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Coordinate control of 3T3 cell proliferation by platelet-derived growth factor and plasma components

(growth control/serum/cell cycle/cell culture)

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ABSTRACT **DNA synthesis and cell division were measured in Swiss mouse 3T3 cells cultured in different concentrations of cell-free plasma-derived serum and increasing amounts of a platelet-derived growth factor. In plasma-derived serum alone, the cells were quiescent and they were arrested in the G_0/G_1 phase of the cell cycle. Addition of a platelet-derived growth factor to quiescent cells maintained in plasma-derived serum stimulated both DNA synthesis and cell division. When plasma components were present at high concentration (5%, vol/vol), the amount of platelet factor added to the cultures determined the number of cell doublings. Plasma-derived molecules were required for the platelet factor to stimulate DNA synthesis and cell division in the maximal number of cells. In addition, plasma components had to be present for recently divided cells to respond to the platelet factor. When 3T3 cells were cultured in excess platelet factor and limiting amounts of plasma-derived serum (0.5%, vol/vol), the cells underwent one doubling and then ceased to proliferate. Addition of fresh plasma-derived serum to these cells induced a second round of cell division. Plasma components and the platelet-derived growth factor acted in a coordinate fashion to regulate the proliferation of Swiss 3T3 cells.**

Culture of most mammalian cells *in vitro* requires the presence of whole blood serum in the culture medium for maintenance of cell viability and for stimulation of cell proliferation (1–3).

Platelets are the source of a potent mitogen present in serum that stimulates the growth of aortic smooth muscle cells, dermal fibroblasts, mouse 3T3 cells, and human glial cells in culture (4–6). The platelet mitogen is a low molecular weight, heat-stable, basic polypeptide (7–10). It is released from the platelets during the process of blood coagulation, when the platelets aggregate. Serum that contains low concentrations of platelet factor can be prepared by centrifuging plasma to remove cellular components prior to coagulation (8). Serum made in this manner does not support the growth of cells that require platelet factor (4, 5, 11–14).

Blood serum contains elements derived from two separate sources: (*i*) molecules present in plasma, and (*ii*) molecules present in platelets that are released during the process of coagulation. Cells maintained in cell-free plasma-derived serum (PDS) remain viable but are unable to grow. They are arrested in the G_0/G_1 phase of the cell cycle (13, 14). Addition of the platelet factor to such cells initiates traversal of the cell cycle. The role of the plasma components in the stimulation of cell proliferation by platelet factor is less clear. Pledger *et al.* (14) have suggested that the platelet-derived growth factor "commits" the cells to undergo a round of cell division and that plasma components allow the "committed" cells to progress through the cell cycle.

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To better define the role of plasma, we measured DNA synthesis and cell multiplication under conditions of excess amounts of the platelet factor and limiting concentrations of cell-free PDS. In this report, we show that plasma-derived molecules were needed for optimal response to the platelet factor. Furthermore, plasma constituents were required for 3T3 cells to undergo multiple rounds of division in the presence of excess amounts of the platelet factor.

MATERIALS AND METHODS

Cells, Media, and Growth Determinations. Swiss mouse 3T3 cells were obtained from Robert Pollack, Department of Microbiology, State University of New York at Stony Brook, NY. The cells were routinely passaged in Dulbecco's modification of Eagle's medium supplemented with 10% calf serum (Gibco), penicillin at 100 units/ml, and streptomycin at 100 μg/ml. Measurements of DNA synthesis and cell number were done as described (15).

Preparation of Cell-Free PDS. Only plasticware or siliconized glassware was used in the preparation of PDS or whole blood serum. Blood (100 ml) was removed by venipuncture from each of four or five adult male monkeys (*Macaca nemestrina*). Both whole blood serum and PDS were made each time blood was drawn. In the preparation of PDS, the blood was collected in prechilled syringes that contained sodium citrate to prevent coagulation [final concentration of sodium citrate, 0.38% (wt/vol)]. The anticoagulated blood was transferred to prechilled plastic centrifuge tubes and spun at 3000 rpm for 18 min at 4° in a Sorvall GSA rotor. The supernatant was respun at 13,500 rpm for 20 min at 4° to remove the platelets. After the second spin, 1.0 M $CaCl_2$ was added to a final concentration of 20 mM, and Ringer's solution was then added in a ratio of 1 vol to 6 vol of plasma. The plasma was then aliquoted into centrifuge tubes and incubated at 37° for 2 hr to allow clot formation. The tubes were spun at 13,500 rpm for 30 min at 4°. The supernatant was dialyzed against 0.1 M Tris·HCl, pH 7.4, at 4° for 24 hr and then applied to a CM Sephadex column (Pharmacia Fine Chemicals). Approximately 8 times the volume of fluid that was applied to the column was collected. The eluate was concentrated back to the original volume in an Amicon concentrator with a PM10 filter (cutoff, molecular weight 10,000). The concentrated PDS was dialyzed against three changes of Ringer's solution over 24 hr. It was then heated to 56° for 30 min, centrifuged at 13,500 rpm for 20 mins, and filtered. The PDS was stored at −70°.

Preparation of Whole Blood Serum. Blood was drawn into syringes containing sodium citrate (final concentration, 0.38%). The anticoagulated blood was transferred to plastic centrifuge

Abbreviation: PDS, plasma-derived serum.

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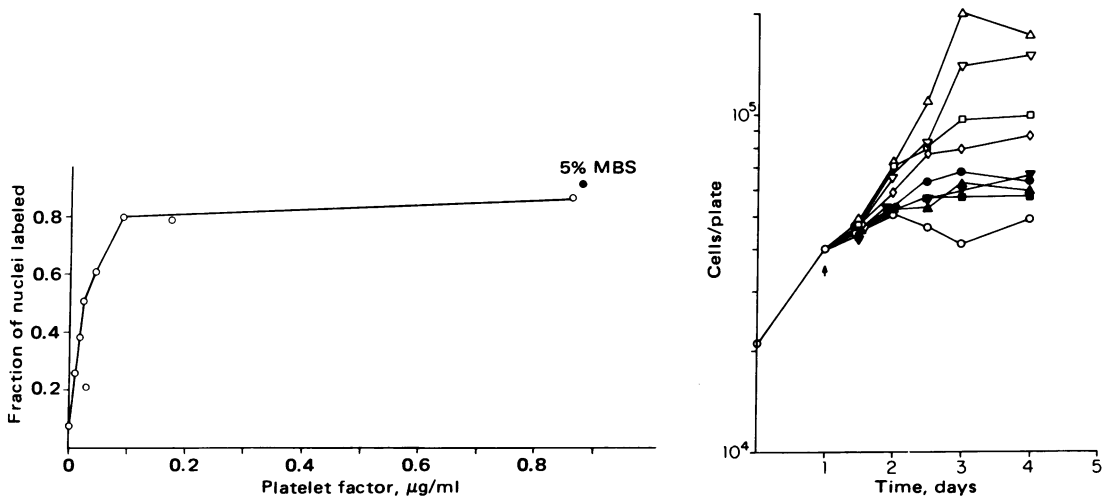

FIG. 1. (*Left*) DNA synthesis in monkey PDS plus increasing doses of partially purified platelet factor. 3T3 cells were plated into dishes containing coverslips in medium containing 5% PDS. After 48 hr, [^{3}H]dThd and different concentrations of platelet factor in 25 μl-aliquots were added directly to each dish. Coverslips were processed for autoradiography 24 hr after addition of the platelet factor. The platelet factor used was material eluted from a Bio-Gel P-100 column (BG-2). MBS, monkey blood serum. (*Right*) Growth of 3T3 cells in 5% PDS and increasing doses of platelet factor. Cells were seeded in 5% PDS into 35 mm culture dishes. Forty-eight hours later, an aliquot of platelet factor (PF) or 5% monkey blood serum was added directly to each dish. ○, 5% PDS; △, 5% monkey blood serum; ▽, PF, 864 ng/ml; □ PF, 173 ng/ml; ◇, PF, 86 ng/ml; ●, PF, 43 ng/ml; ▲, PF, 29 ng/ml; ▼, PF, 22 ng/ml; ■, PF, 17 ng/ml.

tubes and 1.0 M $CaCl_2$ was added to a final concentration of 14 mM. The blood was incubated at 37° for 2 hr and centrifuged at 3000 rpm at 4° for 15 min. The supernatant was respun at 13,500 rpm at 4° for 30 min. It was then heated to 56° for 30 min, centrifuged at 13,500 rpm for 20 min at 4°, and filtered. The serum was stored at −70°.

Preparation of Semipurified Fractions of Platelet-Derived Growth Factor(s). Units of outdated human platelet-rich plasma were frozen and thawed six times and pooled, and the platelet membranes were removed by centrifugation at 27,000 × *g* for 20 min. The membrane pellet was washed with 0.01 M Tris·HCl, pH 7.4/0.39 M NaCl. The supernatant and pellet wash were pooled and defibrinogenated by heating to 55°–57° for 5 min. The precipitated fibrinogen was removed by centrifugation for 30 min at 4230 × *g* at 5°. This pellet was also washed with 0.01 M Tris·HCl, pH 7.4/0.39 M NaCl. The supernatant from the defibrinogenated plasma and the wash from the pellet were pooled. The pH was adjusted to 7.4 by addition of 1.0 M Tris base, and 1.0 M benzamidine was added to a final concentration of 0.02 M.

CM-Sephadex Chromatography. The defibrinogenated platelet-rich plasma was stirred overnight at 5° with CM-Sephadex C-50 that had been equilibrated in 0.01 M Tris·HCl, pH 7.4/0.09 M NaCl/0.02 M benzamidine (50 units of platelet-rich plasma per 500 ml of swollen gel). This slurry was poured into a column containing CM-Sephadex C-50 equilibrated in the same buffer (50 units of platelet-rich plasma per 500 ml of column bed). Stepwise elution included: (*i*) 0.01 M Tris·HCl, pH 7.4/0.09 M NaCl/0.02 M benzamidine; (*ii*) 0.01 M Tris·HCl, pH 7.4/0.19 M NaCl; (*iii*) distilled water; (*iv*) 0.1 M $(NH_4)_2CO_3$, pH 8.9. The growth-promoting activity was eluted with 0.1 M $(NH_4)_2CO_3$, pH 8.9. This fraction was lyophilized and stored at −20°. It is designated CMS III.

Ammonium Sulfate Precipitation and Bio-Gel Chromatography. Fraction CMS III was dissolved in H_2O and then precipitated with 95% ammonium sulfate (wt/vol). The precipitate was dissolved in 1.0 M acetic acid at a concentration of 1.0 mg of protein per ml and was loaded on a column of Bio-Gel P-100 equilibrated in 1.0 M acetic acid (Bio-Gel P-100, 100–200 mesh sieved dry to between 104 μm and 53 μm). The growth-promoting activity eluted in a molecular weight range of 10,000–40,000 as determined with standards. This fraction was pooled and lyophilized. It was 20,000-fold purified from the starting material (outdated platelet-rich plasma). It is designated BG-2. The lyophilized material was dissolved in phosphate-buffered saline for use in cell-growth experiments.

RESULTS

Stimulation of DNA Synthesis and Cell Proliferation by Platelet Factor. Fig. 1 shows the fraction of cells synthesizing DNA and the change in cell number when 3T3 cells were grown in medium containing 5% PDS supplemented with different concentrations of partially purified platelet factor. In the presence of PDS alone, the cells were quiescent. Only 10–20% of the cells synthesized DNA during a 24- or 48-hr period (Fig. 1 *left* and Fig. 2*F*). Addition of platelet factor to such quiescent cells stimulated both DNA synthesis and cell division (Fig. 1 *left* and *right*). Over a dose range of 10–50 ng of platelet factor per ml of culture medium, the number of cells that synthesized DNA was directly proportional to the amount of platelet factor added. Maximal stimulation of DNA synthesis occurred at a platelet factor concentration of 86 ng of protein per ml. The increase in DNA synthesis stimulated by this dose was equivalent to that induced by 5% whole blood serum.

The stimulation of cell proliferation by the platelet-derived factor was also dose dependent, because the final number of cells per dish depended upon the amount of platelet factor added (Fig. 1 *right*). Only a slight increase in cell number was observed in the range of 10–50 ng of protein per ml. The cell number underwent a 2-fold increase at a concentration of 86 ng of platelet factor per ml, the dose required for optimal DNA synthesis. Thus the cells underwent one doubling when sufficient platelet factor was added to induce almost all the cells in the culture to synthesize DNA. Increasing the concentration of platelet factor above 86 ng/ml resulted in a further increase in final cell number. The highest dose tested (864 ng/ml) was almost as effective as 5% whole blood serum in stimulating cell

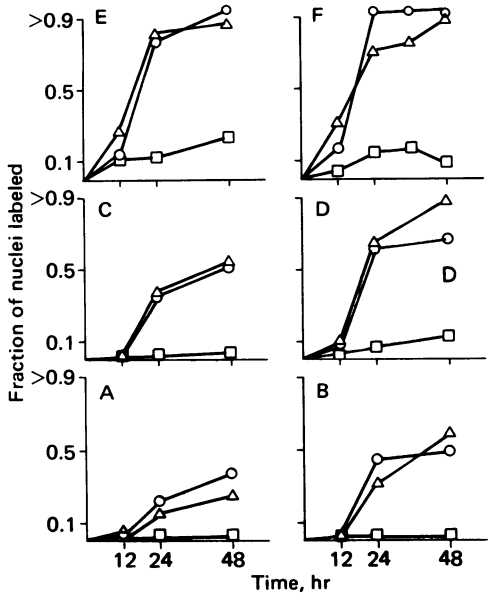

FIG. 2. DNA synthesis in excess platelet factor and decreasing concentrations of PDS. 3T3 cells were plated in medium containing various concentrations of PDS. After 48 hr, platelet factor at a concentration of 864 ng of protein per ml or 5% monkey blood serum was added directly to each dish. [^{3}H]dThd was added at the same time as platelet factor or blood serum. Coverslips were processed for autoradiography 12, 24, and 48 hr after addition of the platelet factor. BG-2 was the source of platelet factor. □, No addition; ○, 5% monkey blood serum; △, platelet factor. (*A*) Serum-free medium; (*B*) 0.02% PDS; (*C*) 0.1% PDS; (*D*) 0.5% PDS; (*E*) 1% PDS; (*F*) 5% PDS.

proliferation. Therefore, in the presence of 5% PDS it was possible to increase the number of cell doublings by adding platelet factor.

Effect of PDS on Stimulation of DNA Synthesis by Platelet Factor. To understand the role of plasma-derived molecules in the stimulation of DNA synthesis by platelet factor, we measured the fraction of cells synthesizing DNA in the presence of decreasing concentrations of PDS and platelet factor concentrations in excess of that necessary for maximal stimulation of DNA synthesis (864 ng of protein per ml). We found that PDS affected the ability of the cells to respond to platelet factor. In the absence of PDS, or in very low concentrations of PDS (0.02–0.1%), 20–50% of the cells synthesized DNA in a 48-hr period (Fig. 2 *A–C*). Recruitment of more than 90% of the cells into DNA synthesis in this experiment required the presence of PDS at concentrations of 0.5% or greater (Fig. 2 *D–F*). Thus, minimal concentrations of plasma-derived molecules appear to be necessary to permit the platelet factor to recruit the maximal number of cells into DNA synthesis.

The requirement of plasma components for optimal stimulation of DNA synthesis by the platelet factor is also demonstrated in dose-response experiments in which increasing concentrations of platelet factor were tested in different concentrations of PDS (Fig. 3). In these experiments the effects of PDS were more pronounced in that fewer cells were labeled at 24 hr in 0.1%, 0.5%, and 1% PDS than in the previous study. For a given amount of platelet factor, increasing the concentration of PDS increased the number of cells that synthesized DNA. The restriction on labeling index imposed by low concentrations of PDS could not be overcome by increasing the concentration of platelet factor. A 5-fold increase in platelet factor concentration only slightly increased the fraction of labeled cells in either 0.1% or 0.5% PDS. The data presented in Figs. 2 and 3 suggest that maximal stimulation of DNA synthesis by platelet factor requires the presence of at least some plasma components.

Effects of PDS on Cell Proliferation. In the previous experiments we examined the effects of PDS only upon stimulation of DNA synthesis by platelet factor. It is equally important to determine how plasma components affect the increase in cell number caused by the platelet factor. To do this, we measured cell numbers in cultures maintained in platelet factor at high (excess) concentration (864 ng of protein per ml) and different concentrations of PDS. As expected from the data on DNA synthesis, we found that PDS is required for optimal cell proliferation (Fig. 4). In the absence of PDS or in 0.1% PDS, a 50–70% increase in cell number correlates well with the fraction of cells that synthesized DNA in these concentrations of PDS (Fig. 2 *A* and *B*). In 0.5% PDS, the cells underwent one doubling and ceased dividing (Figs. 4 and 5). A PDS concentration of 1% or greater was needed for cells to undergo more than a single round of division. Thus, in the presence of excess platelet factor, 3T3 cells required PDS to undergo multiple rounds of division. Restricting the concentration of PDS to 0.5% or less resulted in only a single round of division, despite the presence of excess amounts of platelet factor.

To show that this limitation of proliferation is truly the result of restriction of plasma components, we asked the following two questions:

1. Does addition of excess amounts of fresh PDS to cells that

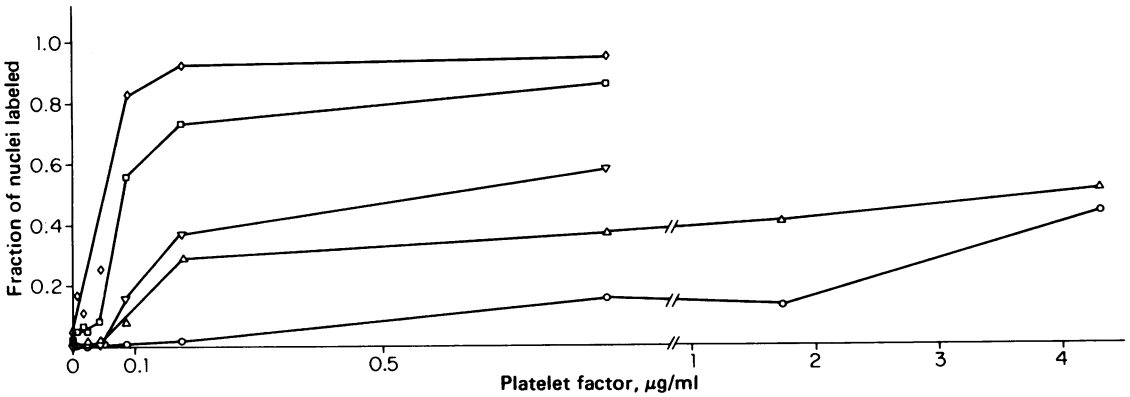

FIG. 3. DNA synthesis in different concentrations of PDS and increasing concentrations of platelet factor. 3T3 cells were plated in different concentrations of PDS. After 48 hr, aliquots of platelet factor were added directly to each plate. Coverslips were processed for autoradiography 24 hr after addition of the platelet factor. BG-2 was used as the source of platelet factor. ○, 0.1% PDS; △, 0.5% PDS; ▽, 1.0% PDS; □, 2.5% PDS; ◇, 5.0% PDS.

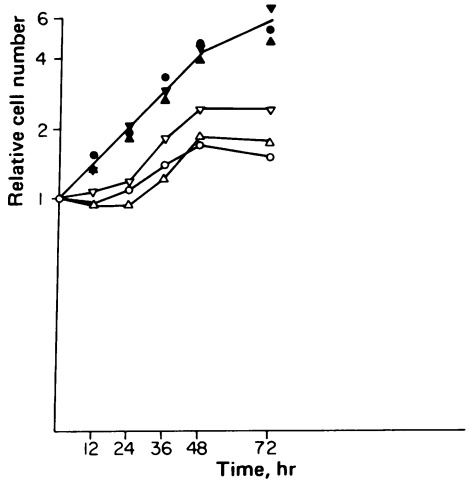

FIG. 4. 3T3 cell growth in excess platelet factor and decreasing concentrations of PDS. 3T3 cells were incubated for 48 hr in medium containing different concentrations of PDS. The medium was then removed and medium containing platelet factor (PF) (864 ng of protein per ml) and the same decreasing concentrations of PDS was added. Cells from two plates per group were removed with trypsin and counted at the indicated times. ○, Serum-free medium + PF; △, 0.1% PDS + PF; ▽, 0.5% PDS + PF; ●, 1.0% PDS + PF; ▲, 2.5% PDS + PF; ▼, 5.0% PDS + PF.

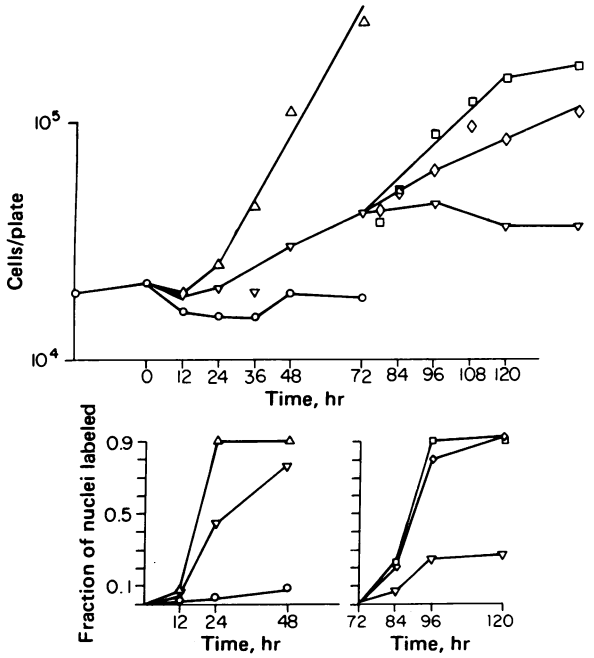

FIG. 5. Stimulation of DNA synthesis and cell growth by PDS in 3T3 cells that have undergone one round of division in 0.5% PDS and excess platelet factor. 3T3 cells were plated in 0.5% PDS. After 48 hr (0 time), the medium was removed and fresh medium containing either 0.5% PDS plus 5.0% monkey blood serum or 0.5% PDS plus platelet factor (864 ng of protein per ml) was added. Cells were counted at various times after the additions. At 72 hr, 5% fresh monkey blood serum or 5% fresh monkey PDS was added directly to plates that had received 0.5% PDS and platelet factor at time 0. Cells were counted at the indicated times. The panels under the growth curve show the fraction of cells synthesizing DNA at 12, 24, and 48 hr after the additions either at 0 time (*Lower Left*) or at 72 hr (*Right*). ○, 0.5% PDS alone; △, 0.5% PDS + 5% monkey blood serum; ▽, 0.5% PDS + platelet factor; ◇, 0.5% PDS + platelet factor at 0 time + 5% PDS at 72 hr; □, 0.5% PDS + platelet factor at 0 time + 5% monkey blood serum at 72 hr.

have already undergone one round of division in the presence of 0.5% PDS and platelet factor at 864 ng/ml induce a second round of division?

2. Does increasing the concentration of platelet factor overcome the growth restriction imposed by a low concentration of PDS?

In the first experiment, 3T3 cells were plated in 0.5% PDS, and excess platelet factor (864 ng/ml) was added 48 hr after plating. As seen in previous experiments (Figs. 2 and 4), the platelet factor stimulated approximately 80% of the cells to synthesize DNA in the following 48-hr period (Fig. 5, *lower left*) and caused a single round of division to occur within 72 hr (Fig. 5, *upper*). Following this single round of division, the cells became quiescent in that only 25% of them synthesized DNA in a 48-hr period (Fig. 5, *lower right*). Addition of fresh 5% PDS to these quiescent cells stimulated both DNA synthesis (Fig. 5, *lower right*) and cell division (Fig. 5, upper).

In a separate series of experiments, very high doses of platelet factor were not able to overcome the restriction on proliferation imposed by 0.5% PDS (Fig. 6). In 0.5% PDS, the cells still underwent a single round of division even in the presence of a 10-fold increase in platelet factor over that used in previous experiments (8.64 μg of protein per ml). The data presented in Figs. 5 and 6 demonstrate that the concentration of plasma components can limit cell proliferation.

DISCUSSION

Plasma and Platelet Factor—Coordinate Control of Cell Growth. 3T3 cells require both platelet factor and plasma constituents to multiply *in vitro*. These two serum components act coordinately to stimulate cell proliferation, and the amount of each present in the growth medium controls the amount of proliferation that occurs. 3T3 cells maintained in medium supplemented only with PDS are quiescent. Platelet factor acts to recruit these quiescent cells to traverse the cell cycle. In the presence of optimal concentrations of PDS, the amount of platelet factor present in the growth medium determines the number of cell doublings that occur (Fig. 1). Optimal amounts of PDS with limiting concentrations of platelet factor permit only a fraction of the cells to synthesize DNA and divide.

3T3 cells require plasma constituents to respond fully to the platelet factor since plasma components must be present for the platelet factor to stimulate the maximum number of cells to synthesize DNA (Figs. 2 and 3). Plasma constituents are also necessary for the cells to undergo multiple rounds of division when high concentrations of platelet factor are present in the medium (Figs. 4 and 5). In the presence of high concentration of platelet factor and limiting concentrations of PDS, some or all of the cells in the culture undergo one round of division and then cease dividing (Figs. 4 and 5).

Plasma Regulates the Number of Cell Divisions. This latter result is of interest because it suggests that cells that have undergone one round of division are unable to respond a second time to the platelet factor in the absence of sufficient amounts of plasma constituents. Thus, one of the functions of plasma molecules is to render recently divided cells capable of responding to the platelet factor. The mechanism by which plasma components accomplish this function is unknown. Plasma may directly supply a molecule, or molecules, required by the post-mitotic cells, or it may induce the cells to synthesize required molecules. The molecules in plasma that are required for cell growth are also unknown.

This report confirms and extends the results of Pledger *et al.* (14). We show that plasma molecules must be present for

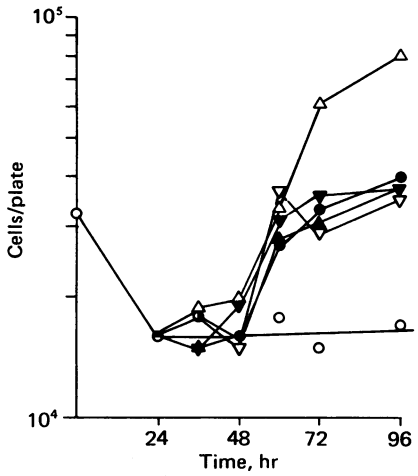

FIG. 6. Growth of 3T3 cells in 0.5% PDS and increasing concentrations of platelet factor. 3T3 cells were plated in 0.5% PDS. After 48 hr, the medium was removed and fresh medium containing 0.5% PDS plus different concentrations of platelet factor was added. ○, 0.5% PDS; △, 0.5% PDS + 5% monkey blood serum; ▽, 0.5% PDS + 864 ng/ml; ●, 0.5% PDS + 1728 ng/ml; ▲, 0.5% PDS + 4320 ng/ml; ▼, 0.5% PDS + 8640 ng/ml.

platelet factor to stimulate DNA synthesis in the maximum number of cells. In addition, we present data that suggest that plasma regulates the number of rounds of division that 3T3 cells undergo in culture. The regulation of proliferation by plasma is reflected in the requirement by recently divided cells for plasma components in order to respond to the platelet factor. Pledger *et al.* (14) conclude that platelet factor "commits" cells to a proliferative response and plasma components allow these "committed" cells to progress through G_1 into S. The function of plasma molecules in the stimulation of proliferation of recently divided cells that we describe here may be the one suggested by Pledger *et al.*—that is, to affect the progression of platelet-factor "committed" cells through the cell cycle. However, because our experiments are designed differently, further experiments are required to confirm their interpretation.

The experimental protocol described in Fig. 5 can be employed to study the molecules in plasma responsible for optimal growth of 3T3 cells. One could allow the cells to undergo one round of division in the presence of high concentrations of platelet factor and limiting concentrations of PDS and then add different plasma components such as purified lipoproteins or hormones, either singly or in combination to determine if these additions initiate a second round of cell proliferation. Ultimately it may be possible to identify all of the molecules in plasma required by 3T3 cells, permitting culture of these cells in a totally defined medium.

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