The Use of the Supravital Method in a Study of Cells Susceptible to Damage in Smears of Normal and Regenerating Bone Marrow

By C. Rosse

Damaged cells or "smudge cells" are a common feature of bone marrow smears, although in clinical reports only rarely is any attention paid to them. In smears of normal marrow at least 10-15 per cent of all cells are unidentifiable because of damage. This represents the greatest source of error in all quantitative estimations of cell populations which are based on counts in fixed smears.

The number of damaged cells varies considerably under various experimental and pathologic conditions. During recovery from sublethal irradiation a threefold increase in damaged cells was reported in the guinea pig and comparable values have been obtained after sublethal doses of nitrogen mustard. Furthermore, a considerable proportion of damaged cells are in DNA synthesis.

An evaluation of what types of cells are most susceptible to damage would be of considerable importance to workers in the clinical, as well as in the experimental field of hematology, particularly since during the process of marrow regeneration the rate of cell damage in smears seems to be increased. The present study was prompted by these considerations and it was intended to answer two questions: 1) What are the cells which are damaged in significant numbers in normal marrow? and 2) Is there any change in the proportion of damage in individual cellular compartments during marrow regeneration?

Materials and Methods

Method

It is well known that there are very few damaged cells in wet preparations. Cell damage seems to occur during the process of making the smear. Consequently, if differential counts prepared on supravitally stained wet preparations were compared with counts on fixed dry smears made from the same cell suspension, the difference in each cell group should represent the numbers of cells damaged in each cell group. Such comparisons can only be made, however, if it can be shown that: a) cell classification and b) cell distribution correspond on the two types of preparations.

A. Cell classification. The correlation in cell morphology was studied by comparing the appearance of cells on wet preparations stained supravitally with neutral red and pinacyanin, and on air-dried methanol-fixed smears stained with NacNeal's tetrachrome stain. The
SUPRAVITAL METHOD AND CELL STUDY

supravital staining method employed was essentially Schwind's modification of Sabin's original technic.

B. Cell distribution. It was assumed that in wet preparations cell distribution was random, and the supravital myelograms were based on the classification of 1000 nucleated cells. In smears, however, there are obvious distortions in cell distribution. In order to test whether 1000 cells were representative of the overall cell distribution in smears, differential counts of 1000 cells from each of 6 animals were compared with the differential counts of 6000 cells in each case.

Animals and Procedure

To answer the first question posed by this study, the bone marrow of 15 normal guinea pigs was examined. To answer the second question 40 guinea pigs were given 150 r total body irradiation from a therapeutic 60Co unit and their marrow was studied over a period of 20 days, 4 animals being killed at 2 day intervals. The guinea pigs were male albinos of Hartley strain, and weighed approximately 400 Gm. at the time of sacrifice. Six of the normal and all of the irradiated animals received an intracardiac injection of 3H-thymidine (1 µc./Gm. body weight; specific activity 3 c./mM) 30 minutes before exsanguination.

A uniform cell suspension was obtained by mechanical agitation of humeral bone marrow in autologous serum. Supravital and fixed smear preparations were made from this suspension.

The incidence of 3H-thymidine-labeled damaged cells was determined on radioautographs coated with Kodak NTB2 emulsion, 500 damaged cells being counted in each animal.

The Supravital Method

The method for preparing the supravitaly stained preparations is very simple. A 0.4 per cent solution of neutral red and a 0.03 per cent solution of pinacyanol, each in absolute alcohol, are kept as stock solutions. One cc. of each solution is added to 5 cc. of absolute alcohol and mixed thoroughly immediately before preparing the slides. Chemically cleaned slides are flooded with this stain. The stain is immediately drained off and the slide rapidly air dried. Only slides with a thin and even coating of dry stain give satisfactory and uniform staining. In order to achieve evenness of the film the excess stain may have to be wiped off the edges of the slide before the solution completely evaporates. It also has to be ensured that dust particles do not adhere to the film because they will prevent the even spread of the cell suspension. They may be whisked off the slide while the film is still wet. Slides prepared in this way may be stored for a number of days.

The cell suspension used in this study contained approximately 50,000-100,000 cells per cmm. A drop of cell suspension is placed on a chemically cleaned coverslip and the coverslip is inverted onto the stain covered slide. The drop must spread rapidly and evenly to the periphery of the coverslip without trapping any air bubbles. The correct size of the drop is important in obtaining the desired depth of the preparation. Uneven spread indicates dirty glassware, dust or too small a drop of suspension.

The preparation is sealed by applying melted wax around the coverslip with a soft hair brush. The preparation is then incubated at 38 C. for 10-15 minutes to allow time for staining and then it is examined under bright field illumination with an oil immersion objective. In a good preparation cells are flattened by the weight of the coverslip to such an extent that there is very little depth in focus over each cell. With experience, in a well-stained preparation a differential count of 1000 cells may be completed in 30-40 minutes. Cells begin to deteriorate within 1½ hours after making the preparation and become overstained in increased numbers.

Statistical Analysis of Data

Mean values and standard deviations were calculated. Student's "t" test was employed for the statistical comparison of data. Differences were regarded as significant if p< 0.05.
RESULTS

A. Correlation of Cell Classification

The appearance of cells on supravitally stained preparations in general corresponded to classical descriptions10-12 with the following exceptions:

a) The "primitive cell"10 could not be identified with any degree of certainty, b) Classification of supravitally stained lymphoid cells was based on descriptions of Rosse and Yoffey.13 c) In view of the variability of distribution and configuration of mitochondria, no special significance was attributed to these criteria, particularly since they did not have any relation to morphologic features of fixed cells.

The comparison of cells stained supravitally and after fixation illustrates in Figures 1-3 that a sufficiently good correspondence exists in the morphologic features of all groups of cells to permit a quantitative comparison on the two types of preparations.

The nuclear morphology of supravitally stained cells with yellow cytoplasm of varying intensity indicated that they corresponded to polychromatic and orthochromatic erythroblasts of the tetrachrome stained smear ("late erythroblasts," Fig. 1). In supravital preparations the identification of "early erythroblasts" was aided by a greenish tinge of their cytoplasm and also by the neutral red precipitation of the basophilic substance (Fig. 1) described by Sabin.14 The close resemblance of these cells to early erythroblasts of the fixed smear is evident in the illustrations.

The very good correspondence in the morphology of supravitally stained

![Fig. 1.—Erythroblasts in supravitally stained (A) and fixed smear preparations (B). X 1300.](image)

Although due to absence of color hemoglobin is not demonstrated, the different maturation stages of erythroblasts are readily recognized in the living as well as in the fixed preparation. E, early erythroblasts. Late erythroblasts in the lower part of A and upper part of B are not labeled. The identification of early erythroblasts in the supravital preparation is aided by the precipitation of the basophilic substance by neutral red. T, transitional cell; U, unknown cell.
and fixed myeloid cells (Fig. 2) indicated that the distinction and different classification of these cells when supravitally stained\textsuperscript{10} is of no practical importance. The same classification was used, therefore, in both types of preparations.

![Fig. 2.—Myeloid cells and monocytes in supravitally stained (A) and fixed smear preparations (B). X 1300.](Image)

The specific granulation and nuclear morphology in all maturation stages of granulocytes shows close correspondence in cells stained supravitally and after fixation. The granules of neutrophil myelocytes (MY) may be compared with those of eosinophil myelocytes in the upper part of Figure 1A and B. MB, myeloblast. M in A is identified as a young monocyte by its nuclear morphology and the rosette of neutral red vacuoles, which are irregular and larger than the granules of neutrophils. Within 10–20 minutes they increase in size to resemble those of the more mature monocyte in Figure 3A. Fixed monocytes are shown in Figure 2B.

The correspondence in the morphology of the two main groups of marrow lymphoid cells (pachychromatic small lymphocytes and leptochromatic transitional cells) in supravitinal and fixed smear preparations is shown in Figure 3. Monocytes are illustrated in Figure 2 and Figure 3A. The rosette of neutral red vacuoles aided their identification on supravitinal preparations. Although numerous criteria have been described from time to time to subdivide the group of blast cells both on supravitinal and fixed smear preparations (see review by Downey\textsuperscript{15}), these criteria were found not consistently reliable. Proerythroblasts, however, were classified with “early erythroblasts” since they possessed distinct features on both types of preparations. Although a numerical comparison of miscellaneous cells (plasma cells, megakaryocytes, reticulum cells and macrophages) was not attempted, their morphology could be readily correlated when stained supravitally or after fixation.

\textbf{B. Cell Distribution On Smears}

The “t” values for the comparison of individual cell groups in myelograms based on 1000 and 6000 cells ranged from 0.01 to 0.6 except in the case of
The high nuclear cytoplasmic ratio of small lymphocytes (L) and transitional cells (T) contrasts with the abundant cytoplasm of the myelocyte (MY) and myeloblast (MB). In both preparations the clumped chromatin of small lymphocytes is readily distinguished from the leptochromatic nuclei of transitional cells. UB, in spite of its similarity to transitional cells, is regarded as an unknown blast because of its more abundant cytoplasm. D, damaged cell.

“eosinophils + basophils” and the group of miscellaneous cells where significant differences were observed (t = 3.2 and t = 5.0, respectively). With the exception of these small cell populations, therefore, a myelogram based on the count of 1000 cells is as representative of the overall cell distribution on fixed smears as a myelogram based on 6000 cells.

C. Comparison of Supravital and Fixed Smear Myelograms

Having shown that cell classification and cell distribution correspond on the two types of preparations the two myelograms may now be compared meaningfully.

Damaged cells (Fig. 4). The number of damaged cells varied greatly in the smears after irradiation, but it remained consistently low in the supravital preparations. In the normal marrow 167 ± 58 cells per 1000 were damaged on smears, compared to 11 ± 3 on supravital preparations (t = 10.4; p < 0.001).

The highest proportion of cell damage was observed on the tenth day post-irradiation (347 ± 130 per 1000). By the twentieth day the number of damaged cells on smears had returned to the normal level (157 ± 55 per 1000). Statistical analysis showed that the number of damaged cells on smears significantly exceeded their number on supravital preparations throughout the experiment.

Erythroid cells. In normal marrow the numbers of erythroid cells closely corresponded in supravital and fixed smear preparations (t = 0.82). After irradiation the same trend was followed in both myelograms up to the tenth
Fig. 4.—Changes in the number of damaged cells in fixed smears and supravitally stained wet preparations in normal and regenerating guinea pig bone marrow following 150 r total body irradiation. (Mean ± standard deviations).

day. Although an obvious difference was observed on the twelfth and fourteenth days, statistically it was not significant (t = 1.9). On the sixteenth and eighteenth days, however, the supravital count (356 ± 27 and 397 ± 51) significantly exceeded the fixed smear count [(220 ± 56 and 202 ± 87) t = 4.4 and 5.3 respectively; 0.01 > p > 0.001]. This indicates that on the fixed smear as many as 40–50 per cent of erythroid cells are missed at this phase of marrow regeneration. Values similar to normal marrow were obtained on the twentieth day, the difference in the two counts being no longer significant (t = 0.9).

Myeloid cells. In normal marrow the supravital count of myeloid cells significantly exceeded their number on smears (t = 2.9; 0.01 > p > 0.001). The data suggest that 20–25 per cent of myeloid cells are not recognized on the smear because they have been damaged. Similar trends were followed after irradiation in both preparations, the supravital count remaining higher throughout the experiment. The difference was statistically significant on the 4th and 6th days (t = 4.2; 0.01 > p > 0.001 and t = 2.8; 0.05 > p 0.02 respectively), on the twelfth day (t = 3.8) and again on the eighteenth and twentieth days (t = 2.8; 0.05 > p > 0.02 and t = 2.5; p < 0.05 respectively). On the eighteenth and twentieth days approximately 55 and 40 per cent of myeloid cells were damaged on the smears.

Small (pachychromatic) lymphocytes. In normal marrow the values for small lymphocytes were the same in the two myelograms. No statistically significant difference was observed between the supravital and fixed smear counts of these cells throughout the experiment, except on the eighteenth day, when the fixed smear count was higher (t = 2.5; p < 0.05). Thus small lymphocytes of the marrow were not susceptible to cell damage during smearing in this experiment.
Transitional cells showed a different pattern of response than the smaller pachychromatic cells. In the normal marrow the supravital and fixed smear counts were in agreement statistically (t = 1.35). The supravital count was significantly higher, however, on the fourth and tenth post-irradiation days (t = 4.8 and 3.4, respectively). On the tenth day nearly 60 per cent of transitional cells damaged on the smears. After the twelfth day identical trends were followed in both myelograms, the two counts remaining in close agreement.

Monocytes. In normal marrow, apart from damaged cells, the greatest difference in supravital and fixed smear counts was observed in monocytes. The number of monocytes in supravital preparations was 49 ± 13 per 1000 cells compared to 24 ± 4 on smears (t = 4.2, p < 0.001). Following irradiation, however, this difference fell below the level of statistical significance and only after the 12th day were significant differences observed again.

Blast cells. In normal marrow the supravital and fixed smear counts of blast cells were comparable (t = 0.52). On the eighth, tenth and again on the fourteenth and sixteenth days after irradiation significantly higher numbers of blast cells were observed in supravital preparations than in smears.

The comparison of supravital and fixed smear myelograms shows that in normal bone marrow the majority of damaged cells are myeloid cells, although the most fragile cell appears to be the monocyte. Erythroblasts, small lymphocytes and transitional cells do not damage in significant numbers. Figure 5 summarizes the findings during recovery from sublethal irradiation. In it the “t” values of the difference between supravital and smear counts have been plotted for those cell populations which showed a significant rate of damage.

![Graph](image-url)

Fig. 5.—“t” values of the difference between supravital and fixed smear counts of erythroid, myeloid and transitional cells in normal and regenerating guinea pig bone marrow following 150 r total body irradiation.
Transitional cells and myeloid cells made up the bulk of damaged cells on the fourth day postirradiation and again on the tenth and twelfth days. On the sixteenth and eighteenth days, however, the majority of damaged cells were erythroblasts. Myeloid cells contributed to the damaged cell pool significantly on the eighteenth and twentieth days.

**D. DNA Synthesis in Damaged Cells**

The percentages of damaged cells incorporating $^3$H-thymidine 30 minutes after its injection are shown in Figure 6. In normal marrow 21.1 ± 1.9 per cent of damaged cells were labeled. There was a decline in this percentage during the first 4 days. On the sixth day a temporary but significant increase occurred (24.0 ± 3.1 per cent). The percentage of labeled damaged cells became markedly elevated again on day 14 (27.9 ± 1.7 per cent) and remained at this level on days 16 and 18, indicating that at this stage of marrow regeneration a large number of cells capable of proliferation could not be recognized in smears because of cell damage. On day 20, 19.3 ± 2.8 per cent damaged cells were labeled, which is comparable to normal marrow.

**DISCUSSION**

The few available reports indicate that the proportion of cell damage in normal human marrow is comparable to that in the guinea pig.\textsuperscript{1,16-18} In a variety of pathological conditions the proportion of cell damage varies within the range of normal\textsuperscript{1} with the exception of monocytic leukemia where 50 per cent of the cells were found damaged. This exception is of interest in view of the present finding that the monocyte is the most fragile cell in normal guinea pig marrow.

The problem created by damaged cells in experimental hematology have been pointed out by a number of investigators.\textsuperscript{2,4,10-22} Previous attempts at comparing differential counts on supravital and fixed smear preparations\textsuperscript{21,23} did not give satisfactory results because an insufficient number of cells were counted\textsuperscript{21} or the cell suspension was not uniform.\textsuperscript{23}

The present study affords identification of damaged cells only on a quantitative basis and not on the individual cellular level. Since it was shown that myelograms could be compared on supravital and fixed smear preparations, it could be determined which cell compartments contributed significantly to the damaged cell pool. This was possible only for the larger cell populations when the myelograms were based on 1000 cells. To determine the cell damage among infrequent cell types (megakaryocytes, reticulum cells, myeloblasts, etc.) or in the subdivisions of the larger cell compartments, a greater number of cells would have to be counted. In spite of these reservations, however, the two questions posed at the beginning of this study could be answered with the use of this method.

The percentage of cell damage in the author's fixed smear preparations was comparable to percentages reported by a number of investigators for the same experimental animal. The absolute number of myeloid, erythroid and lymphoid cells is approximately equal in normal guinea pig marrow when the cells are counted on smears. According to the present study, the
susceptibility to cell damage in these populations varies considerably and therefore they do not contribute equally to the pool of cells unidentifiable because of damage. Although monocytes seemed to be the most susceptible to damage, the majority of damaged cells in normal marrow were, in fact, myeloid cells. About 20 per cent of damaged cells labeled with 3H-thymidine in normal marrow suggesting that a large proportion of the monocytes and myeloid cells which damaged were of the immature type still capable of proliferation.

During recovery from 150 r total body irradiation in the guinea pig the three major cell compartments commence their regeneration at different times. First lymphoid cells regenerate, showing the greatest rate of increase from the tenth to fourteenth day postirradiation. Erythroid regeneration commences on the fourteenth, and myeloid regeneration on the eighteenth-twentieth days. It is interesting to note that the number of damaged cells increased significantly in each cell compartment at the time when that compartment commenced its regeneration. On the tenth day approximately 60 per cent of transitional cells damaged. On the sixteenth and eighteenth days 40–50 per cent of erythroid cells, and on the eighteenth-twentieth days 40–55 per cent of myeloid cells damaged. Although the reason is not clear for the increased numbers of damaged myeloid cells on the twelfth day, their increase on the fourth and sixth days coincided with the occurrence of the abortive peak in this cell group. These observations are borne out by the changes in the percentage of damaged cells incorporating 3H-thymidine during the postirradiation recovery period. At the time of the abortive peak in myeloid regeneration, an increase was observed in the percentage of damaged cells synthesizing DNA (Fig. 6). A greater increase occurred again at the time of final recovery of erythroid and myeloid cells on days 14–18. During this later period around 30 per cent of damaged cells were labeled. In any cell population such a figure indicates a considerable rate of turnover and underlines the importance of the identification of these cells.

Harris et al. found in the guinea pig that on the thirteenth day postirradiation (150 r) 21–22 per cent of labeled cells were damaged. Bond et al. also observed the presence of labeled damaged cells in normal and pathological human marrows. Because of the intensity and frequency of labeling they considered the possibility that these cells may belong to the "primitive precursor pool." It is evident, however, from the data presented here that damaged cells are a changing population. The error introduced into cell quantitations based on fixed smears affects different cell populations during different phases of the recovery.

A correlation between the reappearance of fat and the increase in damaged cells in regenerating marrow has been reported. Histologic examination of the material in the present study confirmed this finding. Unpublished observations of the author showed, furthermore, that the addition of small amounts of fat to the cell suspension increased the proportion of damage on smears three- to fourfold. This would indicate that the suspending medium as well as the individual architecture of cells are determining factors in the susceptibility of cells to damage.
SUPRAVITAL METHOD AND CELL STUDY

Fig. 6.—Changes in the percentage of labeled damaged cells after labeling with \(^3\)H-thymidine in normal and regenerating guinea pig bone marrow following 150 r total body irradiation. (Mean ± standard deviation).

Evaluation of the Supravital Method

The method has been repeatedly reviewed and evaluated. The evaluation, however, was always made on a purely morphologic basis claiming the superiority or otherwise of the supravital method over fixed preparations. These claims have not met with universal acceptance, partly because illustrations of the method were almost invariably camera lucida drawings and not photographs.

Figures 1–3 illustrate that both the supravital and the fixed smear methods are equally suitable for demonstration of cell morphology. Furthermore, the appearance of cells closely corresponds in the two types of preparations. Supravitaly stained wet preparations examined under bright field illumination make cell identification considerably easier than examination of unstained living cells under phase contrast when the cell suspension is a heterologous one.

The disadvantages of a supravital preparation over the fixed smear are considerable: 1) It is not permanent. 2) It is not suitable for radioautography. The present study indicates, however, that the method can serve a useful purpose as a simple and reliable technic in modern quantitative hematology. Its advantages over fixed smears are: 1) the insignificant numbers of damaged cells; and 2) uniform cell distribution. The supravital method, therefore, should be used in conjunction with other technics which have an inherent error in these parameters.
SUMMARY

Cells susceptible to damage during smearing were identified in the different cellular compartments of normal and regenerating bone marrow of the guinea pig by comparing differential counts on fixed dry smears and on supravitaly stained wet preparations. The comparisons are considered valid since 1) the number of damaged cells was negligibly low in supravital preparations; 2) it could be shown that cell classification and 3) cell distribution corresponded on the two types of preparations. In smears of normal marrow the majority of damaged cells were myeloid cells, although the most fragile cell appeared to be the monocyte. In normal marrow, erythroblasts and lymphoid cells did not damage in significant numbers. During recovery from sublethal irradiation the number of damaged cells increased significantly in each cell compartment at the time when that compartment commenced its regeneration.

In contrast to normal marrow, at the time of lymphoid regeneration 60 per cent of transitional cells had damaged and at the start of erythroid and myeloid regeneration 40–50 per cent of erythroid and myeloid cells were concealed on smears by damage. Labeling with $^3$H-thymidine indicated that in smears of normal as well as of regenerating marrow, a large proportion of cells susceptible to damage in the various cell compartments was capable of proliferation.

ACKNOWLEDGMENTS

The author wishes to express his appreciation to Professor J. M. Yoffey for making funds accessible for the initiation of this study and also for his advice. He is also grateful to Dr. N. B. Everett for the generous provision of laboratory facilities, for his maintained interest and critical review of the manuscript. The valuable technical assistance of Mr. Michael Fisher is acknowledged.

REFERENCES

SUPRAVITAL METHOD AND CELL STUDY


The Use of the Supravital Method in a Study of Cells Susceptible to Damage in Smears of Normal and Regenerating Bone Marrow

C. ROSSE