KINETICS OF MEGACARYOCYTES PROLIFERATION*

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L. E. Feinendegen, N. Odartchenko, H. Cottier, and V. P. Bond[1]

Medical Research Center

Brookhaven National Laboratory

Upton, L. I., New York

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Introduction

Studies on the proliferation of megacaryocytes in normal animals have, in the past, been based mainly on characteristics of the cells in various stages of differentiation.[2,8,12] Estimates of megacaryocyte proliferation have been made namely on the basis of the observed pattern of distribution of cells of different maturity following damage by means of physical or chemical agents, such as cytotoxic drugs or irradiation.[7,5,10] The effects of total body irradiation (200 to 450 r) on the megacaryocytes and platelets in the rat manifest themselves as a decline of the number of the megacaryoblasts less than one day after irradiation, and as a fall in platelets observed starting within 5 days thereafter. In the recovery period, the rise in megacaryocytes precedes the rise in platelets by about three days. The fall in megacaryocytes after irradiation was ascribed to a block in cell division and cell death in the "stem" cell precursors of the megacaryoblasts.[10]

DNA is considered to be stable in most proliferating cells. Its specific precursor, thymidine, labeled with tritium has so far little been used to study megacaryocytic proliferation.[5] In most cell systems studied, the short availability time of thymidine was shown to be of advantage for pulse labeling those cells which are in their phase of DNA synthesis, regardless of its duration.[3] If, within a morphologically classified group of cells, incorporation of H^3 thymidine is never observed soon after the injection of the tracer, one may attribute to this group the functional term: "initially non-labeling". As long as one is able to differentiate on morphological grounds, an initially non-labeling group from an initially labeling group of cells, it is possible to

describe the transit of initially labeled precursor cells through a morphological stage corresponding to the initially non-labeling group of progeny cells, and the kinetic behaviour of precursor cells prior to influx.[4] This mode of evaluating kinetics of a cell system in a physiological steady state condition was applied to the megacaryocytic series in rat bone marrow. At least 2/3 of all of these cells were found to comprise the initially non-labeling group, and the data indicate that the cells recognized to belong to this megacaryocytic series renew within approximately 40 hours. The corresponding data from a preceding pilot experiment on 9 rats were confirmatory. The more detailed analysis of the following experiment is presented here.

Materials and Methods

Female Sprague-Dawley rats, 3 months old, were kept in separate cages on Purina Chow and water ad libitum before and during the experiment. Tritiated thymidine obtained from Schwarz BioResearch Laboratories, with a specific activity of 1.9 C/mM, was used in a concentration of 1 mC per ml.

Ten rats were injected in the tail vein, with H^3thymidine 0.5 μc per gram of body weight. The animals were sacrificed under ether anesthesia at 1, 4, 8, 12, 18, 24, 36, 48 hours, 3 and 4 days after injection, one animal per time interval. The tissue was cleaned from the femurs, the bone was split with a scalpel, and bone marrow was brought on a regular glass slide with the help of a small paint brush moistened with rat serum.

The dried smears were fixed in methyl alcohol for 10 minutes and subsequently covered with liquid film emulsion NTB-2 (Eastman Kodak). The autoradiographic exposure time was 10, and in a second set, 20 days. The developed and dried preparations were finally stained with Giemsa buffered at ph 6. The autoradiograms were evaluated by light microscopy. The percentage of labeled cells and the number of grains over each cell nucleus, in the megacaryocytic series was registered. Background was corrected for.

The following subgroups were differentiated on the basis of morphological characteristics.

Group 1. Earliest recognizable immature forms, having a deep basophilic cytoplasm without granulations, a high nucleo-cytoplasmic ratio, and a slightly indented nucleus with a relatively granular chromatin. This cell form is generally the smallest amongst the recognizable cells of the megacaryocytic series, but definitely larger than the rest of the bone marrow cells.

Group 2. More differentiated, still immature forms, having a bluish cytoplasm with occasional azurophilic granules, a higher cytoplasmic nuclear ratio and a more indented nucleus with granular chromatin. The cell size is larger than in Group 1.

Group 3. Further differentiated forms, having a slightly bluish cytoplasm with numerous sequences of granules, a still higher cytoplasmic nuclear ratio and a multilobulated nucleus with denser and coarser chromatin. The cells of this group include also the most mature forms with dark and almost pyknotic nuclei, light cytoplasm and at times irregular contours. The cells of this group are the largest in the marrow.

Group 3a., as a subgroup of Group 3; the most mature cells of Group 3, as described above.

The above classification of cells is arbitrary. It would have been desirable to divide the cells into an "initially labeling" and an "initially non-labeling" group, as has been done with red cell precursors (4). This could not be accomplished as definitively with megacaryocytes because the criteria of maturity are not as well defined. Groups 3 and 3a clearly are "initially non-labeling"; group 1 is clearly "initially labeling". It cannot be said how many of morphological group 2 might belong in either functional category.

Results

Changes in percent labeling and grain count data, as a function of time after H^3thymidine injection are given in Figures 1 and 2. Figure 3 shows the percent labeling on autoradiograms exposed not 10 but 20 days. With increased exposure time the autoradiographic efficiency rises, which is pertinent to the validity of the percentage labeling. However, the relative distribution of grain density is not influenced by a change of autoradiographic efficiency beyond a minimal threshold.

1) Percent of initially labeled cells.

One hour after injection of H^3thymidine, approximately 35% of the cells in Group 1 are labeled, in contrast to only 10% in Group 2. The significance of the labeling of cells in Group 2 is questionable because of possible overlaps with the most immature forms. No cells of Group 3 are seen to be labeled.

2) Change of percent labeling with time after injection of H^3thymidine.

The percentage of labeled cells in Group 1 rises rapidly with time, reaches approximately 80% at 8 to 12 hours and nominally 100% at 24 to 36 hours. The percentage of labeled cells in Group 2 shows a rise similar to the one seen in Group 1, but approximately 8 to 12 hours later.

The percentage of labeled cells in Group 3 begins to increase significantly at 18 hours in a rather linear fashion and reaches nominally 100% at 48 hours. The cells of Group 3a plotted separately, start to show label from 30 hours on. Nearly 100% of them are labeled at 48 hours. From then on, almost all cells registered remain labeled for the entire time of observation, e.g. to 4 days following injection of the tracer.

3) Relative intensity of labeling as determined by grain counts over single cells.

The average intensity of labeling is markedly higher in the most immature megacaryocytic forms than in the rest of the bone marrow cells for a period of several hours following injection (about 2:1 at least). This initially high grain count appears with time in successive stages of maturation. While the labeling intensity of the most immature forms declines as a function of time, a corresponding fall in labeling intensity occurs later in the more mature cells. At three days after injection all cell groups show a markedly lower mean grain count.

4) Mitosis in megacaryocytic cells.

No sequence of mitotic events ranging from prophase to telophase was observed in cells recognized to belong to the megacaryocytic series.

However, chromosome arrangements comparable to multicentric metaphase were occasionally seen within the most immature cell group (Figures 4 and 5), as far as one is able to define stages of maturation in cells undergoing mitosis. It is obvious that the number of chromosomes is greatly increased compared with mitosis in other cell groups in the bone marrow. No mitoses have been observed in the more differentiated and in the most mature forms.

5) Relative number of cells observed in the different groups.

The ratio of the number of cells in Group 1 to all cells counted in the megacaryocytic series was 0.157 $\pm$ 0.039 (1Std. dev.), for Group 2 it was 0.193 $\pm$ 0.041, and for Group 3, 0.646 $\pm$ 0.047. The relative distribution of cells in the 3 groups was therefore, approximately 1:1.2:4.1.

Discussion.

The interpretation of results is based on several assumptions:

a) the availability time of H^3 thymidine is short for the megacaryocytic cell line.

b) all cells synthetizing DNA during availability time of H^3 thymidine become labeled.

c) Labeled DNA is stable until cell death.

d) The administration of H^3 thymidine in a dose of 0.5 μC/gm body weight does not interfere appreciably with the physiological steady state condition. There is no evidence that these assumptions are incorrect.

The evaluation of the data is based largely on the observation of initially labeled cells maturing into a stage corresponding to the initially

non-labeling group of cells.

The initially unlabeled group comprises the more mature elements, which enclose about 2/3 of the total number of cells recognized as belonging to the megacaryocytic series. The figures given here for the size of this group is a lower limit, since a fraction of the cells registered in Group 2 may in fact be closer functionally to Group 3. The origin of a fraction of labeled cells is found within Group 1, the most immature cells among recognizable megacaryocytic elements.

Only about 35% of the cells belonging to Group 1 are labeled initially, while approximately 90 to 100% of all observable megacaryocytic cells become labeled with time. This is most easily explained on the basis of an influx of labeled cells from a group of precursors which is not recognized to belong to the megacaryocytic series. (see 1, 6, 9, 11.).

With consideration of the above interpretation some deductions may be made with regard to the unrecognized precursor cells from the kinetic behaviour of their progeny. The nearly total labeling of the megacaryocytic series by 48 hours after H^3 thymidine injection indicates that the unrecognized precursor cells were essentially all in the phase of DNA synthesis some time before their influx into the megacaryocytic series. Since the labeling index remains 90-100% during days 3 and 4 after injection, the process of DNA synthesis in the unrecognized precursor cells appears to be nearly continuous, at least over the period of 1 to 3 days prior to their entry into the recognizable megacaryocytic series. If there are rest periods during this development, they must be very short.

The grain count data are not considered to be quanititative. Therefore

no attempt is made to differentiate phases of DNA synthesis leading to cell division from those accompanying endomitotic growth. However, the generally high grain count in the recognizable immature cells (Group 1 and part of Group 2) soon after tracer injection, indicates that in these cells more DNA units are reduplicated than in the rest of the immature bone marrow cells at the same time. This is compatible with polyploidy, which may further increase by additional DNA synthesis. Further support for this view is seen in the random distribution of label among the chromosomes in polyploid mitotic figures of recognizable immature megacaryocytic cells as compared to mitoses of other bone marrow cells. The moderate number of labeled cells within Group 1 as observed soon after tracer injection may indicate that not all of the most immature recognized elements undergo DNA synthesis; it may perhaps be then, that the time of DNA synthesis in these cells is short, or that the cells have an unusual mechanism of DNA synthesis.

In a physiological steady state condition, the size of a morphologically defined, non-dividing cell group remains constant. Therefore, the increase of labeled cells per unit of time in the initially non-labeling group allows determination of the transit time within the boundaries of that group. Since the number of labeled cells connected can only be equal to, or underestimate the true number, the values obtained constitute an upper limit. Furthermore, it must be considered that not all cells necessarily mature at the same rate. The majority of the initially unlabeled cells is replaced by labeled cells within approximately 18 to 48 hours. This means that the transit time for the

majority of cells within that group is of the order of 30 hours, or less. The transit time for all cells in this group ranges from 12 to 48 hours. The distances in time between the midpoints of the rising slopes of percent labeling of groups 1, 2 and 3 give the approximate respective median transit times, namely 8-9 hours for group 2 and 25 hours for group 3. These observed transit times correspond to the sizes of the different morphological groups which are 1.2 to 4.1. Knowing the correlation of transit times to size of morphological cell groups, it is calculated that the median transit time of Group 1 is approximately 6 to 7 hours (8: 1.2). The total transit time for the majority of the cells through the entire observable megacaryocytic series is therefore in the order of 40 hours.

Conclusion

The kinetics of the megacaryocytic cell line of the rat bone marrow were studied using tritiated thymidine as a cell label. The changes in the percentage of labeled cells as a function of time after injection of the tracer were registered separately for arbitrarily chosen successive recognizable stages of megacaryocytic differentiation. Emphasis is put on the development of initially labeled cells into a stage of maturation corresponding to initially non-labeling cell forms. The following results were obtained:

1. The transit time for the most immature recognizable stage of megacaryocytic development to megacaryocytic disintegration is approximately 40 hours.

2. Evidence was obtained that the recognizable megacaryocytic elements originate from unrecognized precursors which continuously synthesize DNA for a period of at least 1 to 3 days prior to maturation into recognizable megacaryocytic precursors.

3. The immature megacaryocytic cells able to synthesize DNA take up more H^3 thymidine than the rest of the bone marrow cells, this is consistent with polyploidy.

4. The process of nuclear lobulation is not accomplished by the end of DNA synthesis, thus being comparable to nuclear segmentation in neutrophilic granulocytes. This latest phase of maturation is relatively long (approximately 25-30 hours) as compared to the phase during which recognizable megacaryocytic precursors are able to synthesize DNA (less than 15 hours).

References

1. Ackerman, G. A., Knouff, R. A., Blood, 1960, Vol. 15, 267.

2. Baserga, A., Haematol., 1948, Suppl. 32.

3. Bond, V. P., Fliedner, T. M., Cronkite, E. P., Rubini, J. R., Robertson, J. R., 188, in Stohlman, F. Jr., : Kinetics of Cellular Proliferation, Grune and Stratton, New York, 1959.

4. Bond, V. P., Odartchenko, N., Cottier, H., Feinendegen, L. E., Cronkite, E. P., In: Erythropoietin and regulation of Erythropoiesis, San Francisco, 1962 (in press).

5. Cronkite, E. P., Bond, V. P., Fliedner, T. M., Paglia, D. A., Adamik, E. R., 595, In: Johnson, S. A., Blood platelets, Little and Brown, Boston, 1961.

6. Jones, O. P., Anat. Rec., 1960, Vol. 138, 105.

7. Lindell, B., Zajicek, J., Experientia (Basel), 1957, Vol. 15, 27.

8. Odell, T. T., Jr., Tausche, F. G., Gude, W. D., Amer. J. Physiol., 1955, Vol. 180, 491.

9. Pease, D. C., Blood, 1956, Vol. 11, 501.

10. Simpson, S. M., Int. J. Rad. Biol., 1959, Vol. 2, 181.

11. Yamada, E., 1957, Acta Anat., 1957, Vol. 29, 267.

12. Zajicek, J., Acta Physiol. Scand., 1957, Vol. 40 (Suppl. 138).

13. Zajicek, J., Zeuthen, E., Exper. Cell Res., 1956, Vol. 11, 568.

Footnotes

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Legends to Figures

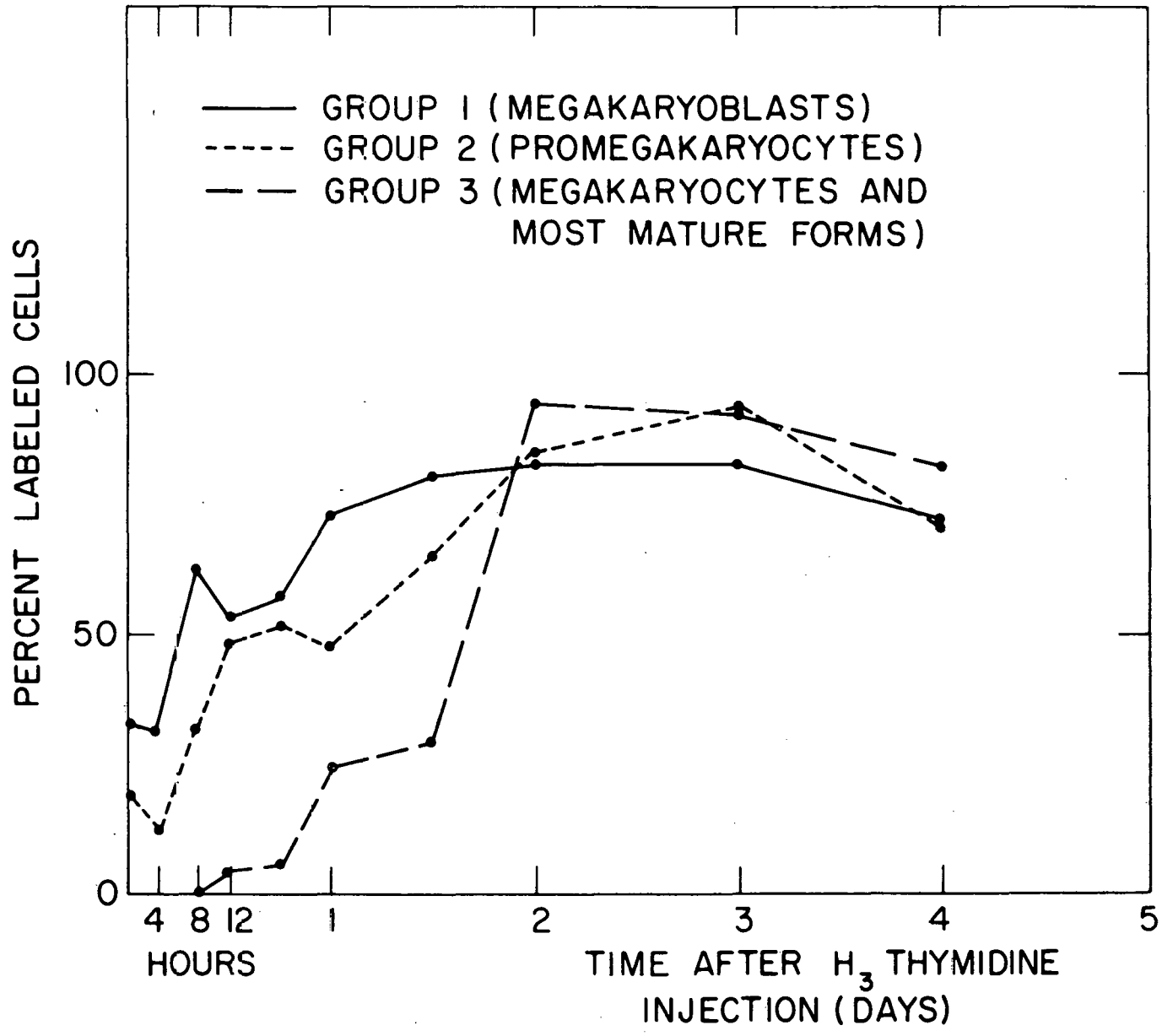
Figure 1: Percent labeled cells in megacaryocytic series, at different times after single i.v. injection of H^3 thymidine. (autoradiographic exposure 10 days.) Note that cells in group 3 do not label initially. Neg. No. 6-608-62

Figure 2: Mean grain count of cells in megacaryocytic series, at different times after single i.v. injection of H^3 thymidine. (autoradiographic exposure 10 days.) Neg. No. 6-605-62

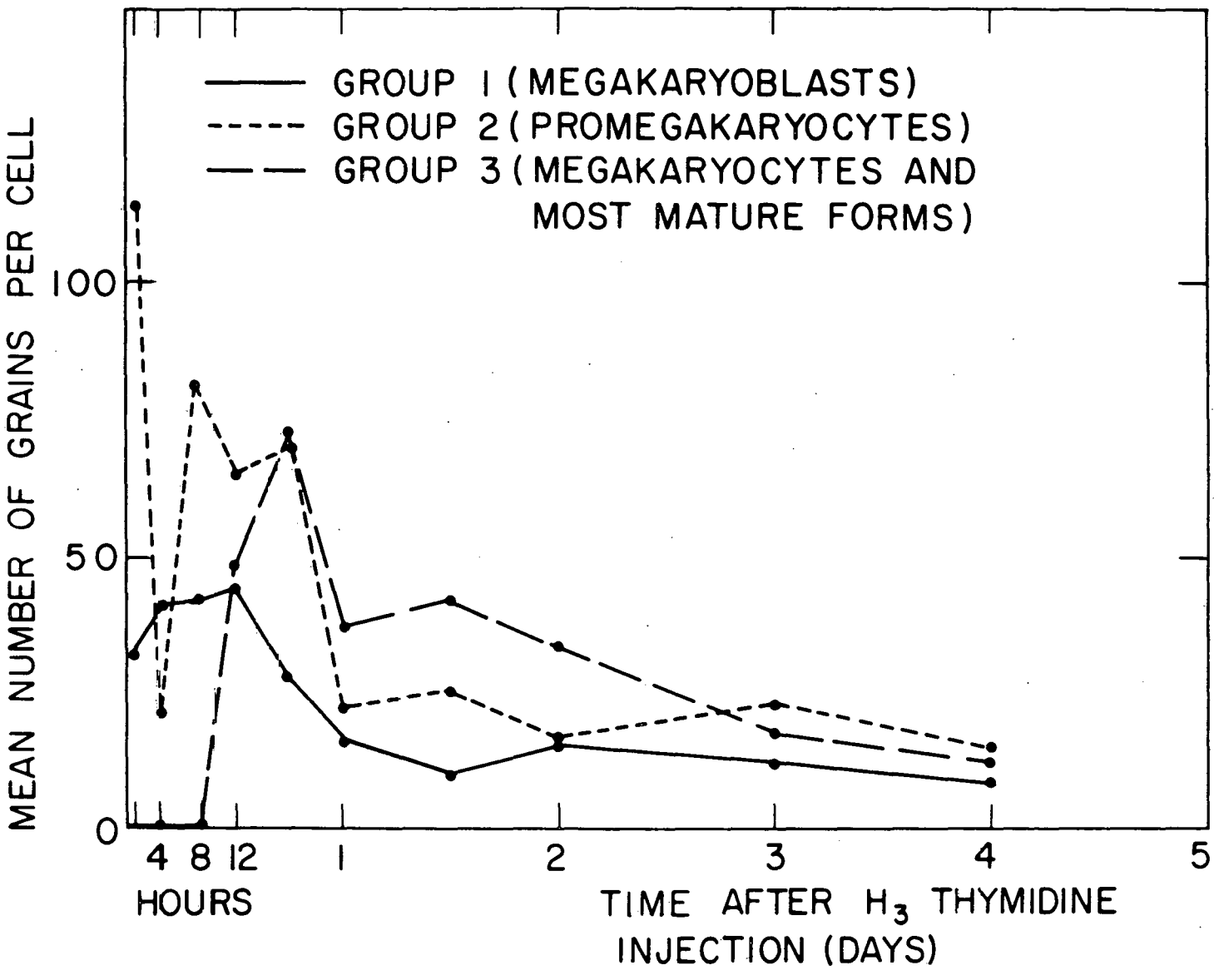
Figure 3: Percent labeled cells in megacaryocytic series, as in Fig. 1, but autoradiographic exposure was 20 days. Neg. No. 6-606-62.

Figure 4: Mitosis in early megacaryocytic cell, note increased number of chromosomes. Neg. No. 6-850-62

Figure 5: Mitosis in early megacaryocytic cell, note random distribution of grains. Neg. No. 6-849-62



BNL Neg. No. 6-608-62 Fig. 1



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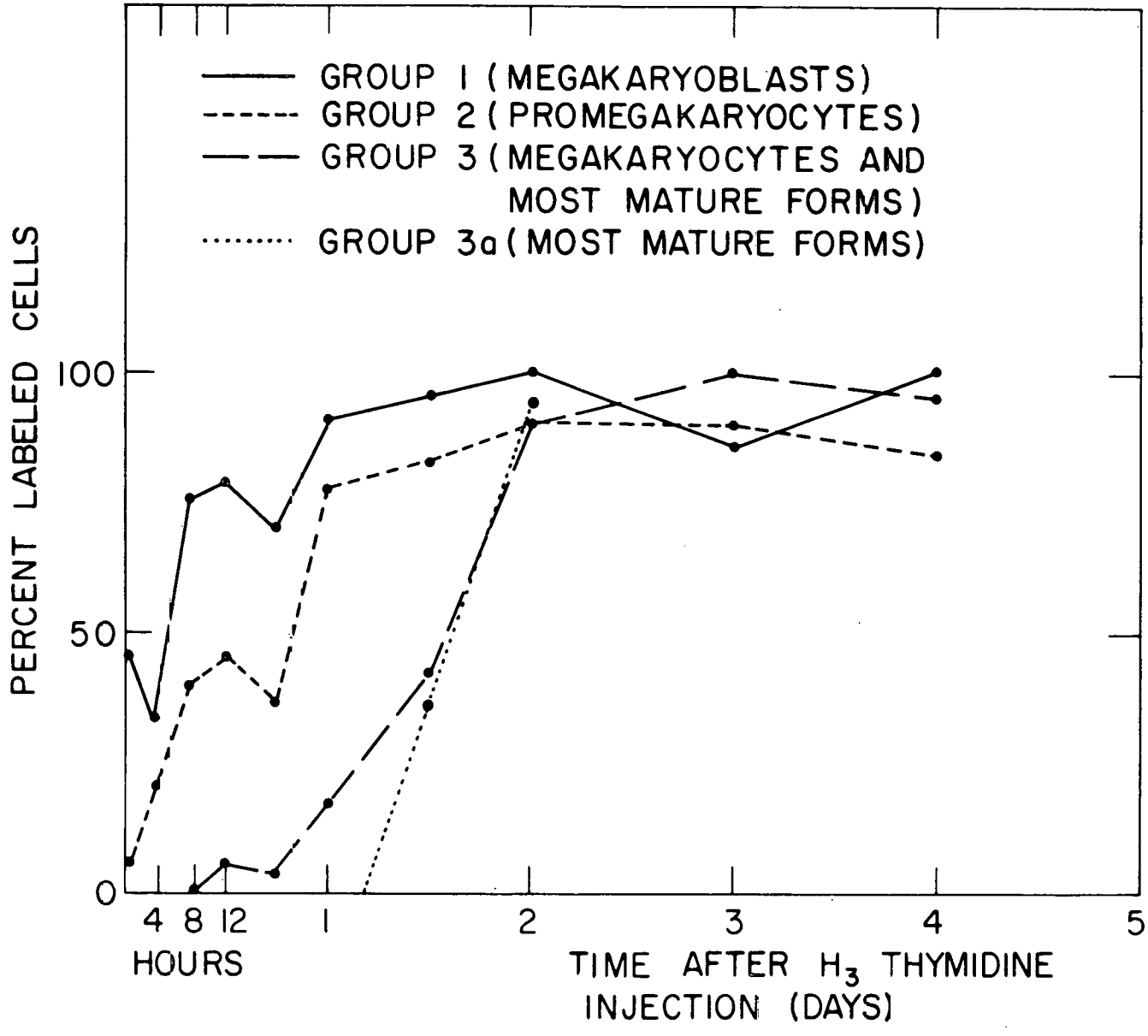

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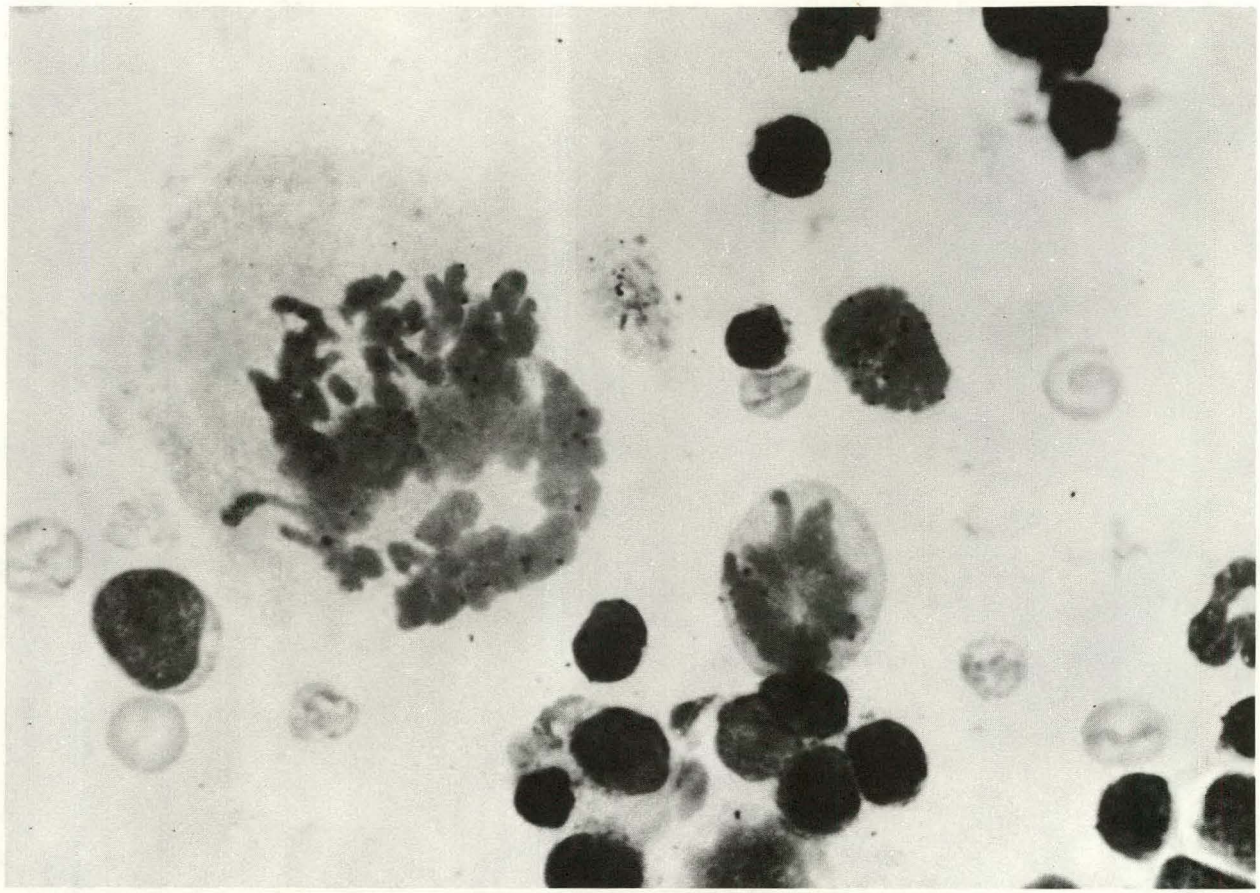
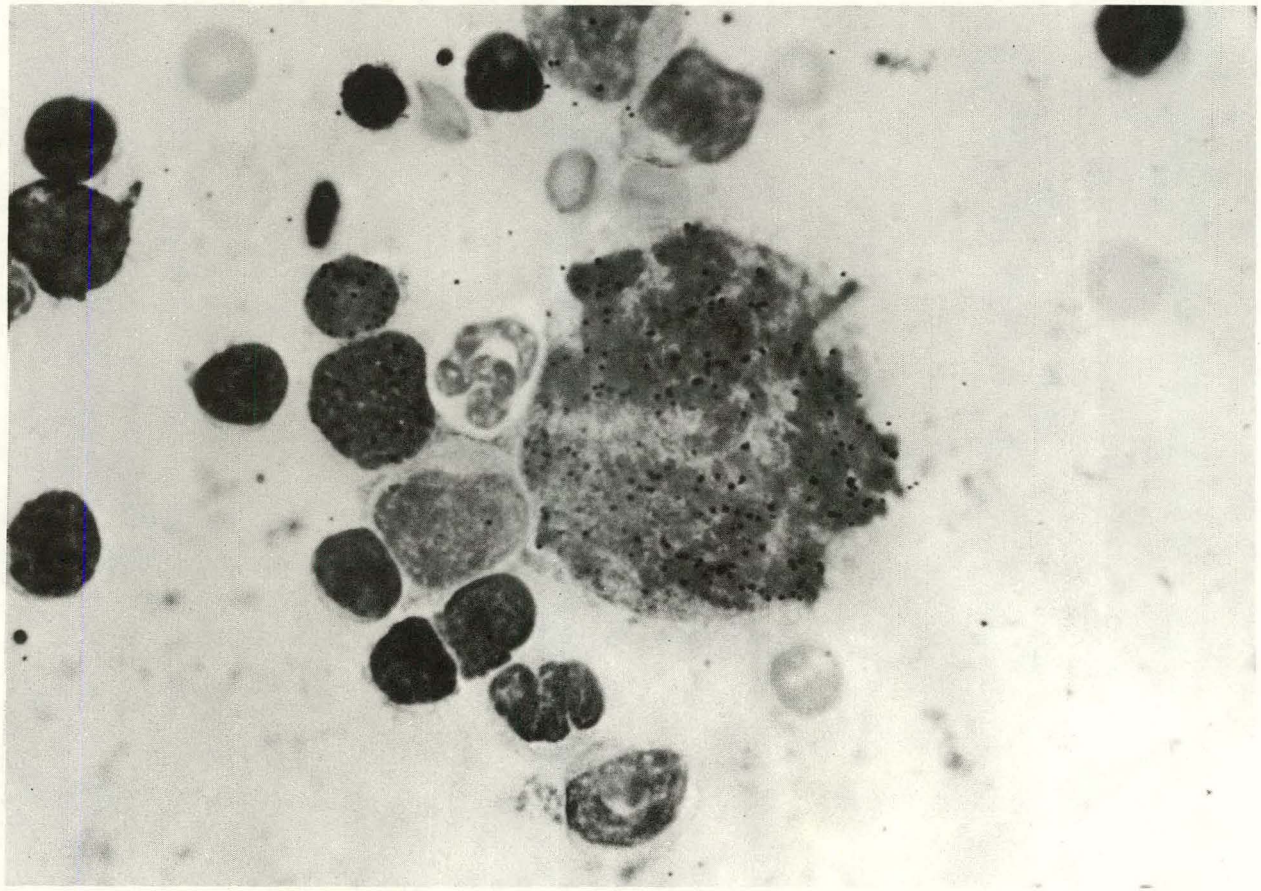


BNL Neg. No. 6-850-62 Fig. 4



BNL Neg. No. 6-849-62 Fig. 5