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Effect of Calcium on Bone Marrow Mitosis *in Vitro** (32956)

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When calcium salts are injected into animals there is a marked elevation of serum calcium (1,2) and increased mitosis in rat bone marrow (3). Furthermore, postirradiation injection of calcium salts is an effective therapeutic measure since it approximately doubles survival (4). If this effect was due to a direct action on the individual marrow cells, serum prepared in this manner should also increase mitosis in these cells in culture and so permit a more detailed study of the mechanisms involved. Therefore, the serum from calcium-treated rats was tested as the nutrient for rat bone marrow cultures under conditions which permit active erythropoiesis for many days (5).

The present study showed that such calcium-enriched serum has a direct effect on the multiplication of isolated marrow cells *in vitro*. A large proportion of the marrow cells undergo rapid, sequential divisions with a very short generation time, and subsequent maturation of these cells seems to occur in a normal manner. The net result is, therefore, a rapid and dramatic increase in the effective marrow cell population.

Materials and Methods. Complete details of the bone marrow culture system have been published (5,6). Cultures consisted of a suspension of femoral marrow cells from 100-gm rats, grown on a coverslip, in a small petri dish, using 5 ml of isogenic serum as nutrient. They were incubated in an atmosphere of 30% CO_2 plus 70% humidified air at 32°C for optimum erythroid maturation (6).

Calcium and magnesium-enriched serum (Ca-serum, Mg-serum) were prepared by injecting adult (>400 gm) rats with 1.0 ml of a 62.5 m*M* aqueous solution of either element as the chloride. Two intraperitoneal injections were given, at 30 min and 3 min, respectively, before the animal was bled to death under ether anesthesia. Serum prepared in this manner contained between 11.5 and 14 mg/100 ml of calcium, or between 3.6 and 5.0 mg/100 ml of magnesium. Normal rat serum contained 9.5–10.5 mg/100 ml of calcium and 1.2–2.9 mg/100 ml of magnesium. These ion levels were determined by the murexide method (7). In later experiments it was found unnecessary to pretreat rats to obtain Ca-serum. Cultures were prepared in normal serum and 0.5 ml of 1% calcium chloride was added directly to the culture. Both forms of Ca-serum produced the same results.

When total nucleated cells were counted, the cells from the supernatant and the coverslip were combined, treated with saponin to lyse the red cells, and counted in a model B Coulter counter. A microscopic check was also made to ensure that there were no clumps of cells. In these cultures the immature cells remained mainly on the coverslip whereas the mature cells were found mostly in supernatant fluid. The supernatant fluid might therefore be considered analogous to peripheral blood *in vivo* and reticulocytes were determined as the percentage of total non-nucleated cells in the supernatant fluid, using brilliant cresyl blue. Some samples were also stained with Wright's stain as described previously

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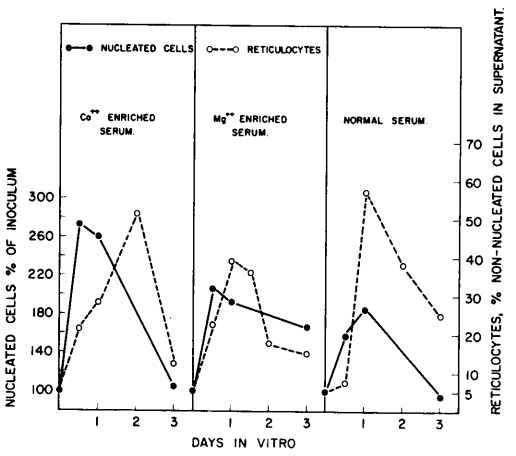

FIG. 1. The effect of enriched sera on nucleated cell multiplication and reticulocyte production in rat bone marrow cultures. ●——● nucleated cells; O- - -O reticulocytes.

(5) for general examination. To determine the rate of entry of cells into mitosis colchicine was added to the culture medium to give a final concentration of 0.062 m*M* during the last 1 or 2 hours of incubation. The cultures were then stained with Delafield's hematoxylin and the percentage of metaphase figures was calculated from at least 1000 total nucleated cells.

Results. All cultures showed a marked increase in the number of nucleated cells during the first day *in vitro*. When enriched sera were compared to normal serum the increase (expressed as percentage of the original inoculum) was greatest in Ca-serum and least in normal serum (Fig. 1). Maximum cell numbers were attained by 12 hours in both of the enriched sera, but not until 24 hours in the normal serum. In each case there was also a marked increase in the percentage of reticulocytes released into the supernatant. In the Ca-serum the reticulocyte increase occurred 36 hours after the increase in nucleated cells, and corresponded with a decline in the latter, suggesting that these nucleated cells had continued their maturation in a normal manner. Cells in normal serum did not show this time relationship. Since calcium was the more effective ion, it was used for all further studies.

The Ca-serum not only affected freshly explanted marrow cells, but also caused a burst of cell multiplication when added to cultures at any time up to 10 days *in vitro* (Fig. 2). Cultures were prepared initially in normal serum, and after different periods of incubation the serum was removed and replaced with either normal or Ca-serum. The manipulations involved in changing the serum caused a loss of many nucleated cells. When the replacement fluid was normal serum no further major changes in cell numbers occurred during the next 3 days. However, when the replacement fluid was Ca-serum, there was a burst of cell multiplication within 18 hours after feeding which more than compensated for the initial cell loss. This burst was then followed by an increase in the percentage of reticulocytes as in freshly explanted cultures. The magnitude of the response to the enriched serum decreased with time, but at any time up to 10 days *in vitro* (the longest time tested) the addition of Ca-serum consistently produced a burst of cell multiplication whereas the addition of normal serum did not.

In the next series of experiments cultures were prepared initially in Ca-serum and then received a second Ca-serum treatment after various intervals *in vitro*. Under these conditions a totally different pattern was obtained (Fig. 3). When a second treatment with Ca-serum was given 24 hours after the first there was virtually no effect; when the interval was 48 hours there was a small effect; but

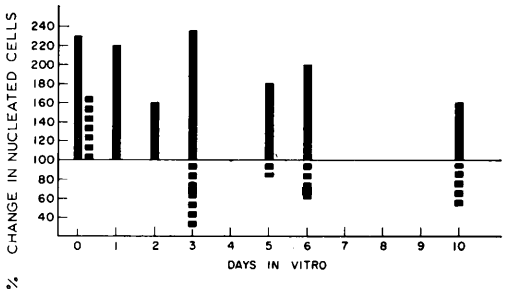

FIG. 2. Maximum change in nucleated cell numbers within 18 hr after feeding with Ca-serum (solid line), or with normal serum (broken line). 100% = number of nucleated cells before feeding. The negative changes represent losses incurred during changing of medium.

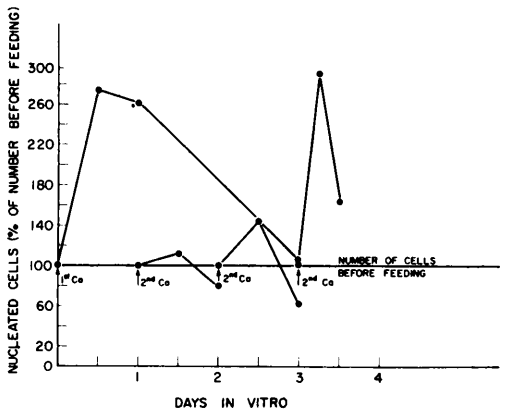

FIG. 3. The effect of a second feeding with Ca-serum at 1, 2, or 3 days after cultures prepared in Ca-serum. 100 % = number of nucleated cells before feeding.

when the interval was 72 hours it had the same dramatic effect as the initial treatment. Thus it was obvious that the cells were almost completely refractory to further calcium treatments until the third day. It would seem that the burst of multiplication after the first calcium stimulation had depleted the culture of cells capable of entering division in response to calcium. The corresponding control cultures receiving normal serum as their second treatment lost many nucleated cells with each fluid change but otherwise showed no response.

The increase of nucleated cells which was seen 12 hours after treatment with Ca-serum on either day 0 or day 3 represented a nearly threefold increase in total nucleated cells, or two complete, sequential division cycles within 12 hours. However, some of the cells in the culture were undoubtedly nondividing erythroid cells in the process of maturation (see discussion of Fig. 1), and some could be recognized as granulocytes in the later (nondividing) stages. Thus, those cells which were dividing must have undergone three to four sequential divisions in 12 hours to produce the net increase in nucleated cells which was observed. With reported generation times for bone marrow cells ranging from .85 to 20 hours (8–10), this, at first, seemed unbelievable. However, it was confirmed by determining the rate of entry of cells into mitosis at hourly intervals during the first 24 hours *in vitro*. Figure 4A shows that cultures treated with Ca-serum increased sharply in the percentage of cells in metaphase 2 hours after explanation, and that this rise was repeated at 4-hour intervals regularly throughout the day. Between each burst, the percentage of metaphase figures usually returned close to the 1.2% level, which is normal for bone marrow from a month-old rat. Therefore the enormous increase in nucleated cells during the first 12 hours after exposure to Ca-serum was due to a remarkably shortened generation time and a high degree of synchrony in those cells capable of division.

The rate of entry into mitosis of cells

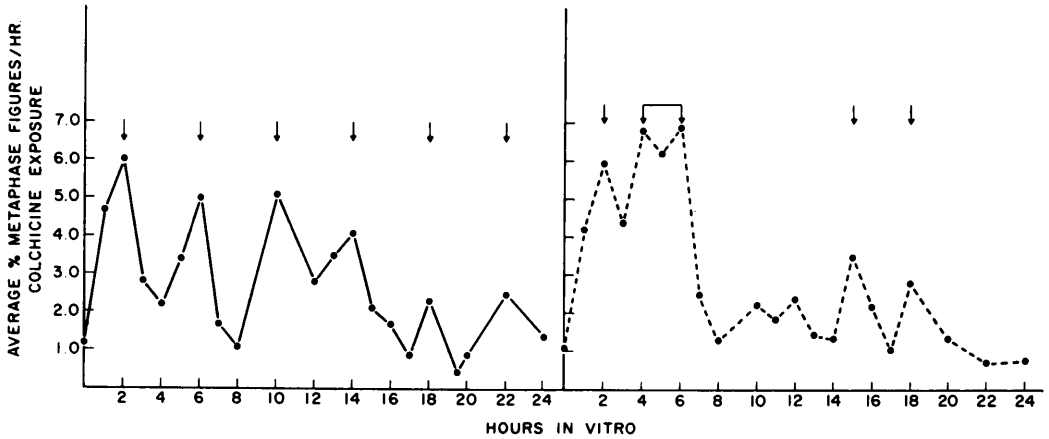

FIG. 4. Percentage metaphase figures accumulated per hour during the first 24 hr *in vitro*. A) In Ca-serum, note the regular bursts of mitosis every 4 hr. B) In normal serum note that the bursts of mitosis are irregular and sometimes resemble plateaus rather than single peaks.

cultivated in normal serum was also determined (Fig. 4B). These cells also showed an initial increase in metaphase figures and bursts of mitosis later in the day. However, unlike the cells in Ca-serum, there was no regular pattern. In normal serum maxima were seen at 2, 4–6, 15, and 18 hours. Such irregularity would suggest that different populations of cells were dividing, each with its own rhythm and a longer generation time than 4 hours. This was also consistent with the increase in total nucleated cells in normal serum at 12 hours, which was much less than that in Ca-serum (Fig. 1).

Discussion. Pretreatment of rats with calcium chloride before bleeding yields serum which stimulates the proliferation of rat marrow cells *in vitro*, resulting in an exceedingly short generation time of 4 hours in most of the dividing cells. The shortest generation time hitherto reported for rodent bone marrow (mouse) is .85 hours (8), and for lymphoid cells (calf) is 5.5– 6 hours (11). Feldman *et al.* (12), however, reported formation of splenic erythroid clones under conditions which suggest a 3-hour generation time, although these authors prefer an alternative explanation.

Marrow cell kinetics are usually determined using techniques devised for short-term work. In the present work the culture technique was specifically selected for long-term continuation of function (5), and embodies some unusual features such as the use of moderately hemolyzed serum (5) and a very high concentration of CO_2 (6). Some features of the culture system may, therefore, have enabled these cells to escape the control mechanisms which operate *in vivo* and in other culture systems. For example, prolonged exposure to high CO_2 increases the calcium ion content of erythrocytes (13). If a high concentration of CO_2 has the same effect on nucleated cells, then the use of 30% CO_2 in the present system, combined with an increase in available calcium, may have increased the intracellular calcium content beyond any level which can be attained *in vivo*.

The 3-day refractory period which occurs after one exposure to Ca-serum suggests that calcium has stimulated all the cells which were either in, or poised to enter, division and that few of the remaining cells are capable of division at that time. The delay of 36 hours between the time when the maximum number of nucleated cells were seen and the time when the highest percentage of reticulocytes were seen in Ca-serum (Fig. 1) approximates the normal interval and suggests that little, if any, acceleration has occurred in the maturation steps as a result of calcium stimulation. However, since the proliferative response to a second Ca-serum treatment at 3 days is proportionally as great as the first response (Fig. 3), it would appear that calcium-responsive cells (prior to the maturation stages) have either accumulated in that time interval or have regained their ability to react to calcium.

In terms of current stem-cell theories, it seems possible that the calcium-responsive cells may be those with doubled DNA in a state of dormancy (14) or poised G_2 (15). Massive calcium uptake by these cells may cause condensation of the chromatin (16) and thereby trigger mitosis. From the present observations it would appear that these cells have a much shorter generation time than other bone marrow cells. This is not unreasonable since their numbers may not be large in comparison to the total dividing-cell population of the marrow. Thus, except when they are all stimulated to divide simultaneously (for example, by excessively high levels of calcium), they would not cause significant deviations from the normal mitotic rate and would not be recognized. The 3-day period in which there is very little response to a second calcium treatment would, therefore, represent the time required for the primitive cells to return to their normal state, and for sufficient cells to accumulate in poised G_2, to produce a measurable burst of cell multiplication.

Summary. The use of calcium-enriched serum as nutrient for bone marrow cultures produces a remarkable mitotic stimulation *in vitro*. The responsive cells may be those normally in a dormant state and under the present conditions they appear to be capable of a 4-hour mean generation time.

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Esterase Activity in Embryonic Development* (32957)

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Although the enzymes of the nonspecific esterase type (carboxylic acid ester hydrolases) are known to be widely distributed in animal tissues, relatively little is known about their activity in different stages of embryonic development. Reports by Rossi *et al.* (1), for example, suggest that esterase activity appears in the early stages of ontogenesis in the gastrointestinal mucosa of human embryos. In addition, Richardson *et al.* (2) observed that the activity in the chicken embryo duodenum increased about 10-fold between days 13 and 22 (second day after hatching).

Introduction of the electrophoretic "zymogram" method by Hunter and Markert in 1957 (3) provided a new, sensitive tool for the study of species- and organ-specific esterase patterns. Using this method for the study of developmental changes in mouse plasma and tissues, Hunter *et al.* (4) found that esterase patterns show distinct changes with a general tendency towards an age dependent increase of the number and intensity of esterase bands.

In this paper, polyacrylamide "disc" gel electrophoresis with and without inhibitors has been used to study esterases in chicken and rat tissues during embryonic development. The electrophoretic studies were supplemented with measurements of the total esterase activity in all tissue specimens used for electrophoretic experiments. The results of these studies show that the changes in esterase patterns were both species and organ specific. The most complex and extensive changes were observed in rat tissues. The wide range of these esterase specific activities during the course of embryonic development, however, suggest some limitations in the interpretation of impure zymogram preparations since protein concentrations may influence the electrophoretic mobility.

Materials and Methods. All tissues were removed immediately after sacrificing the an-

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