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REGULATION OF RAT MEGAKARYOCYTE DEVELOPMENT IN PLASMA CLOT CULTURE SYSTEM

City University of New York PH.D. 1985

REGULATION OF RAT MEGAKARYOCYTE DEVELOPMENT IN PLASMA CLOT CULTURE SYSTEM

by

RUHI UYAR

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INTRODUCTION

Each type of blood cell is replenished from hematopoietic stem cells located in the red bone marrow and the spleen. This pluripotential stem cell compartment remains stable in size under homeostatic conditions. When the specific stimulator is provided, the stem cell will become committed to megakaryocytopoiesis, but does not immediately differentiate further or enter into active proliferation (1). The committed procursor cells (colony forming unit-megakaryocytes:CFU-M) are low in number, about $1/10^4$ bone marrow cells, and slow cycling, 7-20% are in DNA synthesis. Thereafter, the CFU-M appear to undergo cellular proliferation and initiate nuclear replication without cell division before becoming recognizable as megakaryocytes. Therefore, megakaryocytes are polyploid, and, in the normal steady state they have 4, 8, and 16 times the normal diploid amount of DNA (2). The size of megakaryocytes is proportional to ploidy, but the relationship of size and ploidy of a megakaryocyte to the number and the size of platelets it produces is not clear (3). Each cell normally produces a few thousand enucleate platelets by precisely controlled cytoplasmic fragmentations (4).

A major reason for studying megakaryocytopoiesis is to understand and to quantitate platelet production.

However, the nature of these mechanisms and their concequences are poorly understood. The megakaryocytes are of interest to cell biologists and physiologists because of these unusual mechanisms, and to experimental pathologists and hematologists for their perspective on platelet contributions to hemorrhagic and thrombotic diseases. Futhermore, there is increasing evidence of the importance of blood platelets in the pathogenesis of atherosclerosis. The participation of platelets in immune processes and in the spread of cancer is also receiving attention. Finally, a lively and productive interest in platelet pharmacology has developed, in the hope of conceiving and developing new drugs for the control of thrombosis and atherosclerosis.

In vivo studies of megakaryocytopoiesis in humans have been limited because of ethical questions related to a lack of suitable subjects for experimentations, and the limited nature of experiments that could be done on these subjects. The development of in vitro culture systems provided an alternative method for characterizing regulatory activities and analyzing the role of these activities in the proliferation of megakaryocytes. In the last nine years, both agar (5,6,7,8) and plasma clot (9,10,11,12) culture systems have been applied to the study of the biology of megakaryocytes. The application of in vitro culture systems to assay for CFU-M from mouse (10) and human (11) bone marrow have renewed

interest in the cellular and humoral mechanisms that regulate megakaryocytopoiesis and thrombopoiesis. These cloning assays have also allowed megakaryocytes to be isolated (13) and their precursor cells to be studied and monitored.

Although the application of semi-solid culture techniques to the study of megakaryocytes is not relatively new, culture conditions must still be further defined. This is especially in light of stimulation of megakaryocytic precursor cells by prerequisite conditioned media, the average small colony size, and the presence of numerous single megakaryocytes among the colonies (14). It still remains to be determined whether these properties reflect the limited proliferative capacity of megakaryocyte progenitors in vivo, or are caused by less than optimal culture conditions. However, a comparison of the different techniques (Table 1) used to culture CFU-M clearly presented numerous, potentially important differences (20). These differences include the use of different culture media, different conditioned media or other stimulators, and different sources and concentrations of serum. Some of the uncertainities may be due to the uncharacterized components used and the complexity of current culture systems. The first part of this project approached this question of defining culture conditions by

developing a simpler plasma clot culture system. A different species, namely the rat, Ratus norvegicus, was used as a donor of bone marrow cells, spleen cells conditioned medium, citrated plasma and serum.

Changes in marrow megakaryocytes which occur with experimentally induced thrombocytosis indicate that platelet production is subject to negative feedback regulation (21, 22, 23). Recent studies have shown that there are at least two levels of regulation of megakaryocytopoiesis and platelet production (24, 25, 26). At the first level, a compartment of precursor cells was shown to proliferate in response to cell derived products promoted by mitogen stimulation, recognition of decreased marrow megakaryocytes, and/or modulation of the concentration of cholinergic agonists (27). At the second level, however, ploidy amplification in the differentiated megakaryocyte compartment may be promoted by acute changes in platelet demand. In spite of some uncertainties, it is clear that a deficiency of circulating platelets is associated with the stimulation of megakaryocytopoiesis (28) and an excess with its suppression (29). There is some suggestive evidence which supports the idea that ploidy amplification is mediated by the hormone thrombopoietin (30, 31). Thus, separate factors independently influence proliferation and maturation events, but it is not known whether only

a single factor is involved at each level. Therefore, it is possible that platelets themselves and/or their products may well be involved in controlling production and release of platelets from megakaryocytes (32, 33).

A number of investigators have shown that extracts of erythrocytes, granulocytes and lymphocytes have some specific effects on the maturation of these cells (34,35, 36). These extracts have frequently been called "chalones" and their specificity of action for cells of their own kind has been demonstrated. The extracts of these cell types do not appear to affect the expansion and differenciation of their respective progenitor cells, rather these components modulate the late events of the cell division and maturation stages. Similarly, platelet extracts might influence the terminal stages of megakaryocyte maturation, including endomitosis, cell size, and cytoplasmic maturation. In support of this hypothesis, platelet extracts did not appear to affect the number of CFU-M in vivo, or their growth in vitro (37).

Human platelets contain a growth promoting factor called platelet-derived growth factor (PDGF) that stimulates the growth of smooth muscle cells, glial cells and fibroblasts as well as avian, monkey and murine fibroblastic cell lines (38). PDGF is a cationic polypeptide that is synthetized by megakaryocytes and stored in the

alpha granules of platelets (39). It has been purified and characterized as a 28,000-30,000 dalton polypeptide that is heat stable (40). Although PDGF has been extensively studied in investigations concerning inflammatory and repair processes at the site of blood vessel injury, PDGF might also play an important role in the genesis of atherosclerosis. It is a major mitogenic protein in the serum for cells of mesenchymal origin (41).

In the second part of this project, the effects of platelet extract and human PDGF on rat bone marrow cells in plasma clot cultures were investigated. Changes in the numbers of AChE+ cells, the mean diameters of these cells, and white blood cells (WBC) colony numbers were studied. Different concentrations of platelet extracts increased the detectability and the mean diameters of AChE+ cells, but decreased the number of WBC colonies in one series of experiments. PDGF also caused an increase in the cell number, but it had no effect on the cell size.

MATERIALS and METHODS

Bone marrow cells, spleen cells, plasma, serum and platelets were obtained from untreated Wistar rats. Bone marrow was flushed with Leibovitz medium (L-15, from Gibco, Grand Island, N.Y.) into plastic tubes from both femurs of 3-4 month-old female rats. Nucleated bone marrow cells were counted by a Coulter Counter. Blood was drawn into plastic syringes containing 3.8% (w:v) sodium citrate (1:9 citrate:whole blood) by cardiac puncture from 8-12 month-old male rats. The blood was centrifuged at 4 ^{o}C at 1100 x g for 15 min in an angle-head centrifuge to yield plasma. The same procedure was followed for collecting serum except that the anticoagulant was not used, and the blood was allowed to clot in the refrigerator. Both plasma and serum were filtered through 0.45 μm pore size Millipore filters after collection and stored at -70 ^{o}C until use.

A modification of the method of Goldberg et al (42) was used for the collection of platelets from similar rats that were lightly anastethized with ether. Platelet-rich plasma was obtained by centrifugation of the citrated blood at 500 x g for 4-5 min at room temperature. The remaining red blood cells (RBC) were resuspended in sterile isotonic saline to a total volume of 15 ml, and were

centrifuged at 500 x g for 3-4 min. This platelet suspension in saline was combined with the platelet-rich plasma and was sedimented at 1240 x g for 10 min. This yielded a platelet pellet containing visible RBC in the center of the pellet. The supernatant solution were decanted with a polypropylene plastic Pastur pipet and the platelets were removed from the tubes, leaving most of the RBC behind. The platelets were resuspended in saline in fresh plastic tubes. The platelet suspensions were centrifuged at 2200 x g for 12 min, the platelets were decanted as before, and resuspended in saline in order to remove the remaining RBC. The latter step was repeated four more times. The final platelet pellet, without visible RBC, was resuspended in sterile saline. Platelet numbers were determined by the use of both the Coulter Counter and the phase contrast microscope. The total number of platelets was determined from the platelet count and the volume of the final suspension. This suspension was then stored overnight at -20 ^{o}C. The platelets were disrupted by four cycles of freezing at -70 ^{o}C for 45 min, followed by thawing at 37 ^{o}C (43). The thawed suspension was finally sedimented at 3500 x g for 30 min, and the supernatant fraction was used as platelet extract. Calculated amounts of PE in saline were added to the cultures at the begining of each incubation.

Pokeweed mitogen (Gibco)-stimulated rat spleen cells conditioned medium (RSCCM) was prepared by the method of Nakeff (14). The spleens of 2-4-month-old female rats were minced in a culture dish and homogenized in a plastic test tube with a 10 ml plastic syringe and a 22 G, 3.0 cm needle. Nucleated cells were counted and cultured in 35 mm culture dishes for two days. Each dish contained the following ingredients: (a) 60% L-15; (b) 10% bovine embryo extract diluted 1:4 (v/v) with L-15; (c) 10% rat serum; (d) 10% pokeweed mitogen (1/300 final dilution); (e) 10% rat spleen cells (2 x 10^6/ml) in L-15. The medium was collected and filtered through 0.22 µm Millipore filters. Each conditioned media was tested for potency before its final use and then stored at -70 °C for no longer than two months.

Receptor grade PDGF (Seragen, Inc., Boston, MA) isolated in pure form (95%) from human platelets was disolved in 1.0 M acetic acid. Sterile filtered tissue culture grade sodium bicarbonate (Gibco) was added to all the cultures in order to readjust the pH to 7.4 following the addition of acetic acid.

Ten percent citrated rat plasma was used in this study instead of the commonly used 10% citrated bovine plasma. These two plasmas were not compared experimentally.

A modification of a previously described method (9) was used to culture the bone marrow cells in 96-well round bottom plastic plates. Each 0.3 ml plasma clot culture contained the following percentages (v/v) of these ingredients: (1) 40% L-15; (2) 10% RSCCM; (3) 10% bovine embryo extract diluted 1:4 with L-15; (4) 10% rat bone marrow cells (10^6/ml in L-15); (5) Either 20% horse serum (Gibco), 10%, 15% or 20% rat serum, or 10%, 15% or 20% rat plasma; (6) 5% or 10% saline when 15% or 10% plasma or serum, respectively, were used, or either 10% PE or 10% PDGF; (7) 10% rat plasma (in addition to the 10% to 20% plasma or serum).

The bone marrow cells were cultured for one hour to 9 days in an incubator at 37 °C in an atmosphere of 6% CO_2 in air and 97% relative humidity. At daily intervals, plasma clots were rimmed and transferred to glass slides. The clots were partially dehydrated by placing pieces of filter papers on their surfaces. A second piece of filter paper was applied to the first filter paper and allowed to remain long enough for this paper to become moist. With the aid of forceps, the top piece of filter paper was removed and 3 drops of 5% glutaraldehyde (in 0.01 M phosphate buffer, pH 7.0) were placed on the remaining filter paper with a pipet. The first paper with glutaraldehyde was left in place for 10 min and

then gently pressed with a thumb. After removing the remaining filter paper, the slides with fixed plasma clots were rinsed in 0.1 M sodium phosphate buffer, pH 6.0 for one min and air dried with a blower. The fixed and dried clots were stained by the use of the "direct-coloring" thiocholine method for acetylcholinesterase activity (44). For staining, the clots were incubated for 3-4 hours at room temperature in a solution containing: (a) 600 mg of acetylthiocholine iodide, (b) 90 ml of 0.1 M sodium phosphate, pH 6.0, (c) 6 ml of 0.1 M sodium citrate, (d) 12 ml of 30 mM copper sulphate, (e) 12 ml of distilled water, and (f) 12 ml of 5 mM potasium ferricyanide. Following a one min rinse in 0.1 M sodium phosphate, postfixation in absolute methanol for 10 min, and 50% methanol for 30 sec, the cells were counterstained in Harris' hematoxyline for 4-6 min, and then rinsed twice in tap water. The slides were then blued by dipping in a 2% ammonium hydroxide solution, clarified in tap water and mounted.

It was recently hypothesized that the total number of megakaryocytic cells in plasma clots could be used to estimate the number of megakaryocyte colonies, and was a good index of the rate of megakaryocytopoiesis (15). Therefore, the number of AChE+ cells, rather than AChE+ cell colonies, was scored in some of these experiments. Otherwise, an AChE+ cell colony was defined as three or more cells in close proximity. Aggregates of

16 or more cells with horseshoe- or doughnut-shaped nuclei were considered to be a WBC colony.

The final preparations were examined under the light microscope at magnifications of 200X, 300X and 430X to determine the number of AChE+ cells, the number of AChE+ cell colonies and the number of WBC colonies. The measurement of AChE+ cell size was done by use of an occular micrometer at magnifications of 300X and 430X. Two areas that were each the width of one microscope field in the middle of each clot were examined from the top to the bottom of the slide. All the megakaryocytic cells in these areas were measured. The average of the lengthwise diameter and the crosswise diameter was scored.

The Student t test was used for statistical significance.

RESULTS

In each experiment that produced conditioned medium, two sets of spleen cells were incubated with pokeweed mitogen at the same time under identical culture conditions. The conditioned media were then immediately tested for their ability to promote mitotic activity of rat bone marrow cells. Cultures treated with some preparations of the conditioned medium ($RSCCM_3$) showed a 72% greater number of megakaryocytic cells, scored as AChE+ cells/clot, than the controls at day 4 (Fig 1). This was highly significant ($P < 0.001$). Other preparations ($RSCCM_2$) showed about 41% greater mitotic activity than the isotonic saline controls ($P < 0.005$). However, a few preparations ($RSCCM_1$) showed no greater mitotic activity than the control groups, which showed 40% increase in cell numbers (Fig 1). Generally, rat bone marrow megakaryocytes formed colonies, matured fully, and produced cytoplasmic fragmentation when RSCCM was in the medium. Typical small megakaryocyte colonies with varying sizes could be seen as early as 2 to 3 days. Most colonies consisted of 3-6 cells, but at day 5 and later, loose colonies varied between 4 and more than 32 cells in number. Most of the colonies on days 2-4 were tight and small, containing about 8 cells, but they became loose

and larger on days 5-7. The number of single megakaryocytes in the cultures increased as the incubation time increased.

The effects of using 10%, 15% or 20% concentrations of rat serum were compared in Fig 2. The number of AChE+ cells counted was highest on day 4 in all experiments. When the number of AChE+ cells in the 10% rat serum group were compared to the 15% and 20% rat serum groups on day 4, the cultures containing 10% serum showed 17% ($P < 0.01$) and 28% ($P < 0.01$) more cell growth, respectively.

The number of AChE+ cell colonies was also determined when three concentrations of rat serum were used (Fig 3). The largest number of colonies was detected on day 4 for all concentrations of serum. When the maximum cell numbers (Fig 2) were divided by the maximum colony numbers (Fig 3) for all serum concentrations, 10, 11, and 12 were the mean cell numbers/colony for cultures containing 10%, 15% or 20% rat serum, respectively. However, the average number of cells in each colony on day 4 for all concentrations of serum ranged between 4 to 8, and increased further to 16 to 32 cells/colony on day 6. The difference between the actual and the calculated mean number of cells in the colonies emphasized the presence of single cells in the cultures. Tight co-

lonies were rarely seen, since most of the colonies were spread out.

Horse serum, widely used as a supplement in cell culture media, was compared to rat serum in another study (Fig 4). Ten percent rat serum caused 22% more cell growth than 20% horse serum ($P < 0.01$), as determined by the increase in AChE+ cell numbers on day 4. There were no noticeable differences in the appearance of these cells in any of these cultures. Since these results seemed conclusive, 10% rat serum was used in all future experiments that had serum. Furthermore, 10% rat serum replaced 20% horse serum in RSCCM production.

The effects of using 10%, 15% or 20% citrated rat plasma as a substitute for 10%, 15% or 20% rat serum were investigated. These three plasma concentrations were added in addition to the original 10% plasma that was routinely used in plasma clot cultures. Thus, the total amount of plasma in these cultures was 20%, 25% or 30%. Three waves of cell proliferation appeared on days 3, 6 and 9 (Fig 5). Maximum cell numbers were noted on day 3 for all three concentrations, and the increases in all numbers relative to day 0, were 75%, 65% and 40% respectively. There again was a gradual increase in the cell numbers, as the concentrations of rat plasma decreased. The differences between 10% and 15% plasma, and

10% and 20% plasma on day 3 were significant ($P < 0.02$ and $P < 0.01$, respectively). In general, the cells were smaller when plasma was substituted for serum, and cell shapes were oval to round. However, occasional elongated and irregular shapes were seen when serum was used. Sometimes a few AChE+ cells showed formation of pseudopods, and cytoplasmic budding with formation of platelets, but the number of such cells seem to be higher in the serum cultures.

A linear increase in the number of AChE+ cell colonies, with decreased concentrations of plasma was seen (Fig 6). Peaks were noted on days 4 and 8. The size of the colonies on day 9 was relatively larger than on previous days, but all the cells degenerated on days 8 and 9. The presence of single cells in these clots was a little higher than in the serum cultures, except on day 9, when the colonies consisted of more than 32 degenerated cells.

The number of WBC colonies in these cultures was also quantitated in order to compare the occurrance of WBC colonies with AChE+ cell colonies in the plasma studies (Fig 7). The only peak was on day 4 in all groups. This implied that the successive increases and decreases in numbers was unique to AChE+ cells and colonies, and occurred only in cultures that used plasma

as a supplement.

The effects on AChE+ cell proliferation of the addition of either 10% rat serum or 10% rat plasma, were compared (Fig 8). Maximum cell proliferation occurred on day 4, using 10% serum, and on day 3 when 10% plasma was added. The increase in the cell numbers from day 0 to day 4 when serum was added to the medium was 38% greater than the increase in cell numbers found from day 0 to day 3, when plasma was added.

A normal, 10-12 month-old male rat has approximately 900 x 10^6 platelets/ml of circulating blood. Therefore, it was readily possible to prepare PE from the following concentrations of platelets: 450 x 10^6 (PE_1), 675 x 10^6 (PE_2), 900 x 10^6 (PE_3) and 1800 x 10^6 (PE_4) platelets/ml isotonic saline. These concentrations of PE_1-PE_2 were used to study their effects on rat bone marrow cells. The same volume of saline was added to the control groups instead of PE. During the first trial, PE_1 and PE_2 were added to the cultures at day 0. The largest number of AChE+ cells was detected on day 4. The increase in the number of AChE+ cells induced by PE_1 and PE_2 over the control groups was 30% and 108%, respectively (Fig 9) ($P < 0.001$ for both groups). Figure 10 shows the results obtained from the second set of experiments, when PE_3 and PE_4 were added on day 0. The increases in

the number of AChE+ cells over the controls on day 4 were 102% and 40%, respectively ($P < 0.001$ for both groups). The effects of different doses of PE on AChE+ cell numbers on day 4 was compared (Fig 11). This comparison implied that PE_2 (prepared from 675 x 10^6 platelets/ml) was the optimal concentration, since the largest number of AChE+ cells was detected. Larger amounts of PE were less effective for maintaining maximal numbers of these cells in plasma cultures.

The PDGF, known to be present in PE, was investigated to determine whether it was responsible for the increased number of AChE+ cells in cultures that contained PE. Six and 12 ng PDGF/ml culture medium were added to two separate groups of cultures on day 0 of incubation. The control groups had the same volume of 1.0 M acetic acid adjusted to pH 7.4 with sodium bicarbonate, instead of PDGF. The number of AChE+ cells scored on day 4, in the cultures contained 6 ng and 12 ng PDGF/ml medium, were 23% and 20% higher than the control groups, respectively (Fig 12) ($P < 0.005$ for both groups). Two dosage levels of PDGF were about equally effective in causing an increased number of AChE+ cells.

In order to compare the maximum effects of PE and PDGF, results of two series of experiments were

plotted in Figure 13. It was clear that the total increase in the number of AChE+ cells scored on day 4 was 85% higher in cultures containing PE_2, than in the cultures that contained 6 ng PDGF/ml medium ($P < 0.001$).

The presence of WBC colonies in the experiments with PE was also quantitated in order to compare their occurrance to that of AChE+ cells. In both sets of PE experiments, the largest number of WBC colonies was scored on day 5. In the first set (PE_1 and PE_2) the difference in the numbers of WBC colonies between the experimental and the control groups was not statistically significant (Table 2). In the second series using PE_3 and PE_4, the number of WBC colonies/clot was lower in both groups than in the control group (Table 3). These differences were highly significant on day 5 ($P < 0.005$ for both groups). However, the number of WBC colonies in the PE_3 group did not differ significantly from the PE_4 group. The number of WBC/colony in the control groups of both sets sometimes exceeded 64 cells/colony on day 6, but there were never more than 64 cells/colony in all of the groups that received PE.

The mean diameters of megakaryocytic (AChE+) cells were measured in order to determine the effect of different concentrations of PE and PDGF on the size of these cells. On day 3, there were significant increases

in the mean cell diameters of 10% and 14.5% in the cultures receiving PE_1 and PE_2, respectively (Fig 14), when compared to the control groups on day 3 ($P < 0.05$ and $P < 0.03$, respectively). However, the differences between the PE_1 and PE_2 groups was not significant. The results obtained from the second series using PE_3 and PE_4 were shown in Figure 15. Significant increases of 13.8% and 7.4% were noted when the mean diameters of AChE+ cells in the PE_3 and PE_4 groups were compared to the control groups on day 3 ($P < 0.001$ and $P < 0.005$, respectively). The difference between the PE_3 and PE_4 groups again was not significant. The dose dependent effect of PE on the cell size was similar to the results obtained from the counts of the numbers of AChE+ cells. Again, PE_2 was the optimal concentration for producing an increase in the mean cell diameters.

Examination of the cultures in the experimental series that received 6 ng and 12 ng PDGF/ml medium showed no significant differences in the mean diameters of AChE+ cells, when compared with the controls (Table 4).

DISCUSSION

Megakaryocytes have been grown in plasma clot cultures using pokeweed mitogen-stimulated mouse spleen cell conditioned medium (9,11,14). In addition conditioned media have been obtained from 2-mercaptoethanol (5) and phytohemagglutinin (PHA)-stimulated mouse spleen cells (14), a mixture from a leukemia cell line (45), from PHA-stimulated leukocytes (16,19), and from a rat liver cell line (18). Human urinary and sheep erythropoietin have also been successfully used for this purpose (10) (Table 1). Although the target cell(s) and the role of the mitogens in conditioned media production, and the relationship of conditioned media to physiological regulator(s) remain elusive, data were presented here indicating that pokeweed mitogen-stimulated RSCCM could also be used for the stimulation of CFU-M. However, it was essential to produce substantial amounts of conditioned media, since individual lots varied considerably in potency. The reason for this variation has not been explained. The in vitro stimulation of megakaryocyte progenitors by various non-specific conditioned media raised the possibility that these factors might not be of physiological importance. Perhaps they were operating at significantly higher concentrations than would have occurred in vivo,

and a minor degree of hormone receptor cross-reactivity could then have accounted for the stimulation of megakaryocyte growth.

It has been stated that in vitro growth of mouse megakaryocytes depended on the presence of conditioned medium in the cultures (14). It was interesting that megakaryocyte growth occurred in the absence of conditioned medium in this study (Fig 1). An approximate increase of 40% in the number of megakaryocytes relative to day 0 was detected in those cultures that did not have RSCCM. More precise studies would be needed before the relevance of the in vitro finding to the physiological regulation of megakaryocytopoiesis could be regarded as conclusive.

The cyclic growth of megakaryocyte colonies, with peak numbers on days 4 and 8, in the presence of plasma, but not serum, brings about speculations. Studies with agar cultures revealed that the average increase in big cell colony size was due to the appearance between days 5 and 7, of previously unrecognized larger colonies (46). This might have occurred in our cultures. However, precursor cells might have continued to multiply, or perhaps colonies might have resulted from a subset of CFU-M that was slower in cycling status. Furthermore, this slower cycling status might have been due to the

absence of some stimulatory substance(s) in plasma.

Fetal calf, human, bovine, guinea pig and horse sera at varying concentrations ranging from 2% to 30% have been used in semi-solid cultures of megakaryocytes (9,10,11,12,16,17,19,47,48). We have determined that a maximal concentration of 10% rat serum was optimal for plasma clot cultures of rat megakaryocytes in our studies and should be used in future experiments. A medium containing 10% rat serum was shown to be 17% and 28% more effective for support of cell growth and development, than 15% and 20% rat serum, respectively. Various concentrations of rat plasma without any serum were also tested. It was found that 10% rat plasma was more effective than concentrations of 15% or 20% plasma. Thus, it appeared that rat serum and plasma concentrations higher than 10% had an inhibitory effect on megakaryocyte proliferation. Similarly, an inhibitory effect of human serum on the growth of megakaryocyte colonies was reported at low cell concentrations (16). The determination that this inhibitor might be specific for megakaryocytes would require further investigations.

The substitution of fetal calf serum for horse serum resulted, in an earlier study (49), in greater growth of megakaryocytes. This present study also concluded that a 10% concentration of rat serum induced a

22% greater cell proliferation than 20% horse serum. Ten percent horse serum was not tested. However, the greater cell growth, when 10% rat serum was used, implied that cross-species components like serum and plasma might be less compatible to the cultures, then sera or plasma derived from the same species, and might require larger amounts of sera or plasma to be used. The response(s) of cells to foreign proteins present in serum and plasma might also play an important role in the generalized effects of serum and plasma.

Adenosine diphosphate (ADP) is released from platelets during blood coagulation as part of the release reaction (50). Serum is the fluid part of coagulated blood and contains platelet products. Thus, considerably more ADP may be available in serum than in plasma. It was shown that 10 μM ADP caused spherical megakaryocytes to spread and flatten to several times their normal size (51). The smaller size of AChE+ cells and their oval to round shapes in the plasma-alone study might have been caused by the smaller amount of ADP, or by some other component(s) present in serum but not present in plasma.

A major difference between serum and plasma is the absence of several clotting factors in serum. Therefore, 10% extra plasma was added to the cultures to substitute for the 10% rat serum, in the hope of simplifying the

system. However, the number of megakaryocytes in each clot was 38% greater, when 10% plasma plus 10% serum were used, instead of 20% plasma alone. Since platelets break open during hemostasis (52), the greater stimulation of cell growth caused by plasma plus serum, rather than plasma alone, implied that the presence of stimulatory factor(s) in the serum or of inhibitory factor(s) in the plasma. It was likely that the factor(s) came from the platelets.

The consistancy of the concept of inhibition of hematopoietic stem cells by their end products depends on the type and contents of the mature offspring of these cells. This same concept does not also exclude the possibility of a positive feedback mechanism involving factors directly elaborated from the end product of the hematopoietic stem cell. There is at least one clear example of the existence of both positive and negative feedback control systems for granulocyte-macrophage stem cells (colony forming unit-culture, CFU-C) involving a monocyte-derived colony stimulating factor (CSF) and prostaglandin E (PGE) respectively (53). The present study demonstrated that PE and human PDGF significantly increased the numbers and the mean diameters of rat AChE+ cells in vitro.

The effect of hydroxyurea on the cyclic status

of the CFU-M is an open question because of different experimental techniques which resulted in differences in exposure of the cells to toxic levels of the drug (1), and the CFU-M were less sensitive to the drug than were the CFU-C (12). Furthermore, only 7-20% of CFU-M had been shown to be in DNA synthesis (1). Nevertheless, Williams et al (37) have reported that mouse bone marrow CFU-M were not put out of cell cycle by platelets or PE as compared to hydroxyurea treatments in vitro and in vivo. Their results implied that the slow cycling CFU-M compartment cells were not responsive to PE, but did not eleminate the posible responsiveness of small AChE+ or subsequent cell populations to PE and PDGF.

The increase in megakaryocytes as a function of time in culture is a complex function balancing megakaryocyte loss through platelet formation and cell death against new megakaryocyte production from progenitor cells. It has not been easy at this moment to delineate how and where PE influenced the production of new megakaryocytes or inhibited the cell loss through cell death or differentiation. Either of which could lead to a net increase in megakaryocytes/clot. However, it has been previously suggested that the numbers of megakaryocytes and their colonies were likely to be increased by factors that affected endomitosis and cytoplasmic maturation of megakaryocytes (37). A number of these factors might exist in

PE; one of the best known was PDGF. All of them certainly deserve further investigation.

Studies with acute thrombocytopenia induced by platelet antiserum (54) and cyclophosphamide (55) indicated that changes in CFU-M and CFU-C might have occurred in parallel fashion. Therefore, the number of WBC colonies was quantitated in these studies. Previous investigators concluded that PGE inhibited the growth of WBC colonies in vitro (53). Since the synthesis of PGE by platelets was well documented (56), the decrease in the number of WBC/colony and WBC colonies/clot with the higher concentrations of PE in the medium could be explained.

Odell et al (2) demonstrated that the size of megakaryocytes was proportional to their ploidy. Although the ploidy of the cells in this study was not examined, the increase in the mean cell diameters implied a dose dependent increase in cell ploidy with PE in the medium. The nature of the substance(s) and the type of the cells affected are not clear at the present time. However, the relative increase in the cell size for 3 days suggested that the substance(s) present in PE did not act on CFU-M but rather on the successive megakaryocytic generations, as suggested previously (33,37). Otherwise, the increase in the mean cell diameters would have been continuously

detected after day 3, since most mature megakaryocytes in colonies increased in area from days 4 to 7 of culture (11). In these experiments, the relatively small increase in the cell size could be explained, if there were a reciprocal relationship between cell size and the number of AChE+ cells; increases in cell number decrease the size, or vice versa. More direct studies are necessary in this regard.

The present study with PDGF indicated that a commercial preparation of purified human PDGF markedly increased the numbers of megakaryocytes in plasma clot cultures. Although platelets have a rudimentary biosynthetic apparatus, recent findings suggested that megakaryocytes might be the site of synthesis of PDGF (38). Primary Myelofibrosis (PMF) is a chronic myeloproliferative disorder characterized by an abnormal accumulation of collagen in bone marrow and an excessive proliferation of marrow fibroblasts in bone marrow and in extramedullary sites. The pathophysiological mechanisms leading to marrow fibrosis are unknown, but growth promoting activity of PDGF on human marrow fibroblasts has been well characterized (38). Platelet kinetic studies have demonstrated that ineffective thrombopoiesis occurred in PMF, with a highly increased death rate of megakaryocytes (57). The ineffective thrombo-

poiesis due to highly increased death rate of megakaryocytes in PMF might possibly have been due to the presence of PDGF that was elaborated from the dead megakaryocytes in the bone marrow.

Recently it has been reported in 3T3 cells that PDGF acts in G_o phase, rendering cells competent to respond to other serum growth factors, such as somatomedins (58). Also at the onset of G_1 phase immediately after mitosis, PDGF prevented cells from entering the G_o phase (59). However, the question of whether the effect of PDGF on rat bone marrow megakaryocytes is similar to that of 3T3, or some more complex phenomenon is involved, still remains unresolved.

The total percentage increase in the number of megakaryocytes/clot was higher in the PE experiments than in the PDGF experiments. This difference indicated possible effects of (an)other platelet factor(s), in addition to PDGF, in PE. These include ADP, epinephrine, cations, platelet factors 1 through 10, cyclooxygenase, lipids, thromboxane A_2 and prostaglandins E_1, E_2 and $F_{2\ alpha}$ (60). Kurland et at (53), in studies of human granulopoiesis, have shown that PGE inhibited CFU-C growth and $PGF_{2\ alpha}$ enhanced murine CFU-C growth (61). Recently it has been demonstrated that nine PGs added to human bone marrow cell cultures

increased the number of erythroid colonies up to 95%, in the presence of erythropoietin, and also that $PGF_{2\ alpha}$ consistently inhibited erythropoiesis over a wide range of concentrations (62). These results clearly indicated that PGs modulate granulopoiesis and erythropoiesis, and might also be involved in the control of megakaryocytopoiesis. More precise and extensive research is anticipated in this area.

SUMMARY

An improved plasma clot culture system was developed for the growth of rat megakaryocytic cells. These cells were characterized histochemically by an acetylcholinesterase positive (AChE+) staining reaction. The conditioned medium, obtained from pokeweed mitogen-stimulated rat spleen cells, induced megakaryocyte colony formation and full maturation of megakaryocytes to cytoplasmic fragmentation. Ten percent rat serum was more effective than 20% horse serum in supporting cell growth. Greater stimulation of cell growth was provided when both serum and plasma were added to the culture medium, than when plasma was added alone. The use of a single species as the donor of bone marrow cells, spleen cells conditioned medium, plasma, and serum provided a less complex culture medium for the study of megakaryocyte development.

Rat platelet extract and human PDGF were assayed in this culture system, in order to study the ability of these materials to modify the proliferation and/or maturation of megakaryocytic cells. Extracts prepared from 675×10^6 platelets or 6 ng PDGF/ml caused maximum increases over the controls of 108% or 23%, respectively, in the number of AChE+ cells. Higher concentrations of

platelet extracts were found to be less effective in stimulating the maturation of megakaryocytic cells. It was also found that this same dose of PE caused an increase of 14.5% in the mean diameters of AChE+ cells. PDGF had no effect on cell size. The PE also caused reduction in the number of WBC colonies in one series of experiments. It was not clear how and where PE or PDGF stimulated the production of new megakaryocytic cells, or inhibited the loss of these cells by cell death or by differentiation into platelets. However, the increase in the numbers and diameters of AChE+ cells induced by PE implied that substance(s) released by platelets might play a physiological role in the control of megakaryocytopoiesis.

Figure 1. The response of rat bone marrow cells to different lots of rat spleen cells conditioned medium (RSCCM). The control medium contained 10% isotonic saline instead of RSCCM. The number of megakaryocytic cells, scored as acetylcholinesterase positive (AChE+) cells/3 x 10^6 nucleated bone marrow cells, was significantly greater than the controls when $RSCCM_3$ ($P < 0.001$) and $RSCCM_2$ ($P < 0.005$) were used, but was not significant when $RSCCM_1$ was used. Each point was the average of 3 to 4 cultures/day combined from 2 to 5 different experiments. The standard errors of the mean were shown by vertical lines in this and all the following figures.

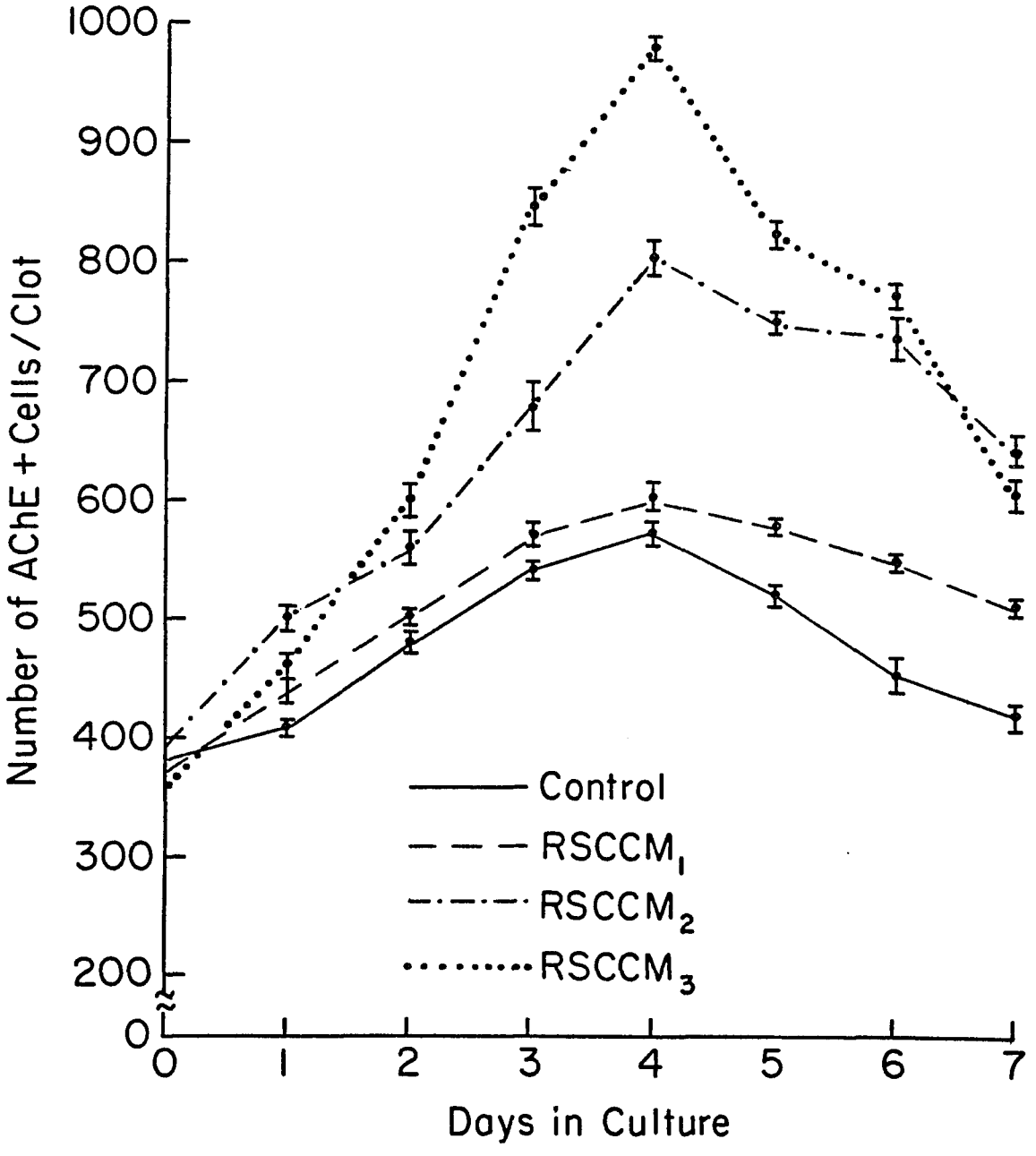
1000
900
800
700
600
500
400
300
200
0
Number of AChE + Cells / Clot
0
1
2
3
4
5
6
7
Days in Culture
Control
RSCCM1
RSCCM2
RSCCM3

Figure 2. The effect of culture media containing 10%, 15% or 20% (v:v) rat serum on rat megakaryocytic (AChE+) cell growth. The cultures containing 10% and 15% serum also contained 10% and 5% isotonic saline, respectively, to compensate for the 20% serum. The results of the groups containing 10% and 15% serum were combined from three different experiments. The data from the 20% serum group was the average of two different experiments ($P < 0.001$ for both 10% vs. 15% and 10% vs. 20% serum groups).

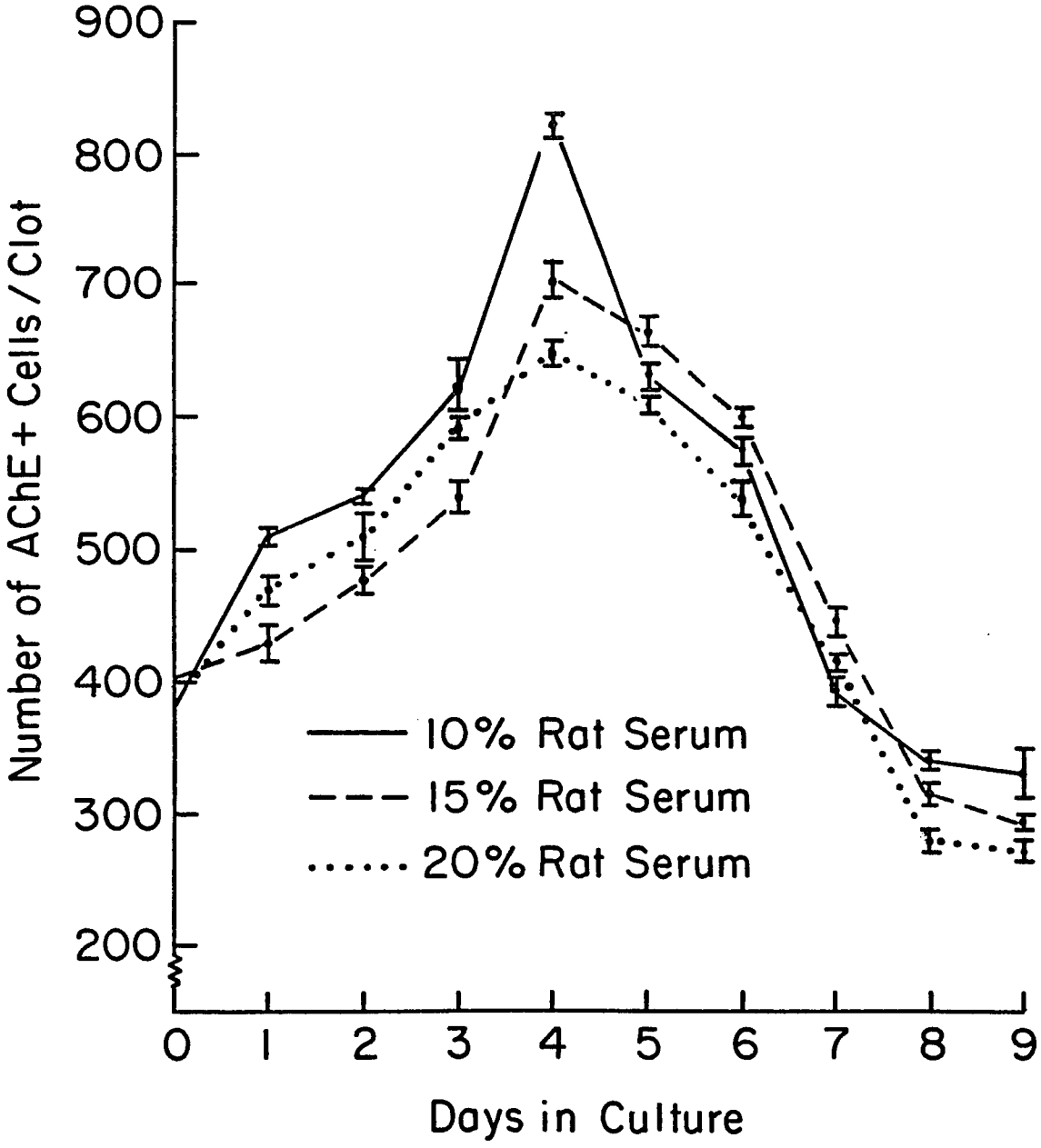
900
800
700
600
500
400
300
200
Number of AChE+ Cells/Clot
10% Rat Serum
15% Rat Serum
20% Rat Serum
0
1
2
3
4
5
6
7
8
9
Days in Culture

Figure 3. The effect of culture media containing 10%, 15% or 20% rat serum on the number of megakaryocytic (AChE+) cell colonies. This was from the same experiments as in Figure 2. The number of colonies was significantly greater on day 4, when 10% rat serum was used, than when 15% or 20% rat serum was used ($P < 0.1$ and 0.05 respectively).

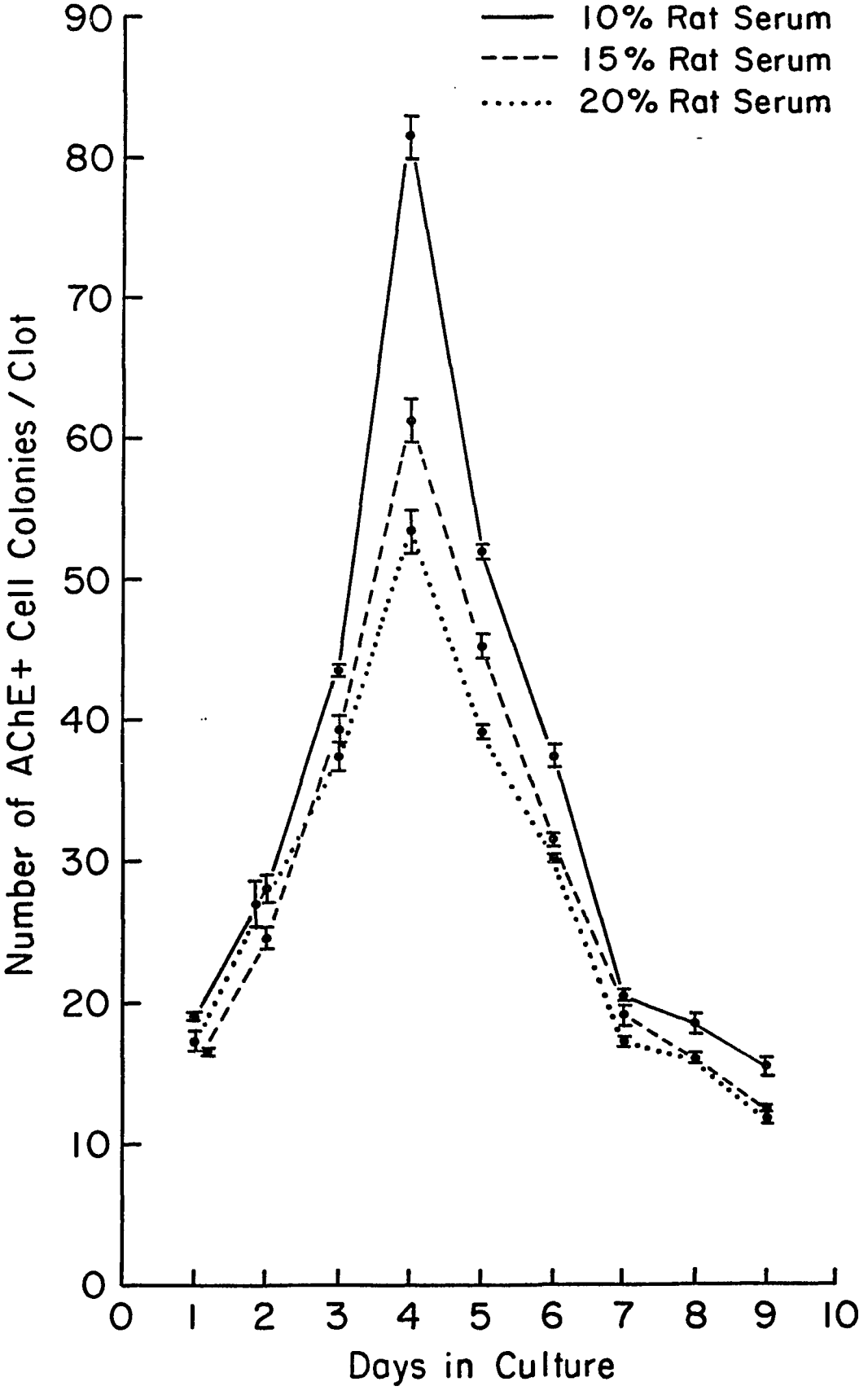
10% Rat Serum
15% Rat Serum
20% Rat Serum
Number of AChE+ Cell Colonies / Clot
Days in Culture
0
10
20
30
40
50
60
70
80
90
0
1
2
3
4
5
6
7
8
9
10

Figure 4. A comparison of 20% horse serum and 10% rat serum on the growth of megakaryocytic (AChE+) rat bone marrow cells. Ten percent rat serum resulted in a 22% greater increase in cell numbers on day 4 than 20% horse serum ($P < 0.01$). The results were averaged from two different experiments.

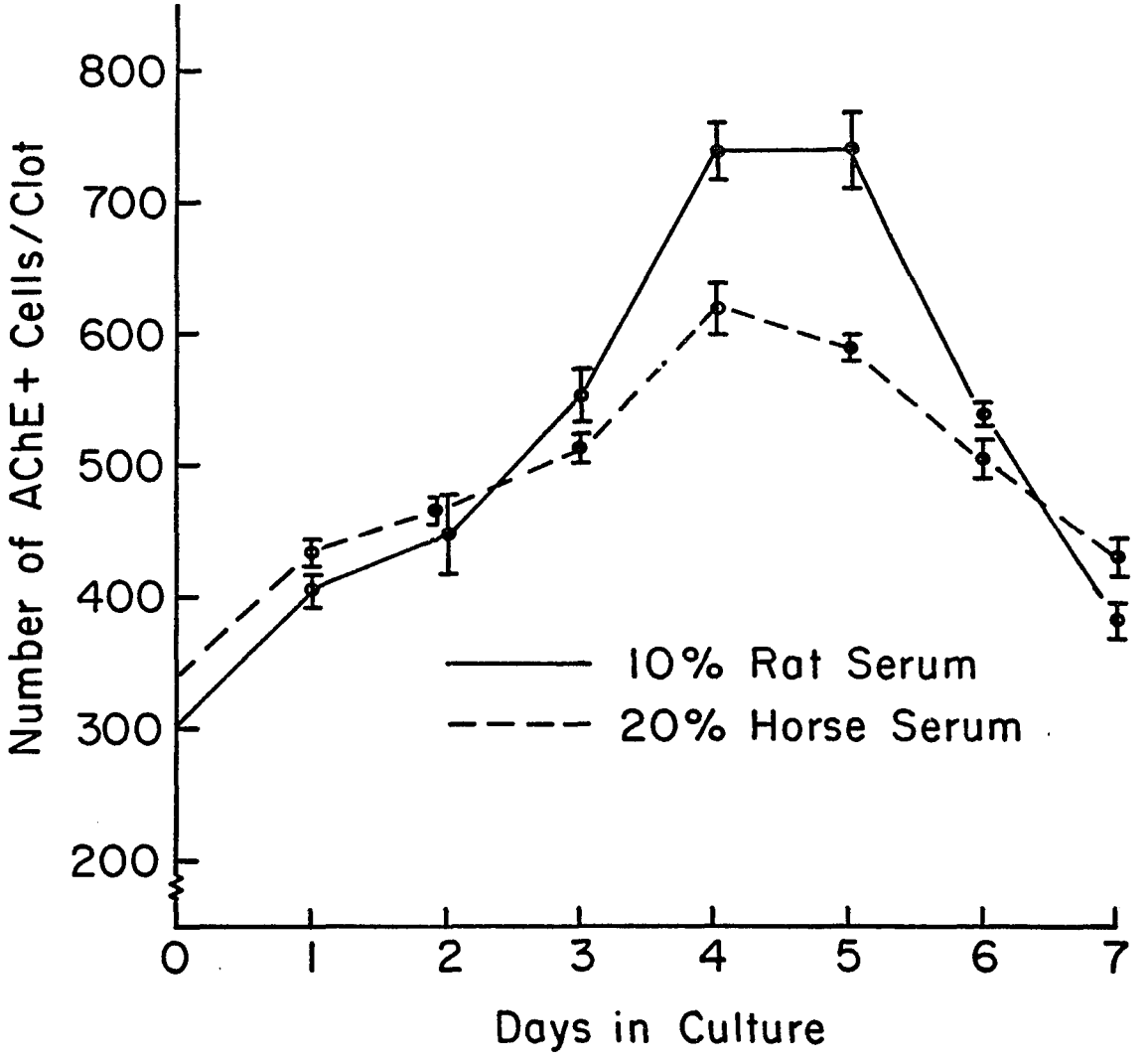
800
700
600
500
400
300
200
Number of AChE+ Cells/Clot
10% Rat Serum
20% Horse Serum
0
1
2
3
4
5
6
7
Days in Culture

Figure 5. The effects of different concentrations of rat plasma in the medium on the growth of megakaryocytic (AChE+) cells. Serum was not added to the medium. The differences between 10% and 15% plasma, and 10% and 20% plasma on day 3 were significant ($P < 0.02$ and $P < 0.01$, respectively). The results were combined from two to three different experiments.

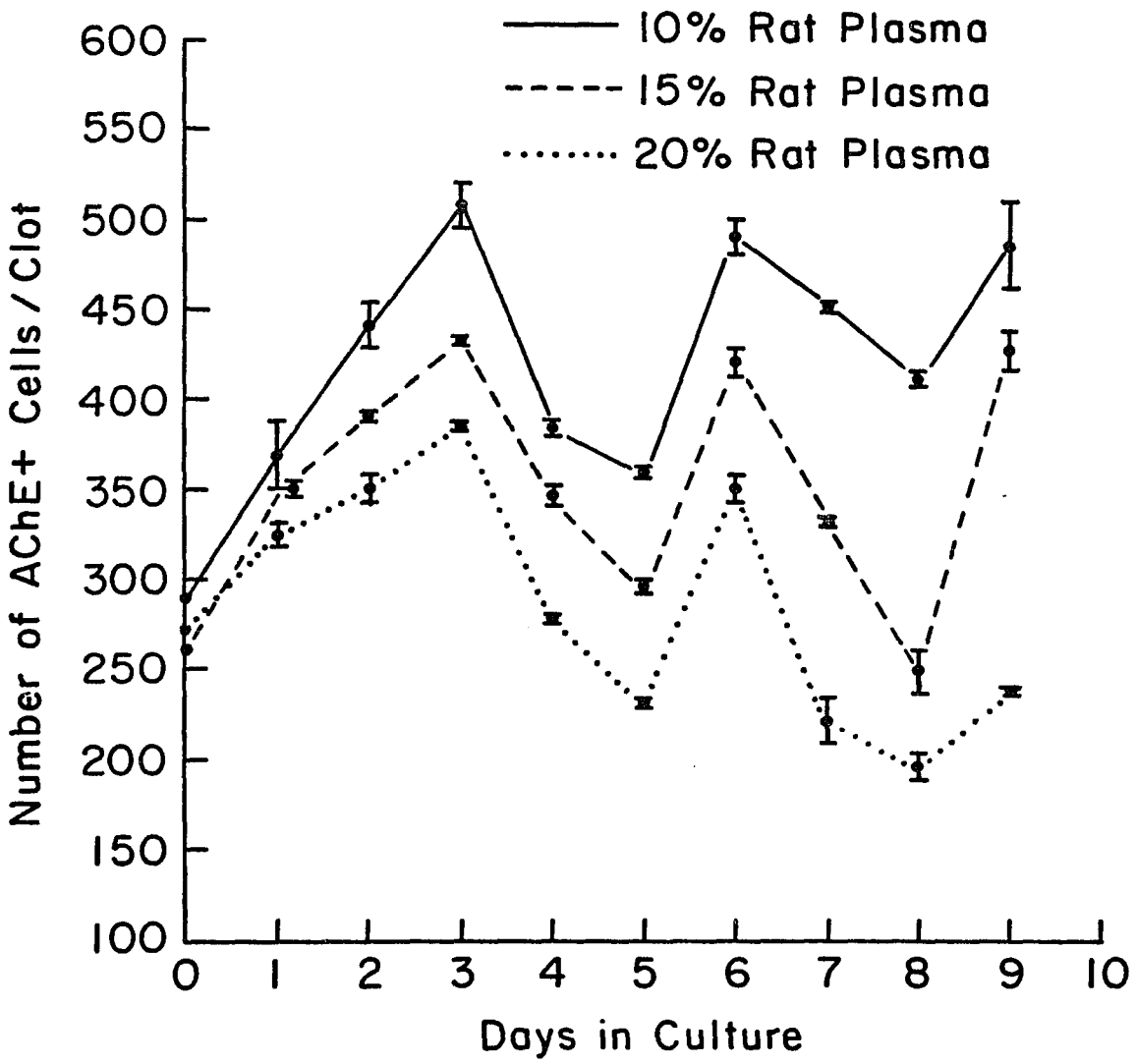
10% Rat Plasma
15% Rat Plasma
20% Rat Plasma
Number of AChE+ Cells / Clot
600
550
500
450
400
350
300
250
200
150
100
0
1
2
3
4
5
6
7
8
9
10
Days in Culture

Figure 6. The number of megakaryocytic (AChE+) cell colonies from the same experiments as Figure 5 were shown. The number of cell colonies were maximum at days 4 and 8 ($P < 0.02$ for 10% vs. 15% plasma groups and $P < 0.005$ for 10% vs. 20% plasma groups).

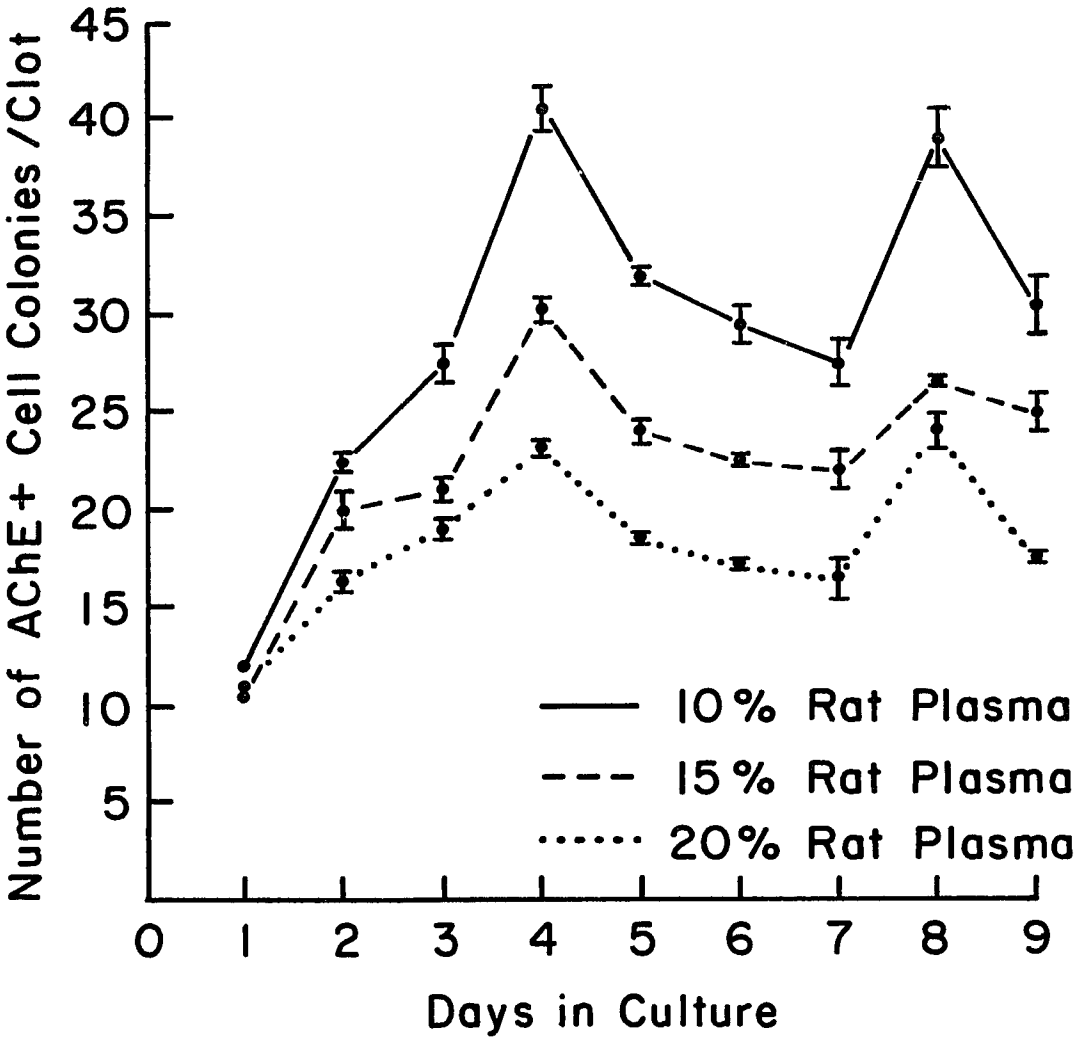
Number of AChE+ Cell Colonies /Clot
45
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
Days in Culture
10% Rat Plasma
15% Rat Plasma
20% Rat Plasma

Figure 7. The number of white blood cell colonies from the same experiments as Figure 5 were shown ($P < 0.01$ for 10% vs. 15% plasma groups and $P < 0.001$ for 10% vs. 20% plasma groups).

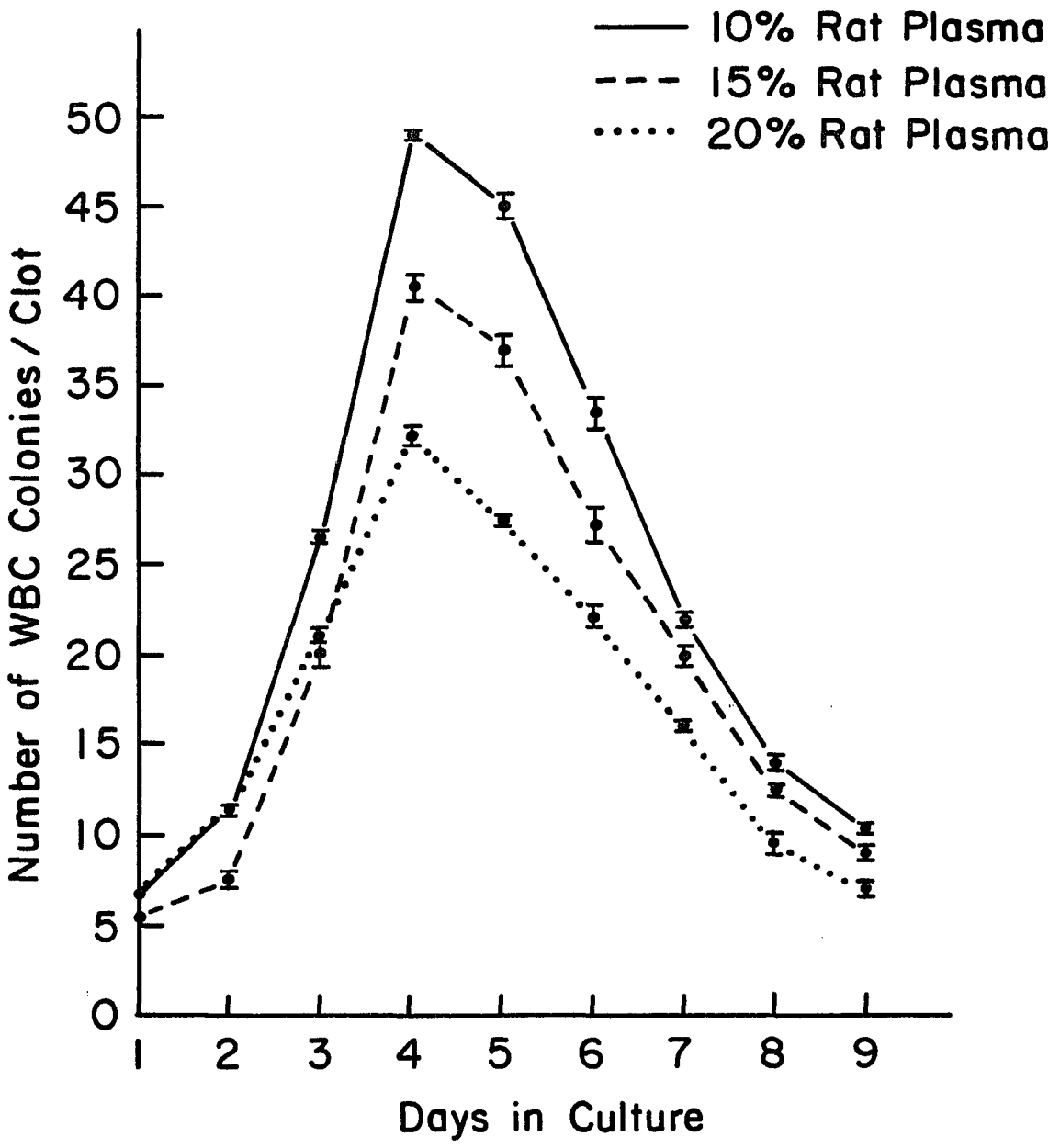
10% Rat Plasma
15% Rat Plasma
20% Rat Plasma
Number of WBC Colonies / Clot
0
5
10
15
20
25
30
35
40
45
50
1
2
3
4
5
6
7
8
9
Days in Culture

Figure 8. The effects of the 10% rat serum from Figure 2 and the 10% rat plasma from Figure 5 on stimulation of the growth of megakaryocytic (AChE+) cells were compared statisticaly ($P < 0.001$ for serum groups at day 4 and plasma groups at day 3).

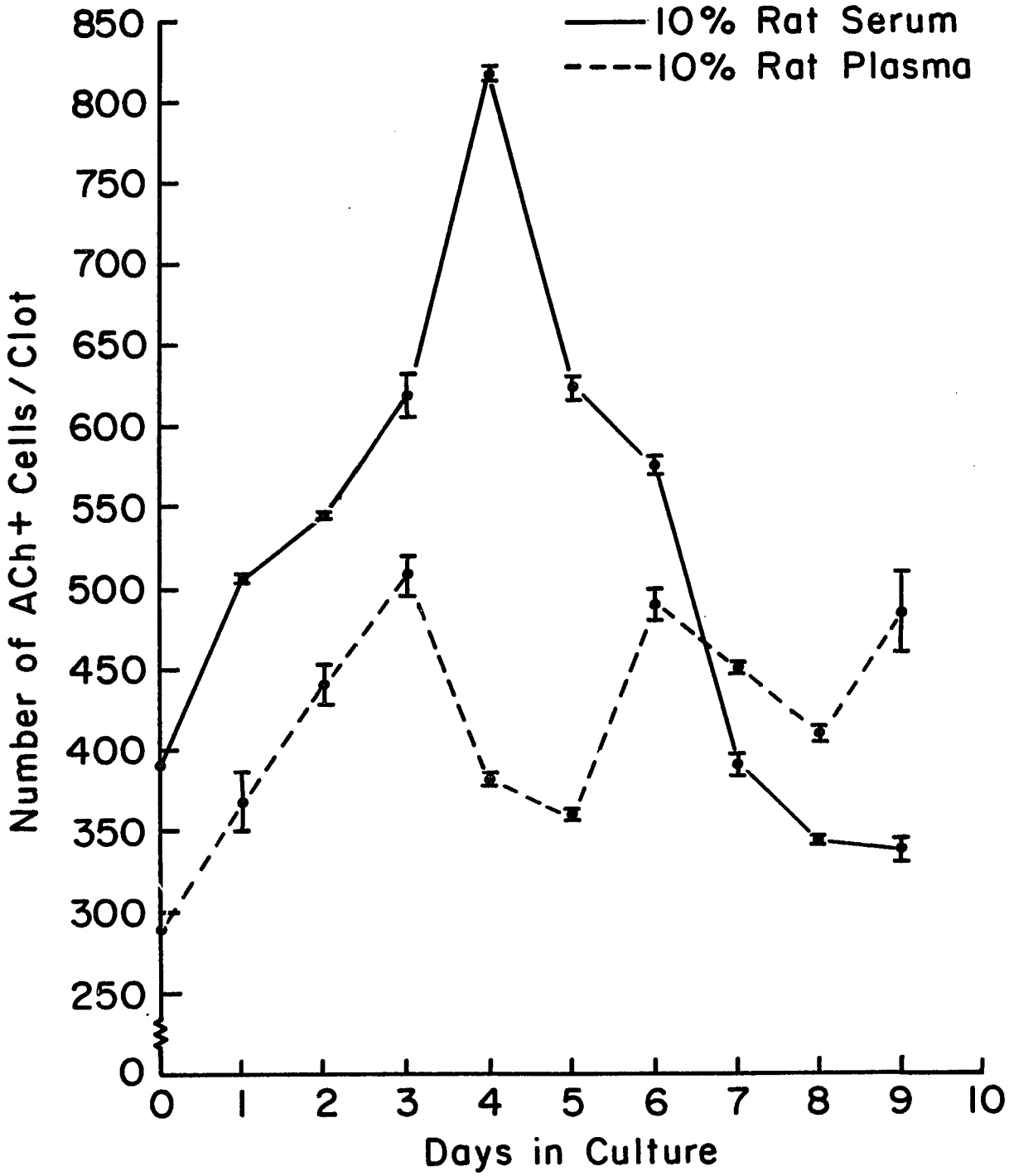
10% Rat Serum
10% Rat Plasma
Number of ACh+ Cells / Clot
850
800
750
700
650
600
550
500
450
400
350
300
250
0
0 1 2 3 4 5 6 7 8 9 10
Days in Culture

Figure 9. The effect of extracts prepared from 450×10^6 (PE_1) and 675×10^6 (PE_2) platelets/ml saline on nucleated bone marrow cells. The control medium contained the same volume of isotonic saline instead of PE. Each plasma clot was 0.3 ml of culture medium with 3×10^5 nucleated bone marrow cells. The results represented the average number of megakaryocytic (AChE+) cells from two (with PE_1) and three (with PE_2) different experiments. The number of AChE+ cells were increased 30% with PE_1 in the medium ($P < 0.001$) and 108% with PE_2 in the medium ($P < 0.001$) over the control groups at day 4.

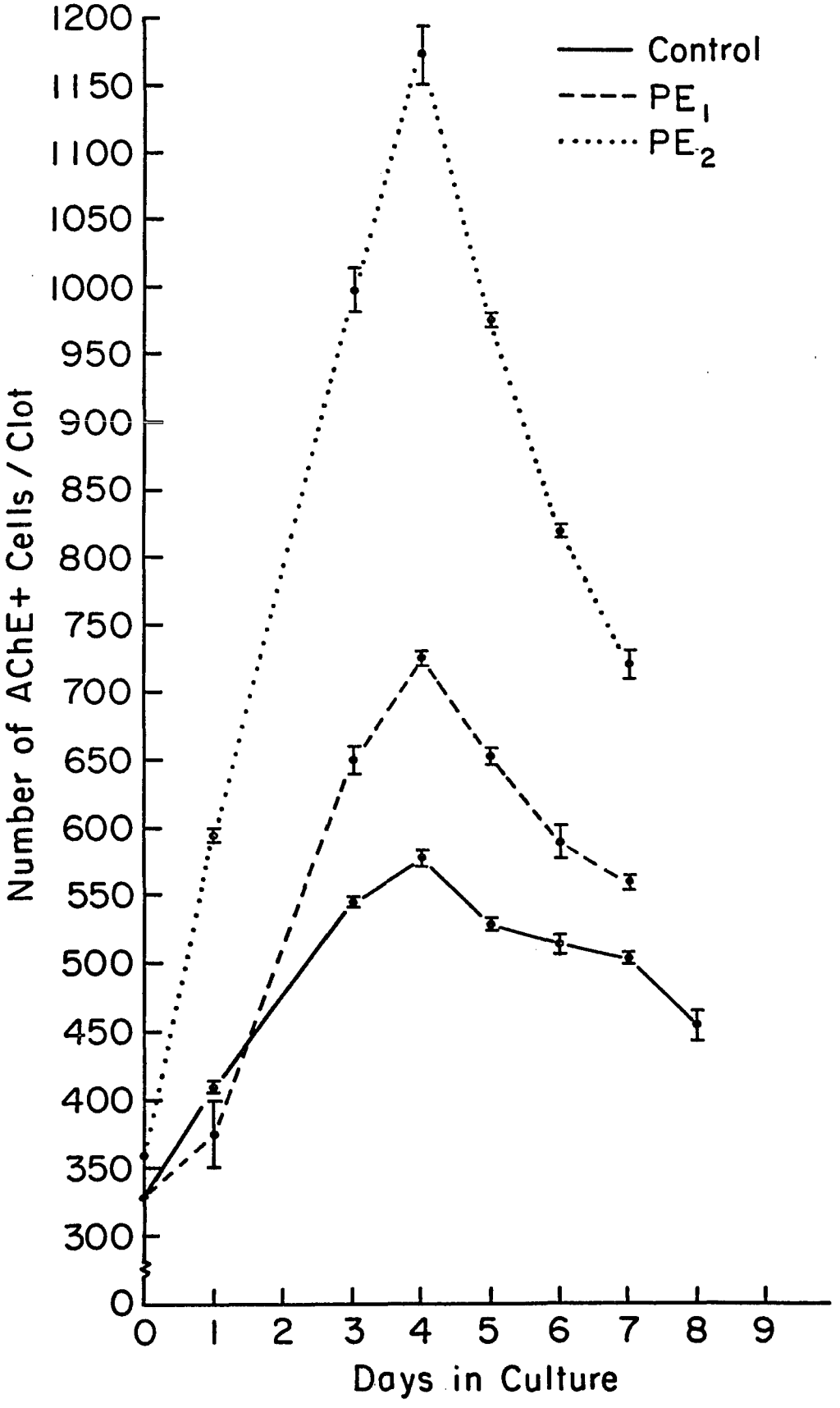
Control
PE1
PE2
Number of AChE+ Cells / Clot
1200
1150
1100
1050
1000
950
900
850
800
750
700
650
600
550
500
450
400
350
300
0
0 1 2 3 4 5 6 7 8 9
Days in Culture

Figure 10. The response of 3×10^3 nucleated bone marrow cells/0.3 ml clot to extracts prepared from 900×10^6 (PE_3) and 1800×10^6 (PE_4) platelets/ml isotonic saline. The control medium contained the same volume of saline instead of PE. The results were the mean number of megakaryocytic (AChE+) cells from two (with PE_4) and three (with PE_3) different experiments. Three to four plasma clots were examined on each day of incubation. The increases in the number of detectable AChE+ cells at day 4, over the controls, were 102% with PE_3 in the medium ($P < 0.001$) and 40% with PE_4 in the medium ($P < 0.001$).

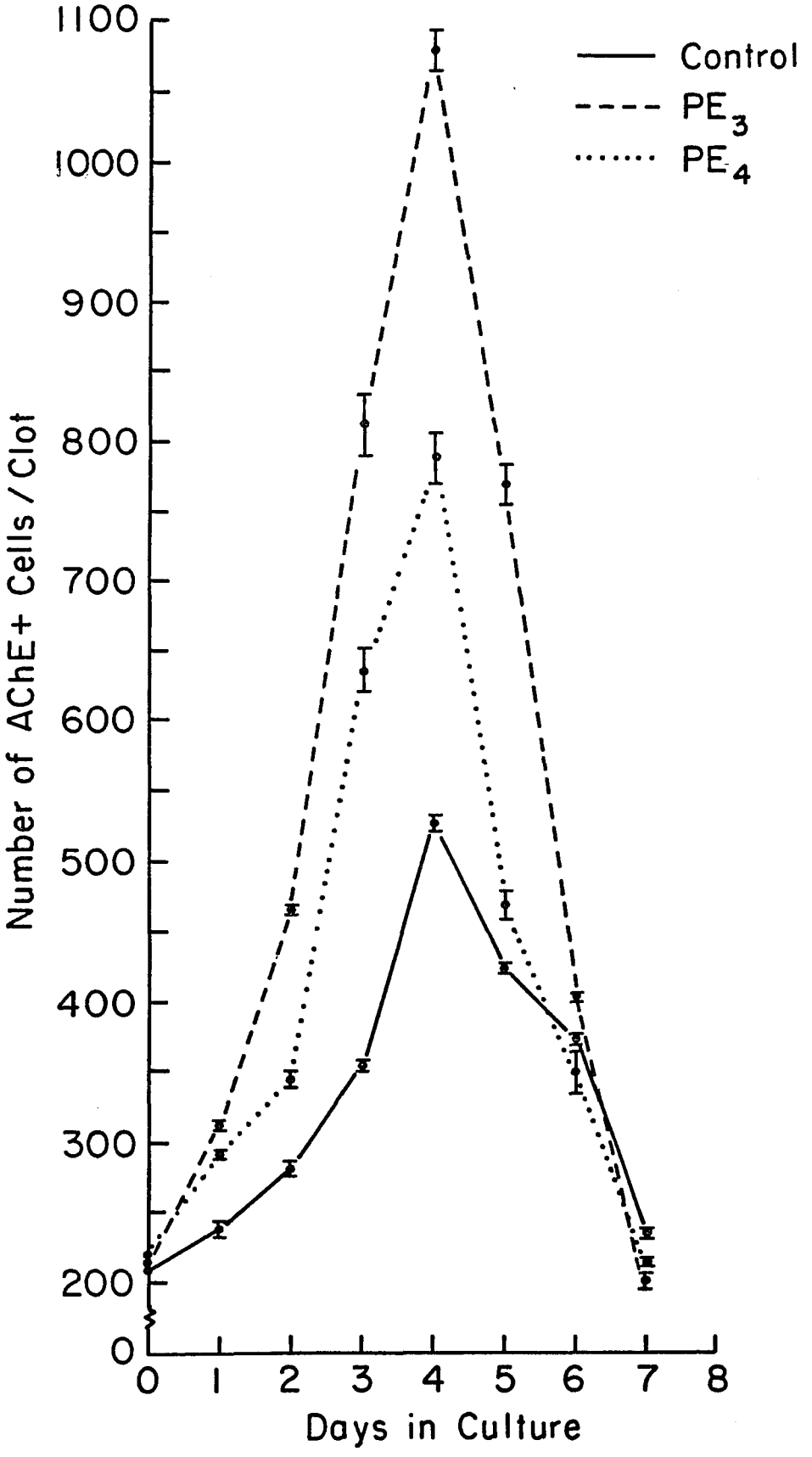
Control
PE3
PE4
Number of AChE+ Cells / Clot
1100
1000
900
800
700
600
500
400
300
200
0
0 1 2 3 4 5 6 7 8
Days in Culture

Figure 11. A comparison of the effect of different doses of platelet extracts on the number of AChE+ cells scored at day 4. The data were obtained from the same experiments presented in Figures 9 and 10.

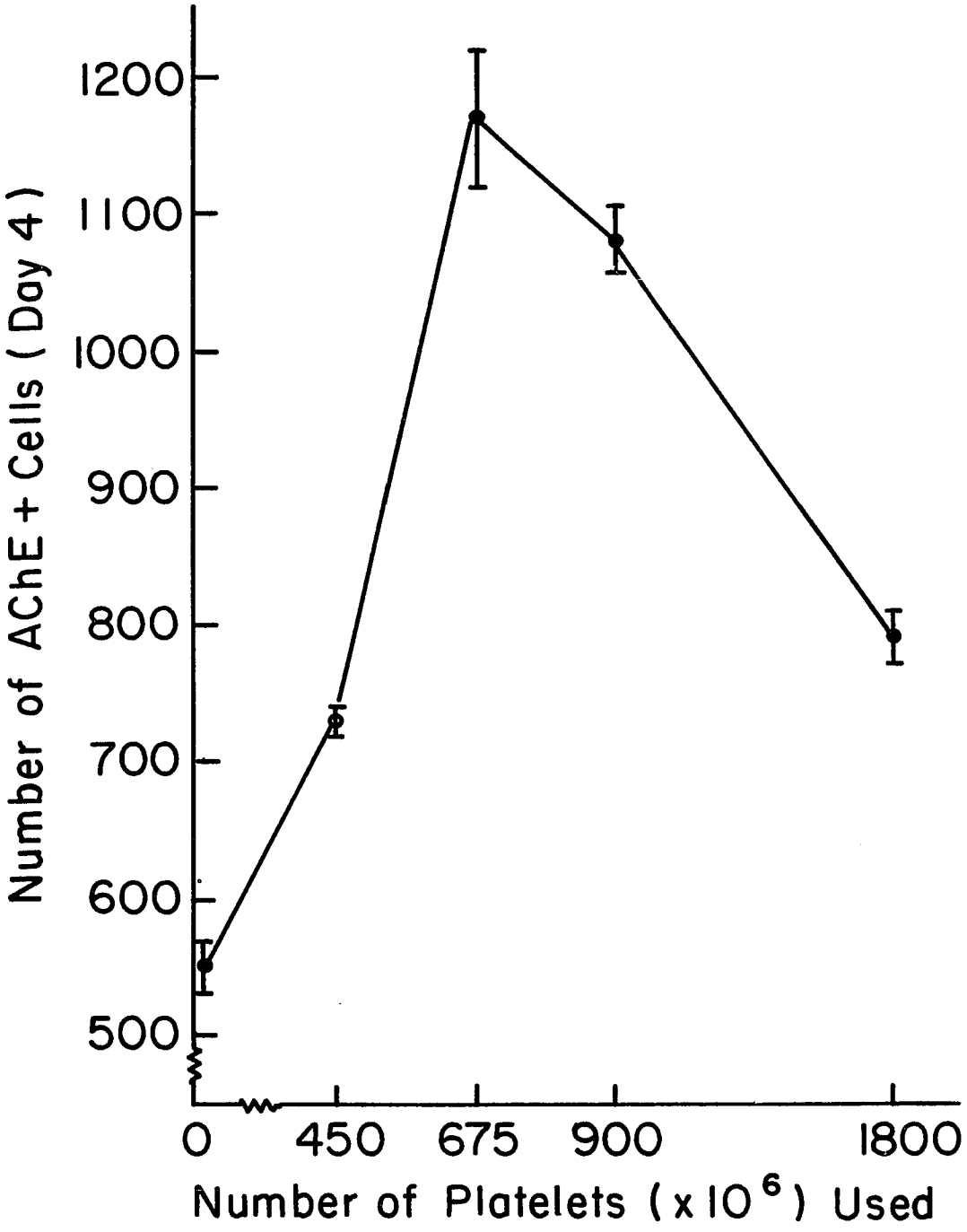
1200
1100
1000
900
800
700
600
500
Number of AChE + Cells (Day 4)
0
450
675
900
1800
Number of Platelets (x 10 6) Used

Figure 12. The effects of 6 ng and 12 ng platelet-derived growth factor/ml medium on nucleated bone marrow cells. The control medium contained the same volume of 1.0 M acetic acid neutralized with sodium bicarbonate. The results represented the average number of megakaryocytic (AChE+) cells from three different experiments. The increases in the detectable cells were 23% with 6 ng PDGF/ml and 20% with 12 ng PDGF/ml ($P < 0.005$ for both groups), when compared to the control group at day 4.

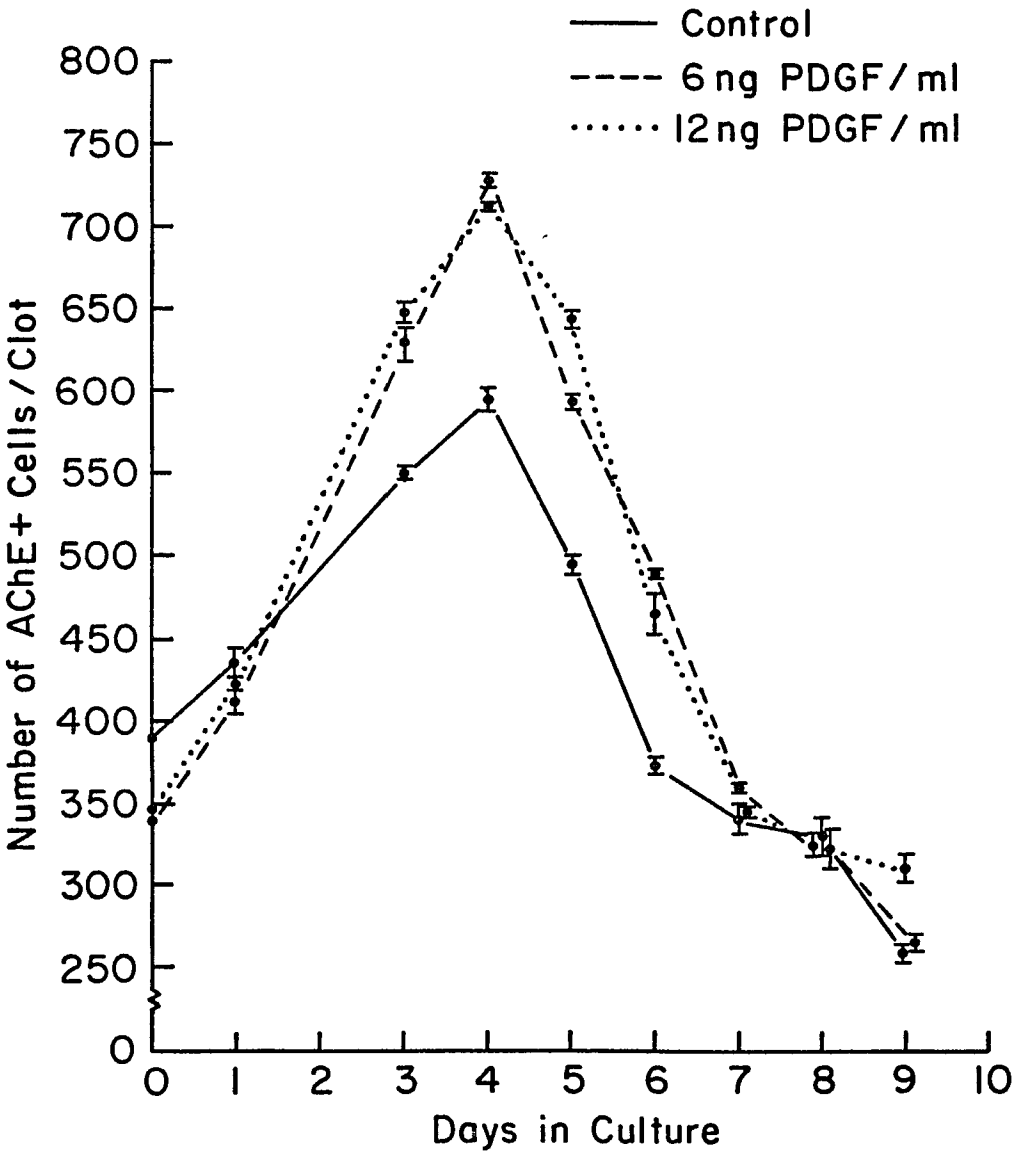
Control
6 ng PDGF / ml
12 ng PDGF / ml
Number of AChE+ Cells / Clot
800
750
700
650
600
550
500
450
400
350
300
250
0
0 1 2 3 4 5 6 7 8 9 10
Days in Culture

Figure 13. The comparison of the responses of megakaryocytic (AChE+) cells to the extracts prepared from 675 x 10^6 platelets/ml and 6 ng PDGF/ml scored at day 4. The data were obtained from the same experiments presented in Figures 9 and 12.

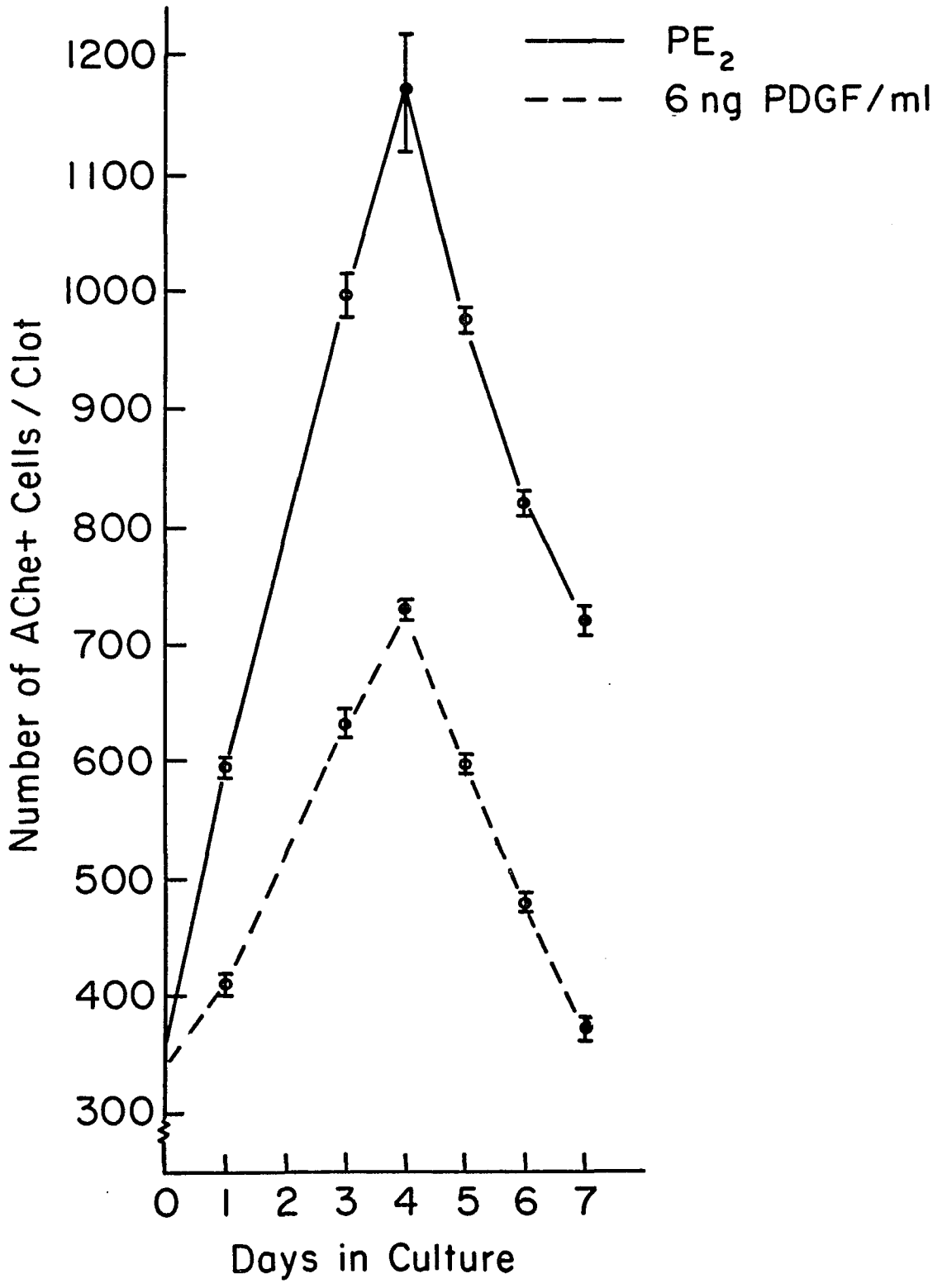
PE2
6 ng PDGF/ml
Number of AChe+ Cells / Clot
1200
1100
1000
900
800
700
600
500
400
300
0 1 2 3 4 5 6 7
Days in Culture

Figure 14. The changes in the mean diameters of megakaryocytic (AChE+) cells in response to 450 x 10^6 (PE_1) and 675 x 10^6 (PE_2) platelet extracts/ml saline on successive days. The results were obtained from the same clots as in Figure 9. One hundred to 140 cells of each group were measured every day. Ten percent and 14.5% increases in the cell size with the presence of PE_1 and PE_2, respectively, were calculated over the control groups at day 3 ($P < 0.05$ and $P < 0.03$, respectively). The differences between PE_1 and PE_2 was not significant.

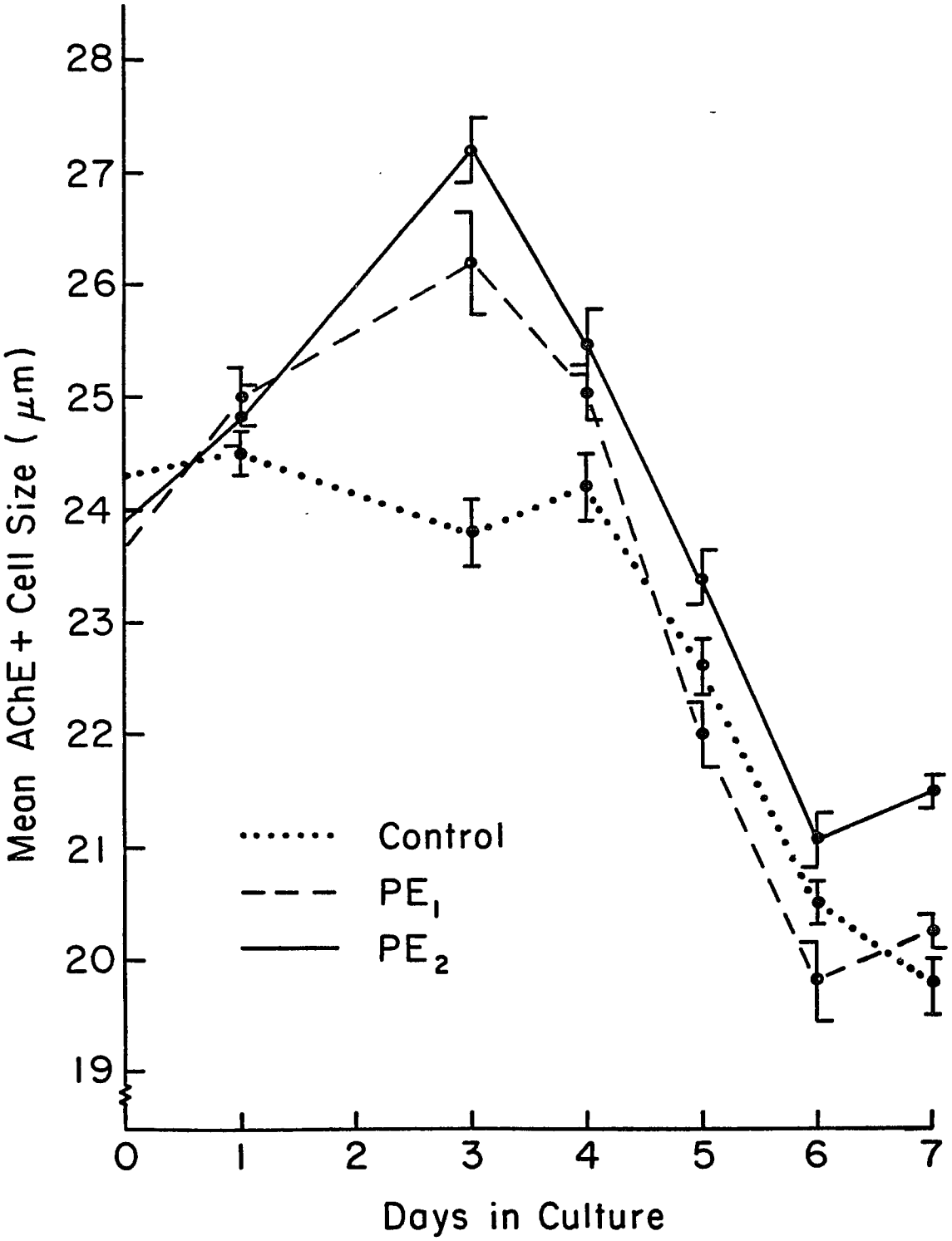
Mean AChE+ Cell Size (μm)
28
27
26
25
24
23
22
21
20
19
0
1
2
3
4
5
6
7
Days in Culture
Control
PE1
PE2

Figure 15. The effects of 900 x 10^6 and 1800 x 10^6 PE/ml medium on the mean diameters of megakaryocytic (AChE+) cells in plasma clot cultures at days 0 to 7. The results came from the same experiments as in Figure 10. One hundred to 140 cells of each group were measured every day. The increases in the mean cell diameters were 13.8% with PE_3 in the medium and 7.4% with PE_4 in the medium, when compared to the control groups at day 3 ($P < 0.001$ and $P < 0.005$, respectively).

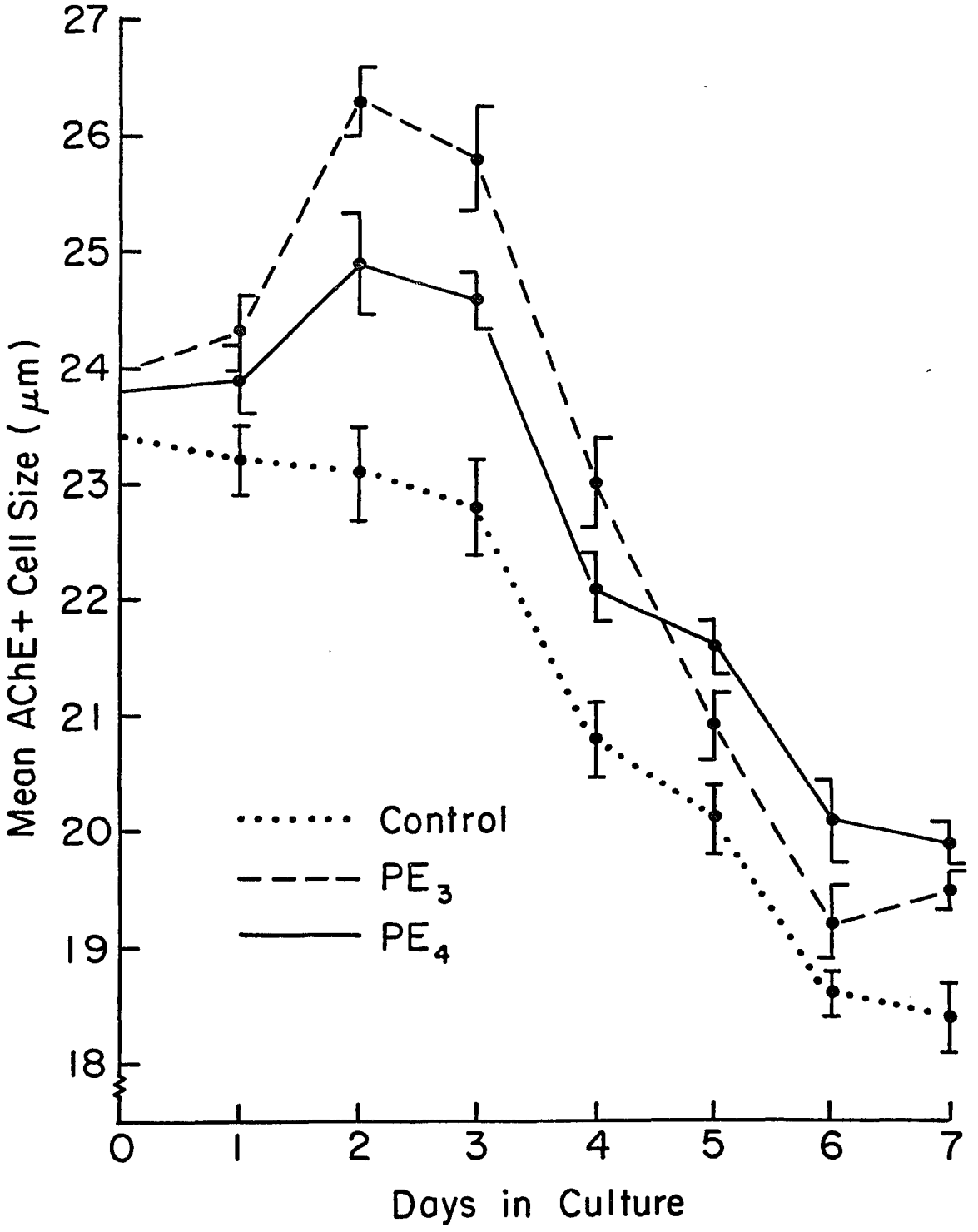
27
26
25
24
23
22
21
20
19
18
Mean AChE+ Cell Size (μm)
Control
PE3
PE4
0
1
2
3
4
5
6
7
Days in Culture

Table 1. Culture conditions for megakaryocyte growth.

Cell source	Culture medium	Serum	Stimulators	Reference
		PLASMA CLOT		
Mouse-BM	L-15	20% HS	MS-PWM	9
Mouse-BM	NCTC-109	20% FCS	Human and sheep-Ep	10
Mouse-BM	NCTC-109	15% HS	MS-PWM	11
Human-BM	Alpha medium	20% human	Human-Ep	12
Mouse-BM	L-15	20% FCS	TSF	15
Human-BM	Alpha medium	2.5-20% human	Leukocyte-PHA	16
Mouse-BM	NCTC-109	20% FCS	Human urine	17
		AGAR		
Rat-BM	Dulbecco's	20% FCS	Rat liver-CM	18
Mouse-BM	Eagle's	15% FCS	MS- 2-ME	5
Mouse-BM	McCoy's 5A	15% FCS	WEHI-3 + BM	6
Mouse-BM	Eagle's	20% FCS	MS-PWM	7
Mouse-spleen	"	"	"	"
Mouse-blood	"	"	"	"
		METHYLCELLULOSE		
Human-BM	Alpha medium	30% FCS	Leukocyte-PHA	19

BM= Bone Marrow
FCS= Fetal Calf Serum
MS= Mouse Spleen cells
Ep= Erythropoietin
PWM= Pokeweed Mitogen
CM= Conditioned Medium
HS= Horse Serum
2-ME= 2-Mercaptoethanol
PHA= Phytohemagglutinin
TSF= Thrombocytopoiesis-Stimulating Factor.

Table 2. The effect of platelet extracts on white blood cell colonies/culture.

Days in Culture	Number of white blood cell colonies/clot		
	Control	450 x 10^6 PE/ml	675 x 10^6 PE/ml
3	14.7 ± 0.6	17.0 ± 0.6	16.7 ± 1.5
4	21.7 ± 1.7	25.2 ± 2.2	26.0 ± 2.1
5	39.2 ± 3.7	41.6 ± 4.0	42.0 ± 2.6
6	26.0 ± 1.4	28.5 ± 2.1	24.5 ± 0.7
7	20.3 ± 2.3	22.3 ± 1.5	18.3 ± 2.5
8	15.5 ± 0.7	16.5 ± 0.7	12.5 ± 0.7

The data were obtained from the same experiments as in Figure 9. Mean ± SEM of 3 to 4 cultures from each of 2 to 3 experiments.

Table 3. The effect of platelet extracts on white blood cell colonies/culture.

Days in Culture	Number of white blood cell colonies/clot		
	Control	900 x 10^6 PE/ml	1800 x 10^6 PE/ml
2	11.3 ± 1.5	11.0 ± 1.0	10.3 ± 1.1
3	18.0 ± 2.1	17.7 ± 2.7	18.0 ± 1.9
4	30.2 ± 1.2	28.2 ± 2.0	26.2 ± 1.7
5	42.7 ± 1.7	36.2 ± 1.7	34.7 ± 1.7
6	33.0 ± 1.4	29.5 ± 2.1	28.0 ± 1.4
7	23.0 ± 1.4	21.0 ± 0.6	19.5 ± 0.7

The data were obtained from the same experiment as in Figure 10. Mean ± SEM of 3 to 4 cultures from each of 2 to 3 experiments.

Table 4. The effect of platelet-derived growth factor on the mean size of megakaryocytic cells.

Days in Culture	Mean size of megakaryocytic cells (um)		
	Control	6 ng PDGF/ml	12 ng PDGF/ml
0	23.9 ± 0.8	23.5 ± 0.8	23.2 ± 0.8
1	22.8 ± 0.8	22.7 ± 0.8	22.9 ± 0.7
2	N.T	N.T	N.T
3	23.0 ± 0.8	22.8 ± 0.8	23.2 ± 0.7
4	21.5 ± 0.6	21.4 ± 0.7	21.3 ± 0.7
5	20.6 ± 0.7	21.0 ± 0.7	20.6 ± 0.6
6	20.9 ± 0.7	20.0 ± 0.7	19.9 ± 0.7
7	20.0 ± 0.6	19.9 ± 0.6	19.5 ± 0.6
8	19.2 ± 0.6	19.8 ± 0.6	19.0 ± 0.6
9	17.4 ± 0.6	16.7 ± 0.5	16.8 ± 0.5

The data were obtained from the same experiments as in Figure 12. Mean ± SEM of 3-4 cultures from each of three experiments. One hundred to 140 cells of each group were measured every day. N.T. = Not tested.

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