Cytochalasin D Inhibits Nuclear Translocation of Nonhistone Proteins Induced by Lectin and Other Agents in Lymphocytes (43045)

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Abstract. **Cytochalasin D (CD), known to interfere with microfilament activity, inhibits RNA and protein synthesis and the cellular uptake of [^{3}H]actinomycin D in concanavalin A-stimulated lymphocytes. It also inhibits the nuclear translocation of nonhistone proteins (NHP) induced by the lectin. Since NHP mediate RNA and protein synthesis and [^{3}H]actinomycin D binding, inhibited nuclear translocation of NHP can explain the inhibition of the former events. CD also inhibits nuclear translocation of NHP caused by NaF or chloroquine. The mechanism of how CD inhibits nuclear translocation of the NHP is obscure. Since NaF and chloroquine enter cells by diffusion, it would appear that CD acts on an intracellular target, rather than abrogating a cell surface-mediated signal generated by binding of the lectin to a cell surface receptor.** [P.S.E.B.M. 1990, Vol 194]

Previous studies have shown that the fungal metabolite cytochalasin B (CB) inhibits lymphocyte mitogenesis induced by lectins (1–4). CB is believed to interact with microfilaments. It appears to act early in mitogenesis since it inhibits RNA and phospholipid metabolism, which occur shortly after lectin-lymphocyte interaction (1). However, it is unknown which essential event of mitogenesis is primarily prevented by CB. Also, the exact mechanism of how lectins induce mitogenesis is not completely understood.

In this report, we have investigated the effect of cytochalasin D (CD) on early events of lymphocyte mitogenesis induced by concanavalin A (Con A), i.e., RNA and protein synthesis uptake of [^{3}H]actinomycin D ([^{3}H]AD) by cells, and the level of nonhistone proteins (NHP) in the nucleus. These events are stimulated very early in mitogenesis (5, 6) and appear to be causally related (7, 8). In addition, the effect of CD on nuclear translocation caused by two lysosomotropic agents, i.e., NaF and chloroquine, was investigated. CD instead of CB was used, since the latter inhibits the transport of sugars (9, 10) and [^{3}H]nucleosides (11) used to determine nucleic acid synthesis, while CD appears to have no such effects.

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Materials And Methods

Cells, Medium, and Chemicals. The methods of isolating lymphocytes from human blood, the preparation of cultures of lymphocytes, the labeling of cells with [^{3}H]uridine and [^{3}H]leucine, and the determination of [^{3}H]actinomycin D binding to cells have been described (8, 12, 13). The medium was minimal essential medium containing 2 m*M* Hepes and supplemented with 5% human plasma or serum albumin, the pH was adjusted to 7.2–7.3 with bicarbonate. Human serum albumin, cytochalasins B and D, and minimal essential medium were from Sigma Chemical Co., St. Louis, MO. [^{3}H]Uridine (20 Ci/mmol) and [^{3}H]leucine (6 Ci/mmol) were from ICN, Irvine, CA. [^{3}H]AD (5.1 Ci/mmol) was from Schwartz-Mann, Cambridge, MA. [U-^{14}C]Glucose (2.9 mCi/mmol) was from Amersham-Searle, Arlington Heights, IL. CD and CB were dissolved in dimethyl sulfoxide; 400 μg of cytochalasin in 1 ml of dimethyl sulfoxide. These stock solutions were kept frozen. They were thawed and aliquots were diluted in Hanks' basal salt solution (HBSS) for experimental use. Similar dilutions of dimethyl sulfoxide had no effect on the paramenters studied here. All other chemicals were of reagent grade.

Isolation of Nuclei and Determination of NHP

and DNA. The procedures have been described in detail previously (13). Briefly, the frozen cell pellet was dispersed in 4 ml of fluid composed of 10 m*M* Tris-HCl (pH 8) in water, 0.32 *M* sucrose, 20 m*M* $CaCl_2$, and 0.5% Triton X-100. The cells were homogenized 20 strokes in Dounce homogenizers of 7 ml. The nuclei were spun down at 500*g* for 5 min, the supernatant was discarded. This procedure was repeated once, followed by washing the nuclei twice in the same fluid. Previous investigators (6) have compared the protein content of nuclei by the present aqueous and a nonaqueous method and could find no significant difference. The nuclei were extracted twice for 10 min with 1.5 ml of 0.25 *N* HCl, to extract the histones. The remaining pellet, containing the NHP and DNA, was dissolved in 3 ml of 0.2 *M* NaOH. This procedure was carried out at 4°C. Cell pellets of one experiment were processed in parallel using one set of homogenizers (Wheaton Glass) for which tight-fitting pestles had been selected. Aliquots of the samples described above were used to determine protein and DNA in duplicate cultures as described previously (13). NHP levels are expressed per unit of DNA.

Transport of Glucose and Uridine. To study transport of glucose and uridine, procedures described previously (10, 11) were followed: $1.5–2 \times 10^8$ lymphocytes were grown in medium containing 5% human plasma and 25 μg/ml of Con A for 96 hr in a beaker. At the end of incubation, the cells were washed twice in medium without either glucose or serum and containing 1 mg/ml human serum albumin. The cells were resuspended in the same medium to a concentration of 10^6 cells/ml. For the experiments on glucose transport, [U-^{14}C]glucose (110 μg/ml [=2 μCi]) was added to the medium and the cell suspension was distributed in replicate culture tubes (1 ml/tube) and incubated at 37°C. Four micrograms per milliliter of CD or CB dissolved in 0.1 ml of HBSS was added to control cultures. At 30-min intervals during incubation, i.e., after 30, 60, 90, and 120 min, cultures of each cytochalasin-treated and control were spun down, the medium was decanted, and the tubes were immersed in iced water. The cells were resuspended in 10 ml of HBSS at 2°C, spun down at 2°C, and the wash fluid was decanted. This procedure was repeated once. After decanting medium or wash fluid, the tubes were inverted on absorbent paper and the open end of the tubes was wiped dry. The cells were placed in 4 ml of cold 10% trichloroacetic acid for 1 hr, spun down at 2°C for 20 min at 2000 rpm, and the radioactivity was determined in 0.5 ml of the supernatant (acid-soluble cell fraction). A similar procedure was followed to determine cellular uptake of [^{3}H]uridine, except that the cells were suspended in medium containing 2 mg/ml of glucose and 5% human plasma. [^{3}H]Uridine was added in the amount of 2 μCi/ml. After obtaining the acid-soluble cell fraction, the pellet was washed once with 5% trichloroacetic acid dissolved in 1 ml of 0.2 *M* NaOH, and assayed for radioactivity. All cultures were in triplicate.

Results

Shortly after Con A stimulation of lymphocytes, the level of RNA synthesis increases (6, 8) with a parallel increase of protein synthesis. Simultaneous addition of Con A and CD to the cultures inhibited the synthesis of both macromolecules: after 48 hr of incubation, 4 μg/ml CD had blocked the synthesis of RNA and protein almost completely (Fig. 1, A and B). Con A activation caused a marked increase in the cellular uptake of [^{3}H]AD. This increase occurred in two steps which were affected differently by CD: an initial fast increase which was not influenced by CD, followed by a further gradual increase which was largely inhibited by CD (Fig. 1C). Similar results were obtained when the cells were incubated with 4 μg/ml CD for 18 hr prior to Con A stimulation, indicating that the failure of CD to inhibit the initial increased uptake of [^{3}H]AD was not due to a short duration of CD action.

Con A induces nuclear translocation of the NHP. The nuclear NHP increase becomes consistently significant after 8 hr of incubation. Subsequently, the nuclear NHP level rises gradually with time to reach levels 80–100% above control values. This increase is independent of protein synthesis: blocking of protein synthesis with cycloheximide does not prevent this increase (6, 13). CD prevented the lectin-induced increase of the nuclear NHP level after 24 and 48 hr of incubation (Fig. 1D). CD was found to have no effect on the events described above in nonstimulated lymphocytes (data not shown).

Several lysosomotropic agents, besides Con A, have been shown to induce nuclear translocation of NHP in lymphocytes (13, 14). CD was tested for an effect on nuclear translocation caused by NaF and chloroquine. The results showed (Fig. 2) that 5 μg/ml CD inhibited the former by 21%, whereas the latter was suppressed completely.

Hume *et al.* (15) have shown that CB may inhibit lymphocyte transformation by its effect on glucose transport. CD has no effect on hexose transport in certain types of cells (16). To exclude the possibility that the present observations may be due to an effect on transport, the influence of CB and CD on the uptake of [U-^{14}C]glucose and [^{3}H]uridine were compared in lymphocytes that had been incubated with Con A for 96 hr. Cells were incubated for 120 min in a medium containing either [U-^{14}C]glucose of [^{3}H]uridine in the presence of either 4 μg/ml CB or CD and parallel to cultures without cytochalasin. At 30-min intervals, the acid-soluble pool was determined in cultures of cytochalasin-treated cells and controls without drug. The

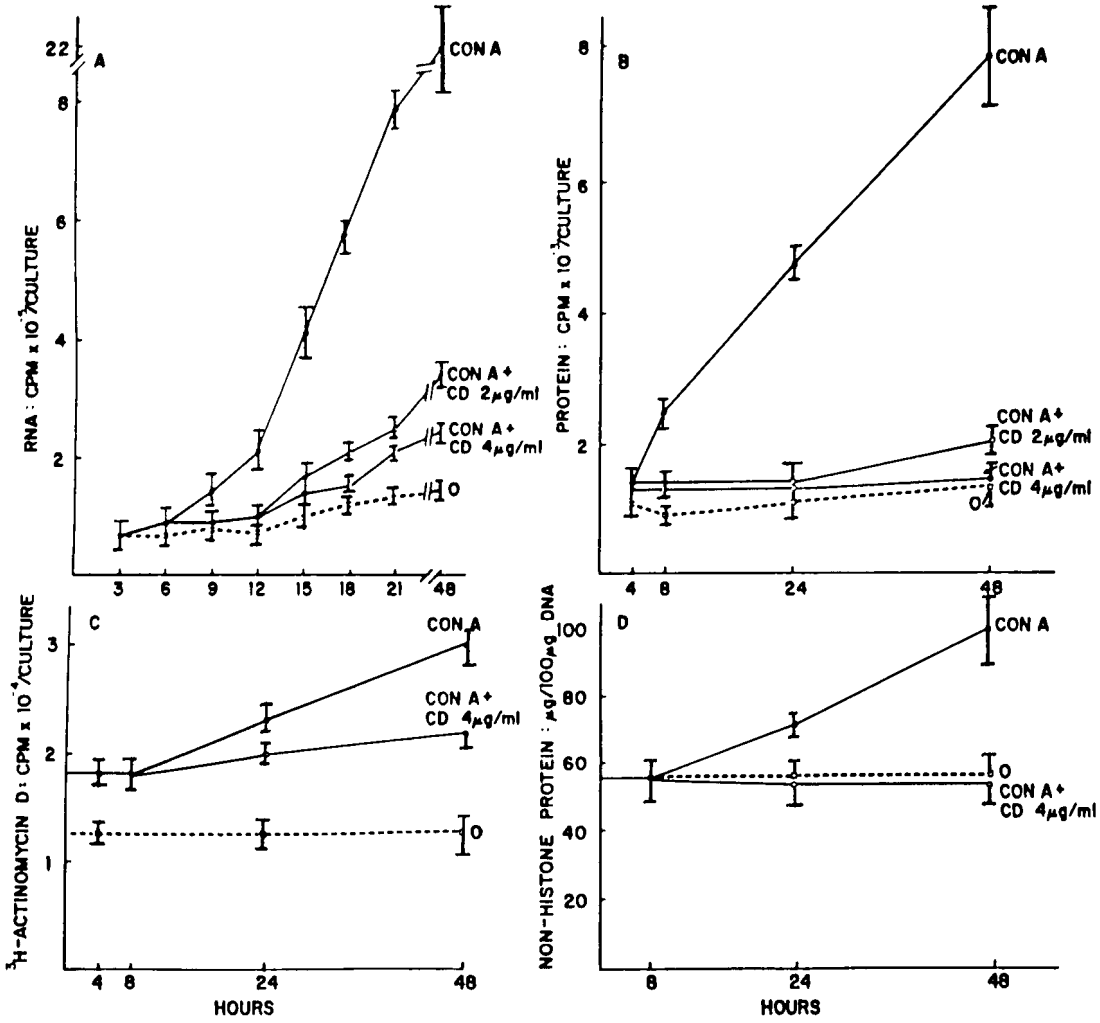

Figure 1. The effect of CD on RNA (A) and protein synthesis (B), the cellular uptake of [^{3}H]AD (C), and the level of NHP (D) in the nucleus of Con A-stimulated lymphocytes. (A and B) Lymphocytes were inoculated in 16- × 125-mm tubes, 2 × 10^6 cells/2 ml of medium containing 5% human plasma. The next day, 25 μg/ml Con A with and without CD were added, parallel to controls without agents. [^{3}H]Uridine or [^{3}H]leucine (2 μCi/ml) was added at 3-hr intervals for [^{3}H]uridine and at 4 hr prior to a determination for [^{3}H]leucine during a 48-hr incubation period. (C) In similarly prepared cultures, 0.1 μg/ml [^{3}H]AD, diluted 3/4 with AD, was added for 4 hr. (D) Purified lymphocytes, incubated overnight in minimal essential medium with 5% human plasma were distributed in 50-ml round bottom glass centrifuge tubes, 20 × 10^6 cells in 10 ml of medium with 25 μg/ml Con A, with and without CD, parallel to controls as shown. All cultures were in duplicate.

results (Table I) showed that CB inhibited the uptake of both [U-^{14}C]glucose and [^{3}H]uridine in the cellular pool about equally at each time interval. On the average, CB reduced the size of the acid-soluble pool of cells labeled with [U-^{14}C]glucose and [^{3}H]uridine by about 54% and 26%, respectively, while CD had no significant effect. The incorporation of [^{3}H]uridine into the acid-insoluble cell fraction (RNA) was reduced to a similar degree. These results clearly showed that the effects of CD on macromolecular synthesis, [^{3}H]AD binding, and nuclear NHP translocation were not due to an interference of CD with cell membrane transport.

Finally, CD does not appear to be toxic to lymphocytes since it was found that unstimulated lymphocytes incubated with 2 μg/ml CD for 48 or 72 hr, followed by removal of CD, responded with DNA synthesis nearly as well as untreated controls when Con A was added to the cultures (data not shown). All of the experiments mentioned above have been repeated at least two additional times, leading to results similar to the ones presented in Figures 1 and 2 and Table I.

Discussion

Stimulation of RNA and protein synthesis occur early in lectin-activated lymphocytes. Increased cellular uptake of [^{3}H]AD and nuclear translocation of NHP are events parallel to stimulated synthesis. All of these events precede DNA synthesis (8). These events appear to be causally related: NHP extracted from chromatin of proliferating cells, when interacting with chromatin of nonproliferating cells, increase template activity (7). NHP alter the structure of chromatin as shown by circular dichroism measurements (17) and increased [^{3}H]AD binding to DNA (8). The increased rate of

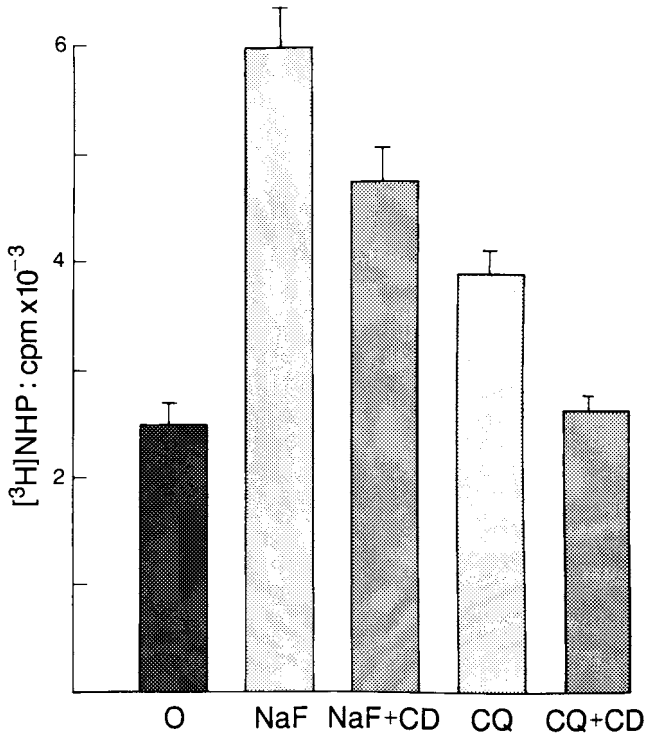

Figure 2. Effect of 5 μg/ml CD on nuclear translocation of [^{3}H]NHP induced by 10 m*M* NaF or 100 μg/ml chloroquine. Lymphocytes were prelabeled with [^{3}H]leucine for 2 hr as described (13), followed by washing and incubation in a label-free medium containing 5% human plasma and 2 m*M* leucine and the addition of agents. Incubation lasted for 4 hr.

RNA synthesis leads to stimulation of protein synthesis. The present results showed that CD inhibited all of these early events in Con A-activated lymphocytes. Although there is no direct proof, the primary of these effects appears to be the inhibition of nuclear NHP translocation, since the other events are mediated by the increased nuclear content of these proteins. NHP have been shown to play a role in gene activation and cellular proliferation (7, 18). Prevention of the nuclear increase of NHP by CD can thus explain the abrogation of mitogenesis by CD, reported previously for CB (1–4). The present results showed that the inhibition of cellular uptake of [^{3}H]AD by CD was incomplete (Fig. 1C). It can be argued that a small, early increase of the nuclear NHP or only a certain species of these proteins, while not measurable, was not affected by CD. Alternatively, part of the increased [^{3}H]AD uptake may not be mediated by NHP.

The results further showed that CD also inhibited nuclear NHP translocation induced by NaF or chloroquine (Fig. 2). The inhibition was marked for the latter but only partial in the case of NaF. NaF is a more potent inducer of translocation than Con A (13) and CD may not act strongly enough to inhibit the action of this strong acting agent.

CD interferes with microfilament activity by inhibiting actin polymerisation (19). However, the mechanism of how microfilaments prevent nuclear translocation of the NHP is obscure. Furthermore, the mechanism of lectin activation of lymphocytes is also incompletely understood. The hypothesis has been proposed previously that lectin binding to a cell surface receptor generates a signal which is transmitted to an intracellular target to initiate mitogenesis and that microfilaments would direct and mediate this transmission (20). This proposal remains to be elucidated. However, neither NaF nor chloroquine bind to cell surface receptors, but traverse the cell surface membrane by diffusion (21, 22). The present results are consistent with the suggestion that microfilaments play a role in

Table I. Effects of CB and CD on the Acid-Soluble Pool Size of Lymphocytes Incubated with either [U-^{14}C] Glucose or [^{3}H]Uridine[a]

Drug	Time interval (min)					%
	30	60	90	120	Average	
	Cellular pools of [U-^{14}C]glucose (cpm/culture)					
0	5,021 ± 120	6,064 ± 112	7,568 ± 768	7,504 ± 32	6,539 ± 258	100 ± 4
CB	2,603 ± 248	2,288 ± 216	3,504 ± 320	3,568 ± 120	2,991 ± 226	46 ± 3.5
CD	5,261 ± 408	6,208 ± 382	7,008 ± 291	7,648 ± 310	6,531 ± 348	100 ± 3.5
	Cellular pools of [^{3}H]uridine (cpm/culture)					
0	96,408 ± 4,760	132,304 ± 3,944	158,464 ± 3,912	152,536 ± 8,848	134,928 ± 5,366	100 ± 4
CB	65,128 ± 2,880	92,184 ± 2,424	117,664 ± 3,312	126,396 ± 824	99,593 ± 2,360	74 ± 2.4
CD	89,504 ± 2,736	124,912 ± 7,024	143,648 ± 4,728	148,645 ± 1,440	126,677 ± 3,982	94 ± 3

[a] Lymphocytes, after incubation for 96 hr with Con A, were washed twice in medium without glucose or serum and containing 1 mg/ml human serum albumin. The cells were resuspended in the same medium to a concentration of 10^6 cells/ml. To determine cellular glucose uptake, [U-^{14}C]glucose, 110 μg/ml (=2 μCi), was added to the medium. The cells were distributed (1 ml/tube) and incubated at 37°C. Four micrograms per milliliter CB or CD were added. At the time intervals shown, three of each control and cytochalasin-treated cultures were spun down, the cells were washed in ice-cold HBSS, and the acid-soluble pool was extracted with cold 10% trichloroacetic acid. A similar procedure was followed to determine the size of the [^{3}H]uridine pool, except that the cells were suspended in medium containing 2 mg/ml glucose, 5% human plasma, and 2 μCi/ml [^{3}H]uridine. For details of the experimental procedure, see Materials and Methods. Data are expressed in average cpm for three cultures with SD and in percentage of the control in the column at the right of the table.

translocation by acting on an intracellular mechanism. It has been proposed that NHP translocation may be the result of inhibited lysosomal degradation of these proteins caused by Con A, NaF, chloroquine (13) or other lysosomotropic agents (14). Such a mechanism remains to be elucidated. The possibility that microfilaments play a role in this mechanism can be considered.

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