical studies have been reviewed recently.[12]

The present studies were initiated in an attempt to determine which of the two mechanisms, microtubule interactions or inhibition of biosynthetic processes, is responsible for the anti-inflammatory action. Binding of all three agents by a protein from the supernatant fraction of human leukocytes occurred at concentrations of drug down to 1×10^{-6} M; vinblastine and colchicine interfere with the fusion of lysosomal

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granules with vacuoles containing ingested matter when present at $2{\cdot}5 \times 10^{-5}$ M. Although incorporation of valine into protein was somewhat depressed by drug levels as low as 6×10^{-6} M in the case of vinblastine and vincristine only, the synthesis of nucleic acids, assayed by incorporation of labeled nucleosides, was not inhibited until much greater concentrations of these agents were employed.

MATERIALS AND METHODS

Radioactive compounds. Vinblastine, colchicine and griseofulvin were custom-tritiated by Tracerlab, Inc., using a catalytic exchange method. After removal of exchangeable tritium, colchicine was purified chromatographically as described previously;[8] in addition, some tritiated colchicine was also purchased from the New England Nuclear Corp. Vinblastine was purified by thin-layer chromatography on silica gel HF_{254} in the solvent systems described later. Griseofulvin was recrystallized from hot ethanol to constant specific activity. Thymidine-^{3}H, uridine-^{3}H, valine-1-^{14}C, glutamate-3,4-^{14}C, and sodium acetate-2-^{14}C were purchased from Tracerlab, Inc., or New England Nuclear Corp.

Incubation of cells with tritiated drug. Leukocytes were prepared by dextran sedimentation and osmotic lysis of red cells as described previously.[13] A leukocyte suspension (about $3{\cdot}8 \times 10^7$ cells, 80% PMNs) in 12% serum–phosphate buffer[13] was incubated with labeled drug ($1–8 \times 10^{-6}$ M; $0{\cdot}5–4{\cdot}9 \times 10^6$ counts/min at 20 per cent efficiency) at 37° for 2 hr. After the incubation, the cells were centrifuged at 250 *g* for 10 min, and then washed with normal saline to remove extracellular radioactivity. The cells were lysed in two ways with similar results: (a) vigorous pipetting after the addition of heparin, about 500 U per ml and (b) homogenization (teflon on glass) for 4·5 min in ice. The disrupted cells were then divided by differential centrifugation into nuclear (400 *g* for 10 min), granule (8200 *g* for 20 min), and cytoplasmic (final supernatant) fractions. In one experiment the nuclear and granule fractions were washed once (Fig. 1), in another experiment not, with similar results.

Electron microscopy. To ascertain that the granule fraction did indeed contain granules, parallel experiments were run with unlabeled drug. The cell fractions were fixed with 1·5% phosphate-buffered glutaraldehyde solution,[14] followed by osmification after rinsing the pellets in the isotonic buffer.[15] The tissue was stained with lead and uranyl salts, embedded in an epoxy resin (Maraglas)[16] and examined in an Elmiscop I. Our best such preparation is shown in Fig. 2.

Distribution of radioactivity. Distribution of radioactivity between the fractions was measured with a Packard Tri-Carb liquid scintillation counter, using a scintillator consisting of 4 g of 2,5-diphenyloxazole (PPO) and 50 mg of 1,4-bis-2-(5-phenyloxazolyl) benzene (POPOP)/l. of toluene and with one-half the volume of ethanol added. Particulate fractions were lysed by sonication (Biosonic II), and all fractions brought to 0·005 M with respect to sodium chloride and applied to 2×30 cm columns of Sephadex G-25 equilibrated with the same solution. The columns were then eluted with 0·005 M sodium chloride and 3-ml fractions collected. Readings of the optical density at 260 and 280 mμ were made with a Zeiss PMQ II spectrophotometer, to assess protein content. In addition, calibration with bovine serum albumin was used to determine elution volumes for proteins in cases where the amount of protein in the fraction was very low.

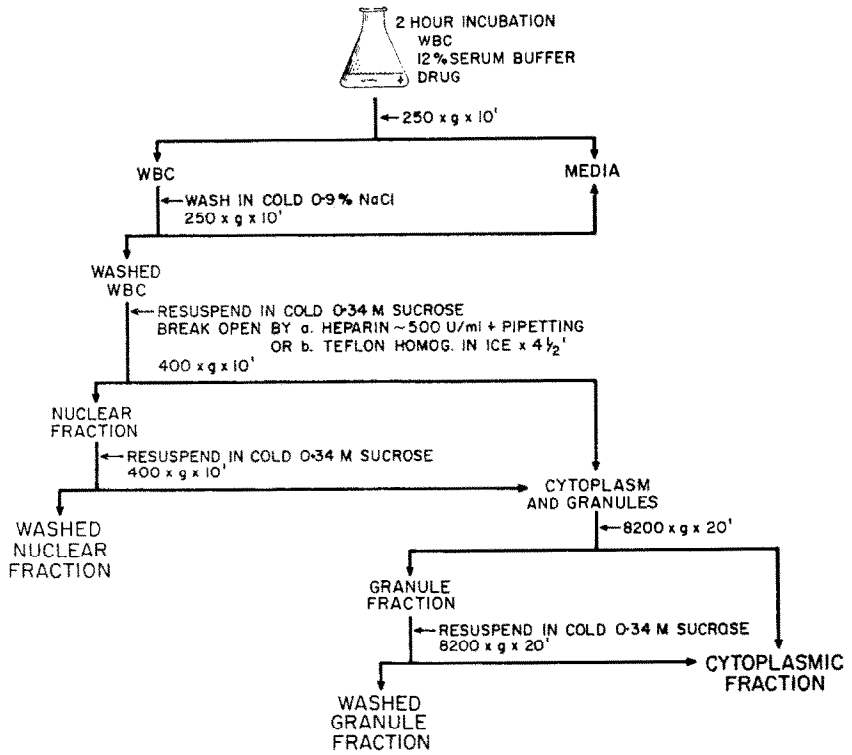

FIG. 1. Procedures used for fractionating leukocytes exposed to tritiated antimitotic agents during a 2-hr incubation.

The ability of vinblastine to cause aggregation of the microtubule protein was assayed by incubating high speed (100,000 *g*) supernatant fraction from leukocytes with colchicine-^{3}H (2000 counts/min/ml: 5 c/m-mole) and unlabeled vinblastine (10^{-3} M).[17] A precipitate formed due to aggregation, and the amount of radioactivity associated with it was measured after its removal by centrifugation at 35,000 rev/min for 30 min.

Kinetic studies. The effects of glutamate upon the uptake of tritiated vinblastine, or of unlabeled vinblastine upon the uptake of glutamate-^{14}C, were studied with similar incubation conditions to those used for binding studies, except that the time course was followed. Samples were removed, layered over 0·25 M sucrose solutions in Shevky–Stafford and McNaught centrifuge tubes, and centrifuged to separate cells from radioactive medium. The cell pellets were resuspended in 0·005 M sodium chloride solution, sonicated, and aliquots counted with the liquid scintillation counter to determine the amounts of intracellular radioactivity.

Thin-layer chromatography for separation of drugs was carried out on 0·5 mm layers of silica gel HF_{254} (Merck). The following solvents were used: methanol (I); *n*-butanol–95% ethanol–concentrated ammonium hydroxide–water (4:1:2:1, by vol.) (II); chloroform–isopropanol (3:1, v/v) (III); and *n*-butanol–formic acid–water (77:10:13) (IV). R_f values for the drugs were as follows: colchicine, I–0·59, II–0·80,

III–0·85, IV–0·65: griseofulvin, I–0·64, II–0·86, IV–0·86; vinblastine, I–0·48, II–0·90, III–0·96, IV–0·26.

Synthesis of macromolecules. The synthesis of nucleic acids, proteins and lipids was measured by incorporation of thymidine-^{3}H (1 μc; 7·6 c/m-mole), uridine-^{3}H (1 μc per flask; 3 c/m-mole), valine-1-^{14}C (0·4 μc per flask; 22 mc/m-mole), and sodium acetate (2·5 μc per flask; 50 mc/m-mole) respectively, using procedures that have been described previously.[18,19] Incubation volumes were 3 ml, and approximately 3·8 $\times$ 10^7 cells were used per flask; time courses of up to 60 min were followed after initial preincubation with drug for 15 min.

RESULTS

Uptake of tritium-labeled drugs. When the labeled drugs were incubated with human leukocytes, they entered the cells rapidly, especially at higher concentrations. The time courses for entry into the cells appear in Fig. 3. Levels of 26 to 71 $\mu\mu$moles per 3·4 $\times$ 10^7 cells were achieved at an external concentration of 2·5 $\times$ 10^{-5} M; at 2·5 $\times$ 10^{-7} M

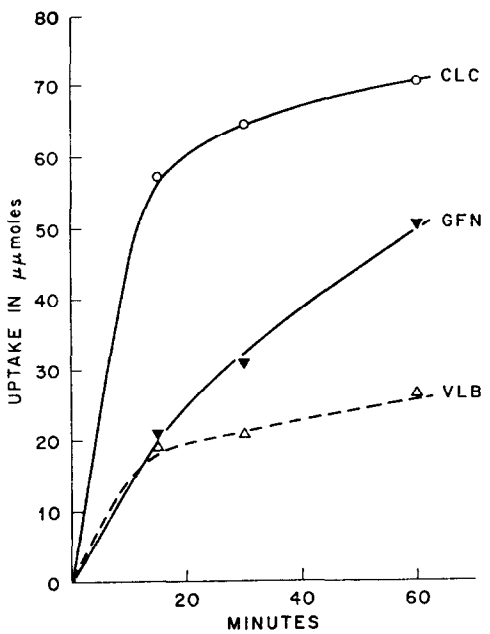

FIG. 3. The uptake of triated colchicine (CLC), griseofulvin (GFN) and vinblastine (VLB) by human leukocytes. Drugs were present at an external concentration of 2·5 $\times$ 10^{-5} M, together with 3·4 $\times$ 10^7 cells in a final incubation volume of 3·8 ml (Ringer saline + 10% calf serum). Cells were separated from medium by centrifugation through 0·25 M sucrose solution, lysed by sonication, and intracellular radioactivity measured.

the intracellular levels were about 2 per cent of these values. Colchicine was taken up more effectively by the leukocytes than was vinblastine or griseofulvin. Although omission of serum from the medium did not affect initial rates of drug uptake, the cells rapidly underwent clumping with decline of intracellular drug levels, so this additive was retained for all experiments.

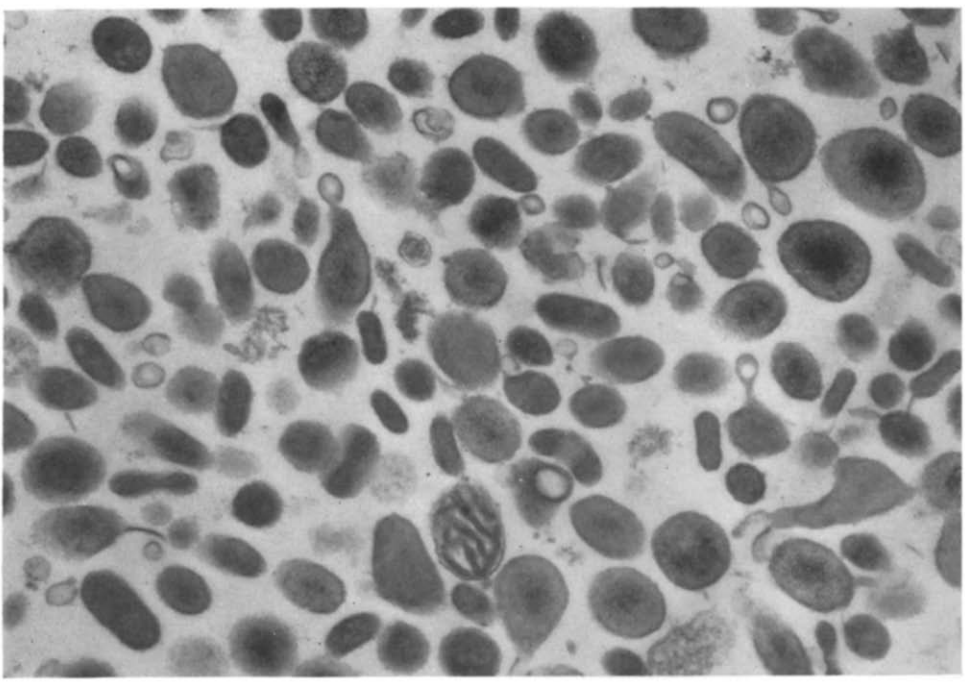

FIG. 2. Electron micrograph (× 40,000) of the glutaraldehyde fixed, O_sO_4 postfixed granule preparation. The fraction contains only rarely other cell structures such as an occasional vesicular membrane fragment or mitochondrion.

Glutamate has been reported to antagonize the biological[20] and biochemical[21] effects of the *Catharanthus* (*Vinca*) alkaloids vinblastine and vincristine. Vinblastine also inhibits the uptake of glutamate by mouse tumor cells *in vitro*.[18] In human leukocytes a similar inhibitory effect of this alkaloid was established (Table 1). In

TABLE 1. EFFECT OF VINBLASTINE ON THE UPTAKE OF GLUTAMATE-^{14}C BY LEUKOCYTES*

Glutamate (M)	Vinblastine (M)	Glutamate uptake ($\mu\mu$moles/hr/10^8 cells)
$8{\cdot}6 \times 10^{-5}$	0	589·5
	1×10^{-5}	288·2
	$3{\cdot}9 \times 10^{-5}$	124·1
$2{\cdot}2 \times 10^{-6}$	0	15·9
	2×10^{-6}	11·9
	2×10^{-5}	7·6

* Cells (approximately $3{\cdot}6 \times 10^7$) were incubated with sodium glutamate-3,4-^{14}C and unlabeled vinblastine at the indicated concentrations in 3·8 ml final volume. Samples were separated from medium at various times and initial rates of uptake determined.

earlier studies using different systems, 100- to 125-fold excess of glutamate did not significantly reduce the entry of labeled vinblastine into Ehrlich ascites carcinoma cells,[22] or the dissolution of the mitotic spindles of *Pectinaria gouldi* oocytes induced by vinblastine.[23] In the leukocyte system, however, when glutamate levels between 100- and 3800-fold greater than those of vinblastine were employed, the uptake of alkaloid by the leukocytes was inhibited. This inhibition followed competitive kinetics (Fig. 4).

Binding of the alkaloids. After incubating leukocytes with labeled drugs for 2 hr, the intracellular distribution of radioactivity was as shown in Table 2. Between 60 and 93 per cent of the tritium was in the supernatant or cytoplasmic fraction, a finding similar to that reported for colchicine[4,5,8] and vinblastine[22,24] in other systems.

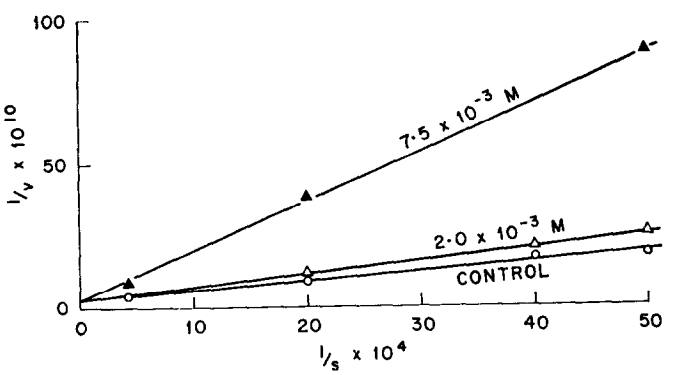

FIG. 4. Lineweaver–Burk plot of the reciprocals of the substrate (vinblastine-^{3}H) concentration and initial velocity of alkaloid uptake by human leukocytes. The data, normalized for 10^8 cells, show competitive inhibition by added monosodium glutamate at the indicated concentrations.

TABLE 2. DISTRIBUTION OF RADIOACTIVITY WITHIN THE LEUKOCYTES*

Drug	Level (M)	Intracellular radioactivity (counts/min)		
		Nuclei	Granules	Cytoplasm
Colchicine	1×10^{-6}	7460	1960	114,490
Vinblastine	1×10^{-6}	220	520	1350
Vinblastine	8×10^{-6}	2590	1760	14,000
Griseofulvin	1×10^{-6}	710	1600	4400
Griseofulvin	4×10^{-6}	4520	2430	21,310

* After incubation of the cells ($3{\cdot}8 \times 10^7$) with tritium-labeled drug for 2 hr, they were washed free of medium, lysed, and separated into three fractions by differential centrifugation.

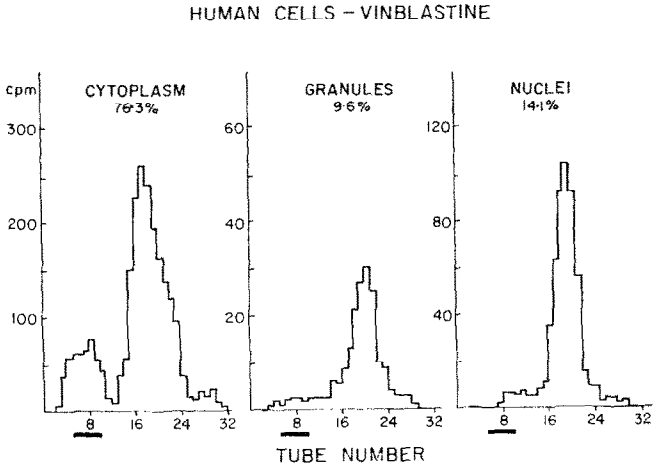

FIG. 5. Elution pattern of tritium label from columns of Sephadex G-25 (2 × 30 cm). Fractions from cells incubated for 2 hr with vinblastine-^{3}H at 8×10^{-6} M were applied to the columns: elution was carried out with 0·005 M NaCl and 3 ml fractions were collected. The dark line below the abscissa represents the region in which protein eluted from the column.

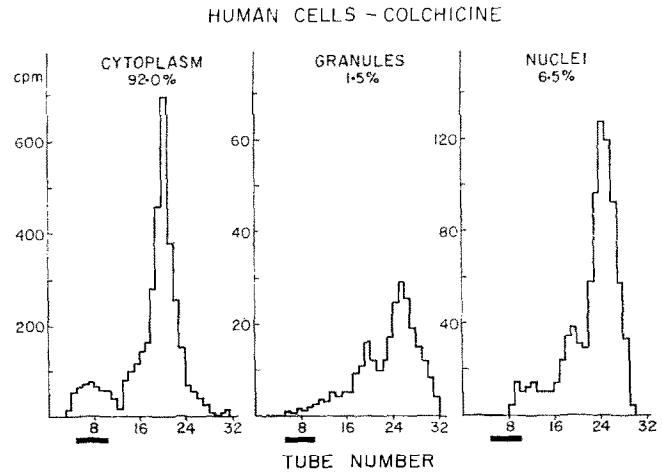

FIG. 6. Sephadex elution patterns for fractions from cells exposed to colchicine-^{3}H (1×10^{-6} M). Procedures were as for Fig. 4.

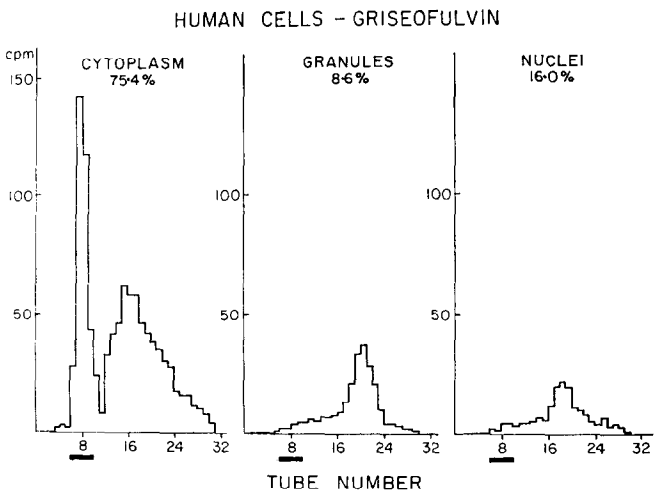

FIG. 7. Sephadex elution patterns for fractions from cells exposed to griseofulvin-^{3}H (4×10^{-6} M). Procedures were as for Fig. 4.

Fractionation of the individual samples on Sephadex G-25 showed some interesting differences (Figs. 5–7). Whereas the radioactivity from the nuclear and granule fractions migrated essentially as free drug, in the cytoplasmic fraction there were two peaks of radioactivity, one associated with protein that eluted from the columns before free drug. Typical results of these studies, showing the amounts of cytoplasmic activity bound to protein, appear in Table 3. Similar findings have been made with rabbit peritoneal exudate cells (95 per cent PMNs).

TABLE 3. BINDING OF DRUGS BY CYTOPLASMIC PROTEIN*

Drug	External concentration (M)	Intracellular level ($\mu\mu$moles/10^8 cells)	Amount bound to protein ($\mu\mu$moles/10^8 cells)
Colchicine	1×10^{-6}	204·0	14·8
Vinblastine	1×10^{-6}	44·7	4·5
Vinblastine	8×10^{-6}	296·7	47·0
Griseofulvin	1×10^{-6}	91·1	37·8
Griseofulvin	4×10^{-6}	329·1	90·4

* Cells were incubated for 2 hr with drugs at the indicated concentrations. After washing and lysis, cell fractions were prepared by differential centrifugation and then subjected to chromatography on Sephadex G-25.

The phenomenon of intracellular microtubule crystal formation, induced by vinblastine and vincristine, has been studied recently[25–27] and has also been shown to occur in cell-free preparations.[17,28] When cytoplasmic fractions from human leukocytes were treated with small amounts of colchicine-^{3}H to label unpolymerized microtubule precursor protein, addition of vinblastine led to precipitation of protein carrying label. There was an accompanying reduction in the amount of tritiated drug

TABLE 4. PRECIPITATION OF PROTEIN-BOUND COLCHICINE-^{3}H ON INCUBATION WITH VINBLASTINE*

Sample	Time (hr)	Control (counts/min)	Vinblastine (counts/min)
Initial preparation	0	10,840	10,900
Supernatant	2	10,870	10,360
Precipitate	2	20	530

* High-speed (100,00 *g* for 1 hr) supernatant (5 ml per sample) from human leukocytes (0·3 ml packed cell volume) was incubated for 2 hr with colchicine-^{3}H (5 c/m-mole) in the absence or presence of unlabeled vinblastine (10^{-3} M). The samples were then centrifuged for 30 min at 35,000 rev/min and the total radioactivity in the precipitate measured. In the vinblastine-treated preparation, essentially none of the counts remaining in the supernatant represented protein-bound colchicine.

remaining in the supernatant (Table 4). Electron microscopic study[28] and gel electrophoresis[17] of such precipitates have established their identity with aggregated microtubule protein.

Synthesis of macromolecules. The incorporation of thymidine-^{3}H into DNA by leukocytes occurred to only an extremely limited extent so that meaningful studies of the inhibitory effect of antimitotics were not possible. On the other hand, the leukocytes incorporated uridine-^{3}H into RNA readily, although uptake was linear for only about 30 min. This incorporation was unaffected by drugs at levels up to 10^{-4} M, but at $1{\cdot}6 \times 10^{-4}$ M vincristine caused a 23 per cent inhibition. Somewhat unexpectedly, both vinblastine and vincristine inhibited the incorporation of valine-1-^{14}C into protein at levels considerably below those that affected RNA synthesis. As can be seen in Fig. 8, small but reproducible effects were observed at concentrations of vinblastine and vincristine as low as 6×10^{-6} M. Colchicine and griseofulvin were inactive at

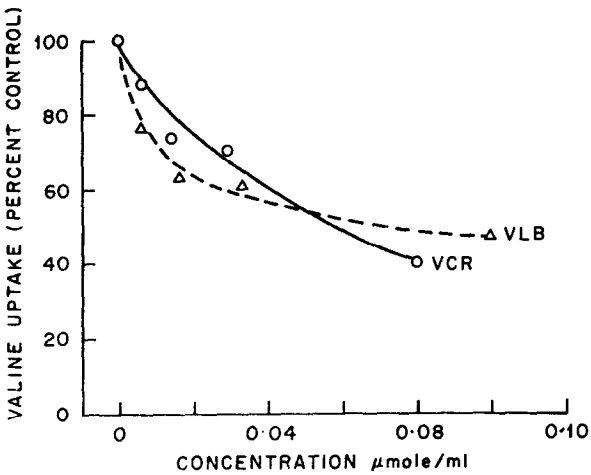

FIG. 8. Effect of vincristine (○——○) and vinblastine (△----△) on the incorporation of valine-^{14}C into proteins by human leukocytes *in vitro*. Data were calculated from initial rates of uptake by $3{\cdot}8 \times 10^7$ cells.

these levels, requiring a concentration of 3×10^{-4} M to give 23·7 and 39·5 per cent inhibition, respectively. The incorporation of acetate-^{14}C into lipids was not affected by vinblastine, vincristine or colchicine at 10^{-4} M. The ability of cells to exclude eosin dye[29] was used to study the possibility that loss of viability might account for the inhibitory effects on biosynthetic processes. In control cells 96 per cent excluded the dye; after a 2-hr incubation with drugs at $2{\cdot}5 \times 10^{-5}$ M, viability was 95·5 per cent for vinblastine and 97·5 per cent for colchicine and griseofulvin. Incubation with vinblastine at 1×10^{-4} M, and colchicine or griseofulvin at 3×10^{-4} M for 1 hr also led to no increase in stained cells (96·5 per cent).

DISCUSSION

These present studies have explored some aspects of the interaction of anti-inflammatory, anti-mitotic agents with leukocytes. In such cells, colchicine interferes with chemotaxis[30] and colchicine and vinblastine interfere with the fusion of lysosomes with vacuoles containing ingested matter at concentrations of the order of $2{\cdot}5 \times 10^{-5}$ M.[13,31] Interference with mitosis is normally seen at even lower concentrations in most mammalian cells. Clearly neither of these effects can stem from inhibition of nucleic acid synthesis, since this was not manifested at concentrations below $1{\cdot}6 \times 10^{-4}$ M. Inhibition of protein synthesis still remains a possibility as a basis for anti-inflammatory action by vinblastine and vincristine since it could be detected at levels below those that inhibit intracellular fusion. However, binding of the drugs by microtubule protein occurred at much lower levels and over a wider range of concentrations than any other interaction, and could be demonstrated with colchicine and griseofulvin as well as the *Vinca* alkaloids. It is also easier to envisage that the drugs act by binding to microtubule subunits needed to assemble tubules for effecting various intracellular rearrangements,[1,32,33] rather than to postulate that the synthesis of a needed protein is inhibited. This is especially probable in view of the lack of effect on valine incorporation by colchicine and griseofulvin at low levels.

In this study as in earlier ones[4,5,8] the protein that bound the antimitotic drugs appeared to be restricted to the cytoplasmic fraction. However, the nuclei and granules from cells exposed to griseofulvin and vinblastine contained relatively larger amounts of radioactivity than did those exposed to colchicine, a finding that calls for some explanation. The associations with nuclei might represent incomplete disruption of cells in those cases. However, alternative possibilities are indicated by examples in the literature suggesting associations of both vinblastine and griseofulvin with various cellular fractions. Association of vinblastine with ribosomes may be needed to give the helical arrays described in *Escherichia coli*,[34] while griseofulvin forms a complex with some types of RNA.[35] If similar associations occurred with these two drugs in our system, we might expect to find radioactivity in the nuclei and mitochondria which contain RNA, while large aggregates of ribosomes might sediment with the granule fraction. If these associations are weak, they may not survive sonication and gel filtration used in our experiments.

The mechanism whereby vinblastine and vincristine inhibit protein synthesis at levels that do not affect incorporation of uridine into RNA is not yet known, but is the subject of continuing investigation. In the murine tumors studied previously,[18,19] protein synthesis was not selectively inhibited. Possibly in leukocytes an additional site of action is present, perhaps at the ribosomal level, or there may be changes in

amino acid transport, for which we have some preliminary evidence. Such an effect could reinforce the depletion of free microtubule subunits due to binding, by preventing their resynthesis.

Lastly, the somewhat perplexing role of glutamate as an antagonist of vinblastine can now be understood. A competitive inhibition of the entry of the drug into the cells, exerted by the amino acid, implies a similar transport mechanism for the two compounds despite great differences in their chemical structure. Since earlier studies have indicated that glutamate antagonizes the biochemical effects of colchicine,[21] this compound, like vinblastine, also may be transported by the same mechanism as the amino acid. Colchicine is known to reach higher levels intracellularly in human leukocytes (10·2–49·5 μg/100 ml cells) than that attained in the plasma (3 μg/100 ml);[36] intracellular concentration of vinblastine has been noted in Ehrlich ascites carcinoma cells[22] and leukocytes.[37] While intracellular binding of drugs must make a major contribution to this phenomenon, active transport processes might also be involved.

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