CYTOCHALASIN B

VI. Competitive Inhibition of Nucleoside Transport by Cultured Novikoff Rat Hepatoma Cells

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ABSTRACT

Cytochalasin B competitively inhibits the transport of uridine and thymidine by Novikoff rat hepatoma cells growing in suspension culture with apparent K_i's of 2 and 6 μM, respectively, but has no effect on the intracellular phosphorylation of the nucleosides. Choline transport is not affected by cytochalasin B. Results from pulse-chase experiments indicate that cytochalasin B has no direct effect on the synthesis of RNA, DNA, or uridine diphosphate-sugars. The inhibition of uridine and thymidine incorporation into nucleic acids by cytochalasin B is solely the consequence of the inhibition of nucleoside transport.

INTRODUCTION

Cytochalasin B (CB) inhibits, as Carter (1) originally observed, cytokinesis (cytoplasmic division) without inhibiting karyokinesis (nuclear division). It also inhibits cell movement and causes changes in cell shape (1). These effects have been attributed to drug-induced changes in microfilament structure (2–5). The inhibition of the secretion of thyroid hormone (6) and growth hormone (7) by CB has also been suggested to be due to an inhibition of microfilament function. Alteration of microfilament structure, however, does not invariably accompany CB effects (8), and it has been suggested previously (9, 10) that direct effects of CB on the plasma membrane may be involved in its biological actions.

Recently, CB has been shown to inhibit deoxyglucose, glucose, and glucosamine transport (10). The inhibition of deoxyglucose and probably of glucose and glucosamine transport is of the simple competitive type, and the affinity of CB for the glucose transport site is at least 1000-fold higher than that of the natural substrates. Since transport is the rate-limiting step in metabolism of glucose and other low molecular weight substances (11, 12), the inhibition of transport results in a proportional inhibition of glucose metabolism and of the incorporation of glucosamine into macromolecules (10).

The present results demonstrate that CB also inhibits competitively the transport of uridine and thymidine, but not of choline, without affecting the intracellular phosphorylation of the nucleosides or nucleic acid synthesis. The inhibition of the incorporation of uridine and thymidine into nucleic acids by CB (13) is a consequence of this inhibition of their transport into the cell.

MATERIALS AND METHODS

Cell Culture

Novikoff rat hepatoma cells (N1S1-67) were propagated in suspension culture in Swim's medium 67 as described previously (14, 15). Cells harvested from the late exponential phase of growth (2.0–2.8 $\times$ 10^6

cells/ml) were suspended in basal medium 42 (BM42; 14) as indicated in the figure legends.

Cytochalasin B

Cytochalasin B (obtained from Imperial Chemical Industries Research Laboratories, Alderley Park, Cheshire, England) was dissolved in 8.2 mM concentrations in dimethyl sulfoxide (Fisher Scientific Company, Pittsburgh, Pa.) or ethanol as a stock solution. In each experiment, addition of appropriate volumes of the solvents had no effect on the processes investigated.

Incorporation of ^{3}H-Labeled Precursors

The incorporation of uridine-5-^{3}H and thymidine-6-^{3}H (Schwarz-Mann, Orangeburg, N. Y.) or of choline-CH_3-^{3}H (New England Nuclear Corp., Boston, Mass.) into total cell material (acid soluble plus acid insoluble) and into acid-insoluble material (macromolecules) was determined as described previously (11, 16, 17). Acid-soluble pools were extracted from labeled cells with perchloric acid, and the acid-extracts were analyzed by ascending paper chromatography with a solvent composed of 3 vol 1 M ammonium acetate (pH 5.0) and 7 vol of 95% ethanol (solvent 28; reference 16). The phosphorylation of uridine and thymidine by cell-free preparations of N1S1-67 cells was measured as described previously (18, 19). The final reaction mixtures contained 250 μM uridine or 100 μM thymidine, respectively.

RESULTS

Effect of CB on Nucleoside Transport

As reported previously (13), CB inhibited the incorporation of uridine (Fig. 1 A) and of thymidine-^{3}H (Fig. 2 A) into acid-insoluble material. The incorporation of the ^{3}H-labeled nucleosides into total cell material (acid soluble plus acid insoluble), however, was reduced to about the same extent as their incorporation into nucleic acids. This effect is typical of that of other inhibitors of nucleoside transport such as Persantin (11, 20), phenethyl alcohol (21), and other nucleosides (20), since transport is the rate-limiting step in the incorporation of various nucleosides into the nucleotide pool and into nucleic acids (11, 20). Chromatographic analysis of acid-extracts from the cells labeled with uridine-^{3}H or thymidine-^{3}H in the absence and presence of CB indicated that CB had no effect on the phosphorylation of either nucleoside after it had entered the cells. The relative distribution of label among the intracellular nucleotides was about the same whether or not the cells were labeled in the presence of CB (Table I). None of the acid-extracts contained significant amounts of labeled free nucleosides, indicating that they became phosphorylated as rapidly as they entered the cells. CB, at a concentration of 160 μM, also had no effect on the phos-

TABLE I

Chromatography of Acid-Extracts from Cells Labeled with Uridine-^{3}H in the Presence and Absence of CB

Nucleoside-^{3}H	CB (μM)	Total ^{3}H* (cpm)	Per cent of total			
			UTP + UDP	UDP-sugars	UMP	Uridine
Uridine	0	11,920	77	14	8	1
	4.1	4,450	77	12	10	1
			dTTP	dTDP	dTMP	Thymidine
Thymidine	0	7,920	91	6	2	1
	4.1	6,150	89	8	2	1

The details of the experiments are described in the legends of Figs. 1 and 2. Acid-extracts were prepared from 1.5×10^6 cells after 120 and 90 min of labeling with uridine-^{3}H or thymidine-^{3}H, respectively.

dTTP, deoxythymidine triphosphate; dTDP, deoxythymidine diphosphate; dTMP, deoxythymidine monophosphate.

*Total radioactivity associated with nucleotides and nucleosides after chromatography of 50 μl of acid-extract with solvent 28 at 30°C for 18 hr.

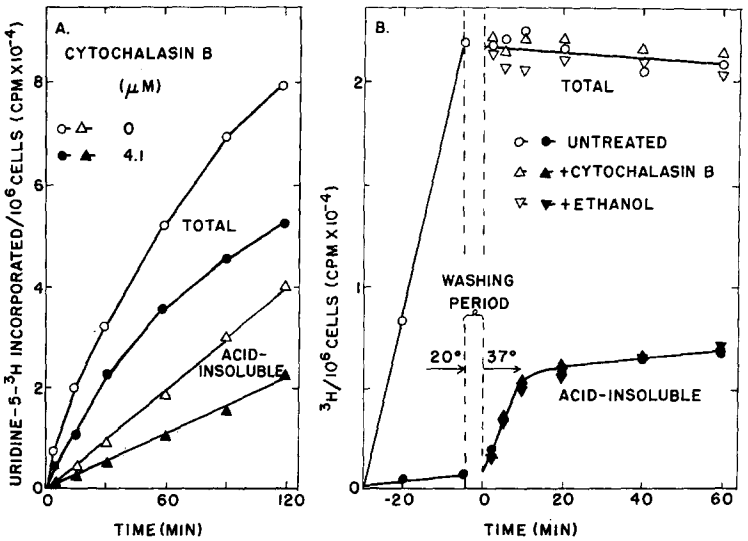

FIGURE 1 Effect of CB on uridine incorporation into total cell material and acid-insoluble material. (A) Samples of a suspension of 2×10^6 cells/ml of BM42 were supplemented with the indicated concentrations of CB and 10 μM uridine-5-^{3}H (95 cpm/pmole). At various times of incubation at 37°C, duplicate 0.5 ml samples of each suspension were analyzed for radioactivity in total cell material (○——○, ●——●) or acid-insoluble material (△——△, ▲——▲). All points represent averages of the duplicate samples. (B) Cells were suspended to 2×10^6 cells/ml in BM42 that had been equilibrated at 20°C and contained 10 μM uridine-5-^{3}H (95 cpm/pmole). The suspension was incubated at 20°C and monitored for radioactivity in total cell material (○——○) and acid-insoluble material (●——●). After 25 min of incubation, the remaining cells were collected by centrifugation, washed once in cold (4°C) BM42, and appropriate portions of the cells were suspended (0 time) to the original cell density in warm (37°C) BM42 containing, where indicated, 4.1 μM CB or 0.05% (v/v) ethanol. The suspensions were further incubated at 37°C and monitored for radioactivity in total cell material and acid-insoluble material. All points represent averages of duplicate 0.05 ml samples. After the pulse and at 60 min, acid-extracts were prepared from 1.4×10^7 cells and analyzed chromatographically (see Table I).

phorylation of uridine or thymidine by cell-free preparations from N1S1-67 cells (not shown).

The results from pulse-chase experiments indicate that CB had no immediate effect on RNA (Fig. 1 B) or DNA (Fig. 2 B) synthesis. Cells were prelabeled with uridine-^{3}H or thymidine-^{3}H at 20°C. At this temperature, macromolecular synthesis is arrested while nucleoside transport and phosphorylation continues at an appreciable rate, and labeled nucleotides, mostly in the form of triphosphates, accumulate intracellularly (18, 21–24; see also Figs. 1 B and 2 B, and Table II). The cells were then collected by centrifugation, washed free of residual labeled nucleosides, and further incubated in fresh medium at 37°C with and without CB. As indicated in Figs. 1 B and 2 B, CB had no effect on the transfer of labeled nucleotides from the pool into nucleic acids.

The synthesis of uridine diphosphate (UDP)-sugars is also almost completely inhibited at low temperatures (23, 24; see Table II). When cells after labeling with uridine-^{3}H at 20°C were chased at 37°C, at least 15% of the accumulated labeled uridine triphosphate (UTP) was used for the synthesis of UDP-sugars whether or not CB was present during the chase (Table II). The results indicate that CB did not interfere with the synthesis of UDP-sugars.

Evidence has been presented previously to indicate that the initial rate of incorporation of various nucleosides into total cell material approximates the rate of their transport into the cell (11, 20). Kinetic analyses of the inhibition of uridine and thymidine transport by CB indicate that the inhibition is of the simple competitive type (Fig. 3).

Lack of Effect of CB on Choline Transport

The results in Fig. 4 show that CB at a concentration of 4.1 μM had no effect on the incorporation

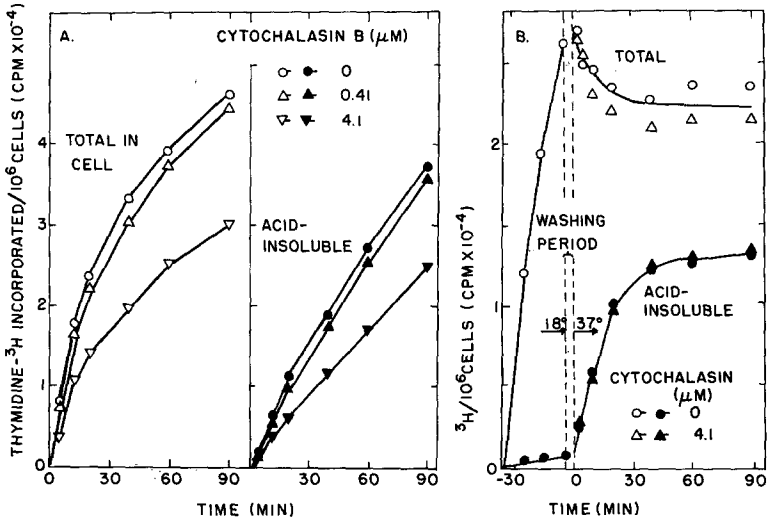

FIGURE 2 Effect of CB on thymidine incorporation into total and acid-insoluble material. (A) Samples of a suspension of 2×10^6 cells/ml of BM42 were supplemented with the indicated concentrations of CB and immediately thereafter with 0.25 μM thymidine-^{3}H (1000 cpm/pmole). At various times of incubation at 37°C, duplicate 0.5 ml samples were analyzed for radioactivity in total cell material (○——○, △——△, ▽——▽) or acid-insoluble material (●——●, ▲——▲, ▼——▼). All points represent averages of the duplicate samples. (B) Cells were suspended to 2×10^6 cells/ml in BM42 that had been equilibrated at 18°C and contained 0.25 μM thymidine-^{3}H (1000 cpm/pmole). The suspension was incubated at 18°C and monitored for radioactivity in total cell material (○——○) or acid-insoluble material (●——●). After 25 min of incubation, the remaining cells were collected by centrifugation, washed once in cold (4°C) BM42, and equal portions of the cells were resuspended to the original cell density in warm (37°) BM42 containing, where indicated, 4.1 μM CB. The suspensions were further incubated at 37°C and monitored for radioactivity in total cell material and acid-insoluble material. All points represent averages of duplicate 0.5 ml of suspension.

TABLE II

Lack of Effect of CB on the Incorporation of UTP into UDP-Sugars

Time	Cytochalasin B (μM)	^{3}H/50 μl of acid-extract (cpm) UTP + UDP	UDP-sugars	UMP
After pulse at 20°C	0	7390	80	190
After chase at 37°C	0	3630	1050	670
	4.1	3880	1040	600

The details of the experiment are described in the legend to Fig. 1 B. Acid-extracts were prepared from 1.4×10^7 cells after 25 min of labeling with uridine-^{3}H at 20°C (pulse) and after a subsequent 60 min chase at 37°C in the presence and absence of CB. Samples of 50 μl of each acid-extract were chromatographed with solvent 28.

of 1 μM choline-^{3}H into total cell material or into acid-insoluble material. Over 95% of the label in the acid-soluble pool of CB-treated and untreated cells was associated with phosphoryl choline. Kinetic analyses of choline transport (17) also failed to detect any effect of CB.

DISCUSSION

Our results demonstrate that CB competitively inhibits both uridine and thymidine transport by N1S1-67 cells without affecting the intracellular phosphorylation of the nucleosides or RNA and DNA synthesis. Glucose transport and glucosamine transport are also inhibited by CB (10). Since glucose, uridine, and thymidine are transported by different systems (11, 12, 18, 20), CB interacts with at least three transport systems. It exhibits some specificity, however, in that it inhibits the different transport systems to varying degrees, and choline transport is not affected. Leucine uptake also does

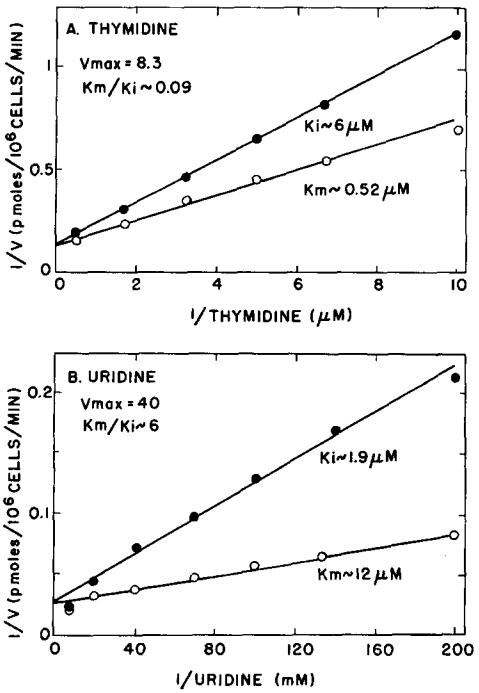

FIGURE 3 Lineweaver-Burk plots of the initial rates of thymidine (A) and uridine (B) transport in the presence and absence of CB. Portions of a suspension of 2×10^6 cells/ml of BM42 were mixed with CB to 4.1 μM (●——●) or remained untreated (○——○). Immediately thereafter, 10-ml samples of each suspension were supplemented (A) with 0.1, 0.15, 0.2, 0.3, or 0.6 μM (1360 cpm/pmole) or 2 μM (78 cpm/pmole) thymidine-^{3}H or (B) with 5, 7.5, 10, 15, 25, or 50 μM (29 cpm/pmole) or 110 μM (13 cpm/pmole) uridine-^{3}H. The suspensions were incubated at 37°C for 5 min and then duplicate 1 ml samples were analyzed for radioactivity in total cell material. These values were considered estimates of the initial transport rates (18, 20). The apparent K_m and Ki values were estimated from the slopes of the lines.

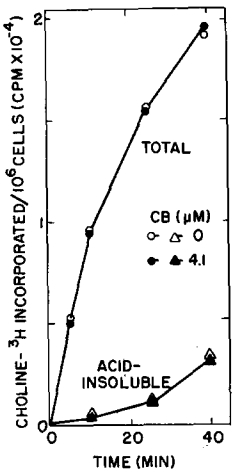

FIGURE 4 Incorporation of choline-^{3}H in the presence and absence of CB. Samples of a suspension of 2×10^6 cells/ml of choline-free BM42 (17) were supplemented with CB where indicated, and with 1 μM choline-^{3}H (93 cpm/pmole). At various times of incubation at 37°C, duplicate 0.5 ml samples of suspension were analyzed for radioactivity in total cell material (○——○, ●——●) or acid-insoluble material (△——△, ▲——▲). All points represent averages of the duplicate samples.

not seem to be affected by CB (13). Glucose transport and glucosamine transport show the greatest sensitivity to inhibition by CB, but uridine transport is also significantly more inhibited by CB than thymidine transport. For instance, at a concentration of 100 μM in the medium, glucose transport and glucosamine transport are inhibited about 90% by 4.1 μM CB (10). At a concentration of 5 μM in the medium, uridine transport is inhibited about 75% by 4.1 μM CB, whereas thymidine transport at 0.5 μM is only inhibited about 40% by 4.1 μM CB (Fig. 3). However, the degree of inhibition depends on the concentration of both the transport substrate and the inhibitor, and additional specificity is indicated by the K_m/K_i ratios. Although the apparent K_i values are similar for the inhibition of the various transport systems, the K_m/K_i ratio of 1800 for deoxyglucose transport is much higher than those for uridine or thymidine transport (6 and 0.09, respectively). The data also show that the inhibition of uridine and thymidine into nucleic acids (13) is solely due to the inhibition of their transport into the cell. Other inhibitors of transport such as Persantin (11, 20), phenethyl alcohol (21), or other nucleosides (20) have a similar effect.

The data do not explain the effects of CB on cytokinesis, cell movement, or changes in cell shape or microfilament structure since mammalian cells grow and divide normally in the absence of nucleosides in the medium. The possibility exists that these effects might be related to a reversible binding of CB to transport sites or, more likely, to other cell surface membrane sites. This conclusion is supported by the finding that most biological effects of CB (1, 13, 25, 26), including the inhibition of deoxyglucose transport (10), are readily reversed by removal of CB from the medium. CB may interact with proteins involved in

transport reactions and/or other membrane proteins. Recent studies have indicated that CB can bind to protein (actin) and change its physical properties (27). While we were unable to demonstrate extensive intracellular accumulation of CB (10), small amounts may be transported into the cell and interact with protein. Such speculation must await studies which conclusively demonstrate an intracellular location of CB.

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