Vol. 97, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 16, 1980 Pages 1140-1145

THE EFFECT OF COLCHICINE ON CHOLESTEROL BIOSYNTHESIS IN CONCANAVALIN A-STIMULATED LYMPHOCYTES

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Received November 4,1980

Summary: Attempting to test the hypothesis that colchicine blocks lymphocyte activation at the commitment point, we determined whether or not colchicine inhibited the ConA-induced increase in the synthesis and content of cholesterol in lymphocyte cultures. Colchicine decreased the incorporation of (^{14}C) acetate into cholesterol by about 50%. However, the decrease affected unstimulated and stimulated lymphocytes equally. The presence of colchicine for as long as 48 hours did not prevent stimulated lymphocytes from accumulating cholesterol. The results suggest that an intact microtubular system is not required for the initiation of blastogenesis and the activation of cholesterol synthesis.

Introduction: Colchicine and other antimicrotubular drugs inhibit DNA synthesis in lectin-stimulated lymphocytes (1,2). Where in the cell cycle these antimicrotubular drugs act to block lymphocyte activation is presently unknown. Kinetic studies by Edelman and co-workers (1,2) showed that colchicine blocks lymphocyte activation at or near the point at which lymphocytes become committed to undergo DNA replication. These authors raised the possibility that microtubules play a crucial role in transmitting the signal from the cell surface to the cell interior. However, a controversy exists in the literature about this hypothesis. Rudd, et. al. (3) recently questioned the validity of Edelman's conclusion by showing the time course of colchicine effect. Other investigators have reported that some reactions considered to be early manifestation of blastogenesis were not inhibited by antimicrotubular drugs. Thus, colchicine does not inhibit the increased incorporation of (^{14}C)oleate into phosphatidylcholine (4), the

0006-291X/80/231140-06$01.00/0

increased RNA synthesis (4,5), and the release of lymphokine and osteoclast activating factor (4,6). The growth of cellular and nuclear volume was only moderately affected (7). It appears that colchicine does not inhibit the initiation of blastogenic response. On the other hand, it may be argued that these early events have not been unequivocally shown to be directly linked and necessary for lymphocyte activation to take place. Furthermore, it has been reported that colchicine inhibits phosphatidylinositol turnover (8) and transport of the amino acid analogue, aminoisobutyric acid (9), which are also early events in blastogenesis.

Colchicine causes a marked inhibition of cholesterol synthesis and HMG CoA reductase activity in cultured glial cells (10,11). For successful blastogenesis stimulated lymphocytes must accumulate cholesterol, presumably for generating new cell membranes (12,13). This present study was initiated in an attempt to determine whether colchicine inhibits cholesterol synthesis in ConA-stimulated lymphocytes and whether colchicine blocks lymphocyte activation because of its effect on cholesterol synthesis. We found that colchicine did not inhibit the ConA-induced increase in cholesterol synthesis and content. Our results support the notion that colchicine does not inhibit the initiation of lymphocyte activation.

Materials and Methods: Lymphocyte Cultures. Human peripheral blood lymphocytes were isolated from heparinized venous blood by the Ficoll-Hypaque method (14). Lymphocytes were cultured in complete media (RPMI-1640 supplemented with 2mM glutamine, 0.1 mg/ml gentamicin and 15% calf serum) at 37°C in a humidified atmosphere of 5% CO_2 in air. They were incubated for 24 hours before the addition of ConA. Colchicine solution was added to the culture just before ConA.

Measurement of (^{14}C)acetate Incorporation Into the Sterol Fraction. Lymphocytes were cultured in glass tubes at a cell density of 2 x 10^6 cells/tube in a volume of 1.0 ml. Lymphocytes were pulsed for three hours with 10 µCi of (1-^{14}C) acetate (54 mCi/mM, New England Nuclear). The incorporation of ^{14}C into the sterol fraction was measured as previously described (13).

Measurement of Cellular Cholesterol Contents. Lymphocytes were cultured in a microculture system using flat bottom sterile tissue culture plates, at cell density of 0.4 x 10^6 cells/well in a volume of 0.2 ml. Thirty min. prior to the harvest α-methyl-mannoside was added to cultures to a final concentration of 0.1M, in order to remove ConA and the attached lipoproteins from the surface of lymphocytes. Lymphocytes were harvested

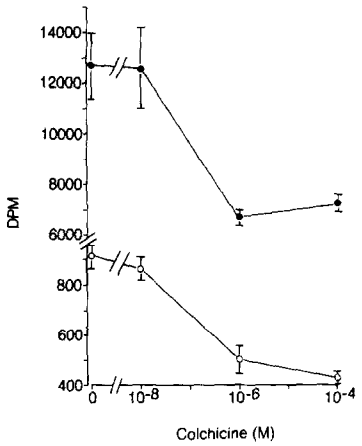

Fig. 1. Effect of colchicine on cholesterol synthesis. Lymphocytes were cultured with colchicine at concentrations indicated on the abscissa, and received 0 (O—O) or 25 (●—●) μg/ml of ConA. Nine hours after the addition of colchicine and ConA, cultures were pulsed for 3 hours with 10 μCi of (^{14}C)acetate. The incorporation of ^{14}C into the sterol fraction was measured. Points and range represent mean ± SEM obtained from triplicate cultures.

with a Multiple Automated Sample Harvester. Cells on the filter paper were washed with 0.3 ml of normal saline for 30 times, and were extracted with 15 ml of chloroform/methanol (2:1 V/V) overnight at room temperature. Lipid extracts were separated into two phases according to Folch procedure (15). The lower phase was washed once with reconstituted pure solvent upper phase (15). The $FeCl_3$-fluorimetric method of Solow and Freeman (16) was used for the determination of cholesterol, except that in the preparation of ferric chloride stock solution 1 gm of anhydrous $FeCl_3$, rather than 0.5 gm of $FeCl_3.6H_2O$, was dissolved in 1 liter of glacial acetic acid. The fluorescence was read 60 min. after the mixing of reagents with a Perkin-Elmer Fluorescence Spectrophotometer (MPF-44B). The excitation wavelength was 528 nm and the emission, 565 nm.

Results: Effect of Colchicine on Cholesterol Synthesis. The incorporation of (^{14}C)acetate into the sterol fraction was measured between 9 and 12 h after ConA stimulation (Fig. 1). At this time ConA increased cholesterol synthesis by about 14-fold. Colchicine at concentrations of 10^{-6} M or above inhibited cholesterol synthesis by about 50%. The degree of inhibition by colchicine in unstimulated cultures was about the same.

Effect of Colchicine on Cellular Cholesterol Contents. The estimate of cholesterol biosynthesis by measuring the incorporation of radioactive precursors is a sensitive assay indispensable for detecting early changes induced by mitogens. However, it may be argued that the reduced incor-

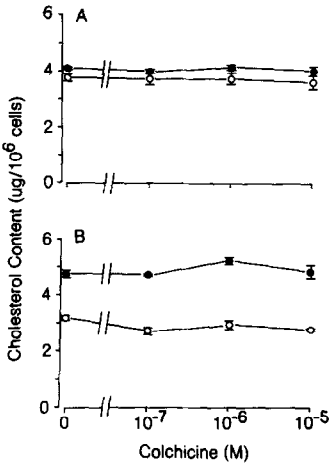

Fig. 2. Effect of colchicine on cellular cholesterol content after 24 hours (upper figure) and 48 hours (lower figure). Lymphocytes were cultured with colchicine at concentrations indicated on the abscissa, and received 0 (O—O) or 25 (●—●) µg/ml of ConA. At 24 h and 48 h parallel sets of cultures were harvested. Cellular cholesterol content per culture was measured. Points and range represent mean ± SEM obtained from triplicate cultures.

poration of radioactivity in the presence of colchicine reflects reduced entry of radioactive precursors into the intracellular pools rather than reduced synthesis. Since the measurement of mass may reflect the activity of synthesis, we also determined the cellular contents of cholesterol. The measurement of cellular cholesterol contents in ConA-stimulated lymphocytes was complicated by the fact that ConA binds both to cell membranes and some serum lipoproteins (17). In order to remove attached lipoproteins from the surface of lymphocytes, we added α-methyl mannoside (0.1M) to lymphocyte cultures 30 min. prior to the harvest. Without this treatment the amount of cholesterol measured in those cultures treated with ConA (25 µg/ml) for only 30 min. was 10% higher than the amount measured in those cultures which received no ConA.

Fig. 2 shows that stimulated cultures had higher cellular cholesterol contents than unstimulated cultures. For as long as 48 hours cellular cholesterol contents in both stimulated and unstimulated cultures were not significantly influenced by colchicine at concentrations as high as

1 x 10^{-5} M. In these experiments parallel cultures were also examined for the effect of colchicine on (^{3}H)thymidine incorporation. In agreement with results reported by others (1,2,4), we found that colchicine at concentrations of 10^{-6} M inhibited DNA synthesis by about 50 to 85% as measured by the incorporation of(^{3}H)thymidine between 48 to 52 h after ConA stimulation (data not shown).

Discussion: The inhibition by colchicine of (^{14}C)acetate incorporation into cholesterol may be due to either reduced entry of (^{14}C) acetate into intracellular pools or reduced cholesterol synthesis. The possibility of a reduced entry of (^{14}C)acetate into lymphocytes seems unlikely, since colchicine at a concentration of 10^{-4} M causes no decrease of (^{14}C)acetate uptake into phosphatidylcholine (4). Furthermore, in cultured C-6 glial cells the decrease induced by colchicine in (^{14}C)-acetate incorporation into cholesterol is accompanied by a decrease in HMG CoA reductase activity (10,11). In vivo studies by Ottery and Goldfarb revealed that colchicine also inhibits hepatic cholesterol synthesis and HMG CoA reductase activity. These observations suggest a role of microtubules in the regulation of HMG CoA reductase and cholesterol synthesis in mammalian cells. However, Fig. 1 shows that the decrease induced by colchicine in cholesterol synthesis affected unstimulated and stimulated lymphocytes equally. This is indicative that colchicine did not inhibit the activation by ConA of cholesterol synthesis.

The 50% inhibition by colchicine of cholesterol synthesis neither led to a cholesterol depletion in resting lymphocytes nor prevented stimulated lymphocytes from accumulating cholesterol (Fig. 2). It is noteworthy that the use of oxygenated sterols to block sterol synthesis in cultured cells will lead to cholesterol depletion only when the cells are grown in the serum-free medium (19,20). When de novo synthesis is decreased by colchicine, lymphocytes probably compensate for the deficit by increasing the uptake of cholesterol from the culture medium. Regardless of the source of

cholesterol supply, the increase in cellular cholesterol content, in spite of the presence of colchicine, is another indication that lymphocytes have been activated.

Synthesis of cholesterol is an essential prerequisite for the onset of DNA synthesis, since treatment of the stimulated lymphocytes with inhibitors of sterol synthesis prevents both the increase in cholesterol synthesis and the increase in DNA synthesis (12,13). Our results suggest that an intact microtubular system is not required for the initiation of blastogenesis and the activation of cholesterol synthesis. The inhibitory effect of colchicine on DNA synthesis probably is at the later stages of blastogenesis.

Acknowledgements: This investigation was supported by a grant from the Veterans Administration.

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