CYCLIC CHANGES IN THE CELL SURFACE

I. Change in Thymidine Transport and Its Inhibition by Cytochalasin B in Chinese Hamster Ovary Cells

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ABSTRACT

Cytochalasin B (CB) shows a marked concentration-dependent inhibition of the incorporation of \[^3H\]thymidine into Chinese hamster ovary cells. This inhibition was shown to result from an inhibition of thymidine uptake, not from an inhibition of DNA synthesis. Cells normally acquire the capacity to transport thymidine as they move from the G1 stage of the cell cycle into the S phase. If CB is added to cells while they are in G1, they do not acquire the ability to transport thymidine as they enter S. However, the addition of CB to cells that are already in S has no effect on their ability to transport thymidine. These results are discussed in terms of a model in which elements involved in thymidine transport enter the cell surface membrane as the cells move from G1 to S. It is proposed that CB prevents this structural transition by binding to the cell surface.

INTRODUCTION

Many lines of evidence suggest that the control of proliferation in mammalian cells somehow involves the cell surface. (See reviews of this literature by Burger, 1971 and Pardee, 1971.) Furthermore, this control appears to be exerted in the G1 stage of the cell cycle, before the initiation of DNA synthesis. (For a review of this literature see Prescott, 1973.) Much of this evidence implicating the surface membrane comes from the study of normal, non-transformed cells which become arrested in G1 when they come into contact with one another in confluent cultures (Stoker, 1964; Todaro, et al. 1965). Normal cells differ from transformed cells, which do not show contact inhibition of growth, in many properties of the cell surface (Burger, 1969; Benjamin and Burger, 1970; Eckhart et al., 1971; Fox et al., 1971). Normal cells can be stimulated to divide by a variety of agents which are thought to react with the cell surface (Burger, 1970; Sefton and Rubin, 1970; Weston and Hendricks, 1972; Sivak, 1972; Vaheri et al., 1972). Other types of cells whose progress through the cell cycle has become temporarily arrested in G1 can also be stimulated to divide by agents which are presumed to interact with the cell surface. For example, Powell and Leon (1970) have shown that human lymphocytes can be stimulated to synthesize DNA and divide by concanavalin A. Greaves and Bauminger (1972) have used phytohemagglutinin and pokeweed mitogen bound to Sepharose beads to stimulate mouse lymphocytes. MacManus and Whitfield (1972) have demonstrated that cyclic AMP
activates thymic lymphocytes without entering the cells and Byron (1973) has found that cholinergic agents are initiators of DNA synthesis in the hemopoietic cell line. If the cell surface plays a role in the initiation of steps leading to DNA synthesis, then one might expect to see changes in the cell surface which coincide with this initiation point. A number of structural changes have been observed, although the relationship between these changes and the initiation of DNA synthesis is not known (Kuhne and Bramson, 1968; Cikes and Friberg, 1971; Pasternak et al., 1971; Scott et al., 1971; Fox et al., 1971; Porter et al., 1973).

The present paper considers surface changes that occur during the cell cycle, specifically during the transition from G1 to S. Changes in the uptake of thymidine (a membrane function) are described. Cytochalasin B (CB), a drug which presumably reacts with the cell surface (see Discussion section), can inhibit these changes if added during G1 but not during other stages of the cell cycle.

MATERIALS AND METHODS

Chinese hamster ovary cells, a hypodiploid, spontaneously transformed cell line, originally isolated by Puck, was obtained by us from D. F. Petersen, Los Alamos Scientific Laboratory, Los Alamos, N. M. Stocks were maintained in monolayer culture. For collecting cells synchronized by the mitotic shake-off technique, cells were seeded in Blake bottles at a density of 2.5 x 10^6 cells/bottle and allowed to grow for 48 h. The mitotic shake-off procedure was modified from that described by Petersen et al. (1968). After six preliminary shakes each of 5-s duration, at intervals of 10 min, cells were collected at 30-min intervals. Cells were collected in 50-ml aliquots of Ham's F-12 medium supplemented with 10% fetal calf serum omitting thymidine. 2-ml aliquots (about 10^6 cells/ml) were pipetted into 35-mm Falcon Plastics tissue culture dishes (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.) containing 25-mm diameter glass cover slips. The mitotic index of these preparations ranged from 95 to 99.5%.

CB was obtained from Imperial Chemical Industries, Ltd. (Cheshire, England). Stock solutions of 1 mg/ml were prepared in dimethyl sulfoxide (DMSO) and stored at 4°C. In all cases DMSO controls were run. DMSO had no effect on the uptake or incorporation of [3H]thymidine.

In early experiments the effect of CB on the incorporation of [3H]thymidine was measured. [3H]thymidine (17.6 Ci/mmol, Schwarz/Mann Div., Becton, Dickinson and Co., Orangeburg, N.Y.) was added to cover slip cultures at 1 μCi/ml for 15 min. The pulse was terminated by washing the cells in balanced salt solution and then fixing in 1:3 acetic acid to ethanol. After fixation cover slips were extracted with 1 N HCl and dehydrated through ethanol. The dried cover slips were then counted in a windowless gas-flow planchet counter. The techniques for measuring incorporation of radioactive precursors into macromolecules in cover slip cultures is described in detail by Everhart et al. (1973).

The uptake of thymidine into acid-soluble pools and acid-insoluble material (DNA) could be differentiated by the following technique, described by Hauschka et al. (1972). After labeling with [3H]thymidine at 5 μCi/ml the cover slips were washed through six changes (100 ml each) of F-12 salt solution and then air dried for determination of total counts. For the determination of acid-insoluble counts the cover slips were washed through three changes of F-12 salt solution, then extracted for 10 min with 0.5 M perchloric acid at 4°C. They were then washed through six changes of salt solution and air dried. Dry cover slips were counted in the gas-flow counter. Acid-soluble counts were calculated by subtracting acid-insoluble counts from total counts.

RESULTS

Effects of CB on Incorporation of [3H]Thymidine

In the initial experiments CB was added at 1 h to cells in G1 which had been synchronized by mitotic selection. The drug was added at different concentrations ranging from 1 to 10 μg/ml and left on the cells for the duration of the experiment. At intervals after adding the drug, cells were pulse labeled with [3H]thymidine and the incorporation of acid-insoluble counts was measured. The results for this experiment are shown in Fig. 1. The control cells begin to incorporate [3H]thymidine at about 4 h after shake-off. The rate of incorporation increases up to 12 h, reflecting the continued entry of cells into S from G1. The CB-treated cells show a dramatic dose-dependent inhibition of incorporation. 1 μg/ml has little effect on the incorporation of [3H]thymidine while 10 μg/ml almost completely inhibits incorporation.

The simplest interpretation of these results is that CB inhibits DNA synthesis by arresting cells in the G1 stage. To see if this were so, synchronized cells were treated for 12 h with CB. The increase in cell number in cultures that were released at 12 h, and the formation of binucleates in cultures that were not released were examined. These results are shown in Fig. 2. In Fig. 2A it is seen that
in continuously treated cultures binucleates first appear around 12 h after shake-off. By 20 h, almost all cells are binucleate. Control populations double their cell number during this same interval. Fig. 2 B shows that cells released after 12 h of inhibition proceed to divide normally, two to three population doublings occurring in the next 42 h. These results indicate that while CB inhibits incorporation of [3H]thymidine, it has no delaying effect on nuclear division. A 12-h treatment with CB caused no delay in cell division in released cells. It therefore seemed unlikely that CB inhibited DNA synthesis, especially since two to three population doublings occurred.

Fig. 3 shows the effect of adding CB at different times in the cell cycle on the incorporation of [3H]thymidine. The rate of incorporation in control cultures increases from a low level at 3 h to a very high rate at 9 h. When CB is added to cells at 1 h, the cells do not incorporate [3H]thymidine as they move into S. This same finding was already demonstrated in Fig. 1. However, if CB is added to cells that are already in S, at 6 h or at 9 h, it does not inhibit incorporation. Thus, whatever the effect of CB on thymidine incorporation, it is dependent upon the stage of the cell cycle at which it is added. If added in G1 it inhibits incorporation, but if added later it has no effect.

The inhibition of incorporation of [3H]Tdr could result from effects on (a) uptake, (b) phosphorylation, and (c) supply of thymidine from other pathways. We initially investigated the uptake of thymidine since this seemed to be the most likely site of action and since we have demonstrated in another paper the sensitivity of this step to inhibition by another compound, dibutyryl cyclic AMP (Hauschka et al., 1972).

**Effects of CB on Thymidine Transport**

The kinetics of total uptake and the incorporation of [3H]thymidine into acid-insoluble material at five different times after mitotic shake-off are shown in Fig. 4 A-E. At 2 h (Fig. 4 A), when all cells are in G1, there is negligible incorporation of acid-insoluble counts. The rate of uptake at this
FIGURE 3. The effect of the addition of CB at different times in the cell cycle on the incorporation of [3H]thymidine. CB was added to replicate cover slip cultures of cells synchronized by mitotic shake-off at a final concentration of 5 µg/ml. In the lower curve (A) the drug was added at 1 h after shake-off. The incorporation of [3H]thymidine into acid-insoluble material was then determined at 3, 6, and 9 h. In the upper curve (B) CB was added at 3 h, 6 h, or 9 h and the rate of incorporation determined after 1 h. Incorporation in controls (○) was determined in the presence of DMSO. In all cases cells were labeled for 10 min with 5 µCi/ml [3H]thymidine (15 Ci/mmol).

FIGURE 4. Uptake and incorporation of [3H]thymidine over the cell cycle. Total uptake of [3H]thymidine (○) and incorporation into acid-insoluble material (●) are shown in A-E. Cells were synchronized by mitotic shake-off and plated in replicate cover slip cultures. At intervals [3H]thymidine was added to a final concentration of 5 µCi/ml and the kinetics of uptake and incorporation determined as described in the text. The difference between the two curves at each time reflects the uptake of acid-soluble [3H]thymidine. The rate of uptake in the initial 10 min was determined for each time after shake-off and is shown in (F).

Leighton P. Everhart and Robert W. Rubin Cytochalasin B and the Cell Surface. I 437
of thymidine uptake by cells which have been in the continued presence of CB is shown in Fig. 5, lower curve (△). The uptake of thymidine by these cells is blocked. On the other hand, the addition of CB to cells at 3, 6, or 9 h has no effect on the uptake measured at these times. Cells treated in G₁ with CB do not acquire the ability to take up thymidine as they enter S the way control cells do. However, when CB is added to cells that have already begun to enter S there is no effect on the rate of uptake.

**Reversibility of CB Inhibition**

Finally we have examined the reversibility of the CB inhibition. CB was added at 1 h to shake-off synchronized cells and was left on for 4, 7, or 11 h. Cells were released by washing twice and then were placed in conditioned medium. Samples were labeled with [³H]thymidine during the inhibition and after release. These results are presented in Fig. 6 A–C. After the 4-h block, cells go through a 3-h lag and then begin to incorporate [³H]thymidine in the same manner as the controls. Cells treated for 7 or 11 h show a similar lag followed by a period of increased incorporation. Regardless of the time of treatment the lag is about 3 h, and the shapes of the curves follow the shapes of the control curves.

**DISCUSSION**

The results are interpreted in the following manner. Cells normally acquire the ability to transport thymidine as they enter S. If CB is added to cells while they are still in G₁, they do not acquire the ability to take up thymidine when they enter S. However, once a cell has entered S and acquired this ability to take up thymidine, CB has no effect. When cells are released from CB inhibition, they reacquire the ability to take up thymidine in the same way that normal cells acquire this ability as they progress from G₁ into S. Increased thymidine transport is normally coupled with cell cycle progress. CB dissociates these two processes.

We have shown that the rate at which cells take up thymidine varies over the cell cycle. Thymidine transport probably occurs by a mechanism which involves several components. One of these is a saturable transport system specific for thymidine. Because the cells have an excess of thymidine
kinase, thymidine at low concentrations is phosphorylated as rapidly as it enters the cells. Thus it is difficult to rule out facilitated diffusion as a mechanism of uptake. Thymidine can also enter cells by simple diffusion, but at the low concentrations used in this study diffusion plays a very small role. Plagemann and Estensen (1972) have studied the effect of CB on thymidine transport and report an inhibition of uptake. They found no inhibition of thymidine kinase and concluded that the effect resulted from an inhibition of the saturable membrane component. On the other hand, it should be pointed out that known variations in thymidine kinase over the cell cycle (Stubblefield and Murphree, 1967) do correspond well with the variations in thymidine transport demonstrated here. However, it is difficult to attribute the inhibition of thymidine uptake by CB to an inhibition of thymidine kinase unless the kinase becomes associated with the membrane as the cell moves through the cell cycle. This association could be responsible for the increased rate of uptake. An in vitro assay of thymidine kinase activity, such as performed by Estensen and Plagemann, may not measure membrane-associated enzyme. If CB binds to the cell membrane, it would not be expected to affect the soluble form.

Additional evidence suggests that CB reacts with the surface membrane of cells. In an accompanying paper (Rubin and Everhart), we show morphological evidence that the primary site of action of CB is at the cell surface. This evidence includes (a) the nature of the response i.e., an alteration of cell surface morphology, (b) the speed of the response, within minutes, and (c) the speed of the recovery, again within minutes.

The idea that CB binds to the cell surface membrane is not a new one. Indeed, in the original paper describing the effect of cytochalasin on cells Carter (1967) suggested that the drug might act by preferential absorption to biological membranes. A similar suggestion was made by Bluemink (1971). Recent findings of many workers confirm the idea that the primary site of action of CB is on cell surface activities. CB inhibits the uptake of glucose, deoxyglucose, and glucosamine in a wide variety of cultured cell types (Cohn et al., 1972; Estensen and Plagemann, 1972; Kletzien and Perdue, 1973; Mizel and Wilson, 1972; and Zigmond and Hirsch, 1972). CB also inhibits phagocytosis (Zigmond and Hirsch, 1972), endocytosis (Wagner et al., 1971), and surface contractility (Bluemink, 1971). It has been reported that radioactively labeled CB preferentially binds to isolated cell membrane fragments (Hauschka, 1973, unpublished data). In short, the evidence that CB interacts with the surface membrane is almost compelling.

The following model of thymidine uptake over the cell cycle and its inhibition by CB is proposed. During mitosis there is a rearrangement of the surface membrane of the cell which leaves the cell in early G1 with little ability to transport thymidine. As the cell moves through G1 and into S, new molecules are inserted into the membrane which are involved in thymidine transport. CB can bind to the surface of cells in G1 and block the insertion of these elements. However, once the molecule is in place CB has no effect on its activity. Thus CB does not inhibit uptake by cells that have entered S and acquired the activity.

One question that is raised is, what is the relationship between the acquisition of thymidine transport activity and initiation of DNA synthesis? Exogenous thymidine is not an essential precursor for DNA synthesis in the CHO cell because the cell can make TMP by other pathways. It is possible that the pattern of incorporation of [3H]thymidine seen in controls does not really correspond to DNA synthesis at all but rather reflects changing ability to transport thymidine. Surely, this is the case in cells released from a CB block where the cells are making DNA at a time when there is little thymidine incorporation. We are in the process of measuring DNA replication by other techniques to compare with patterns of incorporation of thymidine.

Another question is what is the significance of the fluctuation in uptake of nucleosides over the cell cycle? Can this be a control mechanism? While thymidine is not required by CHO cells, changes in its uptake may reflect changes in the uptake of other molecules which are important in regulation. For example, Sander and Pardee (1972) have shown that uptake of amino acids increases as cells move through G1. The importance of essential amino acids in the regulation of cell cycle traverse has already been pointed out (Everhart and Prescott, 1972). It should be noted that cyclic AMP, which is demonstrated to play a role in control of cell growth (Otten et al., 1971), inhibits uptake of small molecules such as thymidine and uridine (Hauschka et al., 1972). Furthermore, there is a good correlation between levels of cyclic AMP over the cell cycle and the rate of uptake of small molecules i.e., cyclic AMP level is higher in early
G₁ when uptake is low (Sheppard and Prescott, 1972; Burger et al., 1972; Zeilig et al., 1972). The possibility that growth is regulated by controlling the uptake of critical molecules is evident (Holley, 1972).

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