The Effect of Nitrogen Mustard on Granulocytic Cells
as Observed by the Marrow Culture Technic*

EDWIN E. OSGOOD, M.D. AND I. T. CHU, M.D.†

(From the Division of Experimental Medicine, University of Oregon Medical School, Portland 1, Oregon)

The action and effect of the nitrogen mustard methyl-bis (β-chloroethyl) amine hydrochloride (HN₂) on hemopoietic and other tissues have been studied (3–6, 9–11, 13, 14) through in vivo observations and animal experiments, and the apparent similarity of the drug to roentgen rays and ultraviolet radiation has been noted. From studies made according to the marrow culture technic (15) reported in this paper, the effect of HN₂ on marrow cells of the granulocytic series has been compared under the same conditions with the observed effects of ionizing radiation (16–19) and of urethane (20). While the ultimate effects of these agents are similar, as seen in the peripheral blood of patients under treatment, observations of their mode of action in cultures of blood and marrow indicate that ionizing radiation, of all modalities tested, in doses under 400 r, inhibits or decreases the rate of cell division; urethane produces striking morphologic changes; while HN₂ kills all cells of the granulocytic series except the segmented neutrophils, without the occurrence of morphologic changes similar to those produced by urethane.

MATERIALS AND METHODS

Marrow cultures were set up as previously described (21), using sterile ascitic fluid as a source of protein, instead of human cord serum. A marrow culture was prepared from each of seven patients with miscellaneous diseases displaying essentially normal marrow pictures. About 10 ml. of human marrow obtained by sternal puncture was introduced into a 50 ml.-vaccine vial containing 2.5 ml. of citrated, balanced salt solution. After manipulations to eliminate most of the non-nucleated erythrocytes, the final culture contained about 100,000,000 nucleated marrow cells suspended in about 50 ml. of medium, which consisted of 35 per cent human ascitic fluid and 65 per cent balanced salt solution, similar in composition to cerebrospinal fluid. Each culture was first thoroughly shaken in a vial until a uniform suspension was obtained; then samples of equal volumes were transferred aseptically to a series of vaccine vials, one of which was left as a control to which only the solvent was added. Equal volumes of varying concentrations of the nitrogen mustard in isotonic saline were added to the others. All transfers, additions, and withdrawals were made with sterile syringe and needle through rubber vaccine vial caps which were sterilized each time with 70 per cent ethanol. A total nucleated cell count and a differential cell count of at least 1,000 cells were done on each control and each nitrogen mustard-containing culture at 3, 24, and 48 hours, and from these data the absolute number of each type of cell was computed.

The nitrogen mustard was dissolved in sterile saline and immediately serially diluted so that the amount necessary to give the desired concentration was contained in 0.2 to 0.5 ml.; a corresponding volume of sterile saline was added to the control culture. This manipulation was carried out very rapidly, the maximum time from opening the vial until the nitrogen mustard was mixed with the culture being 3 minutes. The final concentrations of HN₂ in the cultures studied ranged from 1:500,000–1:10,000,000, but since no changes in either the number or the morphology of the cells were observed in concentrations of less than 1:2,000,000, only the data from cultures containing a concentration of 1:500,000 are reported in this paper.

RESULTS

No morphologic or significant quantitative changes in the cells were observed in the cultures containing concentrations of 1:2,000,000 nitrogen mustard or less. The only morphologic change not-

* Aided by a grant from the Medical Research Foundation of Oregon.
† Now Assistant Professor of Medicine, Shanghai National Medical College, Shanghai, China. His fellowship in the Division of Experimental Medicine was made possible by the American Bureau for Medical Aid to China Inc.
‡ The nitrogen mustard used was kindly furnished by Merck & Co., Inc., Rahway, N.J.

Received for publication, September 21, 1949.
ed in the cultures containing greater concentrations of HN\textsubscript{2} was pyknosis of the nuclei of the progranulocytes, myelocytes, metamyelocytes, and band cells, affecting 0.82 per cent, or 7 of 856 cells counted at 3 hours; 12.4 per cent, or 57 of 461 cells counted at 24 hours and 15.7 per cent, or 42 of 267 cells counted at 48 hours. The criteria for cell identification and the nomenclature are those recommended (8) by the nomenclature committee.

The absolute numbers of each type of cell in each of the seven controls were added and their sums divided into the sums of the absolute numbers of the same type of cells from the seven corresponding cultures containing 1:500,000 dilution of nitrogen mustard. Each value was multiplied by 100 to give the weighted mean of the numbers of that cell type, expressed in the percentage of the numbers of the same type of cell in the control at the same time. These results were then plotted on graph paper, and it was apparent there were no significant quantitative deviations from the control in any but the 1:500,000 culture. The data from the 1:500,000 culture are plotted in Figures 1 and 2. Preliminary plotting of the curves for each cell type showed no significant differences among the curves for any of the cells less differentiated than the segmented neutrophils; the curves for all these cells fit the same straight-line curve on semi-logarithmic paper, as shown in Figure 1. In this figure, the cells capable of division—progranulocytes and myelocytes—and cells incapable of division—metamyelocytes and band cells—have been plotted in groups to decrease the statistical error. Note that there is no difference between the data for the less differentiated and more differentiated cells; and that on semi-logarithmic paper, both sets of data fit a straight-line curve which intercepts the control line at zero time when the nitrogen mustard was added, and intercepts the 1 per cent line when the number of these cells had decreased to practically zero, after approximately 70 hours. The data on the segmented neutrophils from the same seven cultures, plotted in the same way, however, fit a straight-line curve on arithmetic graph paper, as shown in Figure 2. This curve intercepts the control at 4 hours and the zero line at about 50 hours, giving a life-span within the normal limits for these cells, as previously determined by the marrow culture technic (22).

**DISCUSSION**

The granulocytic series of cells in marrow cultures makes an ideal cell population for studies of the effects of therapeutic agents on living cells (23), because cell division is normally only mitotic and occurs exclusively in the progranulocytes and early myelocytes, with occasional divisions in the relatively small number of myeloblasts present. The more mature cells contain no nucleoli and have never been observed to divide either mitotically or amitotically. The stages of differentiation within the cell series are easily and sharply identifiable, and the length of life of the segmented neutrophil, the most differentiated stage of the granulocytic series, has been determined (22) to be about 48–90 hours under the conditions of the marrow culture, with a mean value of about 60 hours.

To facilitate comparison of these results of HN\textsubscript{2} in marrow cultures with the results previously obtained (18) with roentgen irradiation, the data from Figure 1 in the earlier paper have been re-plotted on arithmetic graph paper to the same scale as the other figures in the present paper, as shown in Figure 3. Note that after ionizing radiation only the cells capable of division leave the control line at zero time, and that all cell types give a straight-line curve on arithmetic graph paper for at least the first 72 hours. Also note that there is a time lapse of 6–8 hours before the metamyelocytes and band cells begin to decrease and a time lapse of approximately 60 hours before the segmented neutrophils begin to decrease, in contrast to a time lapse of only 4–5 hours before segmented neutrophils decrease when HN\textsubscript{2} is added.

Colchicine in a concentration of 1:100,000 showed almost identical effects (18) to those illustrated in Figure 3 for 400 r of ionizing radiation, as did the combination of identical amounts of colchicine and irradiation. However, in the cultures containing colchicine alone, approximately 50 per cent of those cells capable of division were arrested in the metaphase of mitosis at 20 hours, whereas none of these cells was arrested in the metaphase in the irradiated cultures containing colchicine, and only about 0.5 per cent of cells capable of division were found to be in mitosis in the control cultures which were not irradiated and had no added colchicine.

These previously reported experiments showed clearly that ionizing radiation, in doses of 400 r or less, inhibits mitosis but does not kill cells of the granulocytic series in marrow cultures. After ionizing radiation, the segmented neutrophils do not begin to decrease until after those present at the time of irradiation have lived out their life-span and died.

It is evident from Figure 1 that the progranulocytes and myelocytes—cells capable of division—begin to decrease immediately, but so also do the metamyelocytes and band cells which are inca-
Fig. 1—Immature Cells of the Granulocytic Series in % of Control in Marrow Cultures with 1:500,000 Nitrogen Mustard.

Control

Fig. 2—Segmented Neutrophils in % of Control in Marrow Cultures with 1:500,000 Nitrogen Mustard.

Control

Fig. 3—Cells of the Granulocytic Series in % of Control in Marrow Cultures Receiving 400 r Irradiation. (Data replotted from Fig. 1 in "Is the Action of Roentgen Rays Direct or Indirect?")

Control

- Segmented Neutrophils
- Metamyelocytes + Band Cells
- Progranulocytes + Myelocytes

0 3 6 9 12 24 48 72 96 120 hours

% of Control

on April 20, 2017. © 1950 American Association for Cancer Research.
sible of division. The decrease for both groups of cells is exponential and not on a straight-line curve on arithmetic graph paper. This can be explained by the fact that nitrogen mustard instantly stops further cell differentiation and kills equal percentages of the surviving cells in equal time periods, in the interval following exposure to the drug. This is the usual effect of cell-killing agents. The manner of cell death is associated with pyknosis of the nucleus, a common form of cell death seen in these cells in untreated cultures. The action of HN2 does not interfere with autolysis of the cells after death, as evidenced by the fact that the number of disintegrated cells parallels the number of persistent living cells and after the first few hours does not increase in either the controls or the nitrogen mustard cultures.

The well-established genetic effects (1, 2, 11, 12, 24) of HN2 and ionizing radiation are statistically too rare events to explain the quantitative therapeutic action of these agents. One of the most difficult observations to explain in these experiments is the very narrow range of concentration within which the effects of HN2 could be demonstrated. With colchicine (18), effects are observable in concentrations of 1:100,000,000 in marrow cultures and increase steadily with concentrations up to about 1:100,000, at which concentration essentially all mitoses are arrested in the metaphase. With ionizing radiation (16–19), effects are demonstrable in the marrow cultures with as little as 10 r of irradiation. More and more divisions are inhibited progressively, until dosages of about 400–500 r are reached, at which levels mitoses are almost completely inhibited. There is no evidence of an increased rate of cell death until dosages of 1,000 to 2,000 r are reached, when killing effects on cells become apparent. The striking morphologic changes in the granulocytes produced by urethane (20) in marrow cultures were evident in concentrations of 1:40,000 and progressively increased up to concentrations of 1:200. The foregoing observed effects of colchicine, ionizing radiation, and urethane represent ratios for each of these agents of 1:1,000, 1:40, and 1:200, respectively, between the lowest concentration where demonstrable effects were observed and the highest concentration where increasing effects of the same type were still being observed. For nitrogen mustard, the ratio between the concentration producing the minimum observable effect and the concentration that kills all cells appears to be not more than 1:2 to 1:4, suggesting that there is some type of "all or none" relationship between the concentration and effect. This, perhaps, explains why the clinical dosage has to be so accurately based on body weight, and why it has been impossible to find an effective therapeutic dose that does not also produce toxic effects. The known very short life of the active cation ion from nitrogen mustard indicates that the action must take place almost instantaneously and must produce an effect which is long persistent. The effective concentration is probably present with the customary dose clinically used, but it seems unlikely that this effective concentration would be present for longer than a very few minutes. The customary dosage of 0.1 mg. HN2 per kilogram of body weight is equivalent to 0.1 mg. per 45 ml plasma, or an HN2 concentration of 1:450,000. So it would seem that the effects must all take place within a very few circulation times, after which the HN2 would be diluted to an ineffective concentration. This would explain the observation of Karnofsky, Graef, and Smith (18), that by means of occluding the circulation to the lower extremities by means of clamps on the abdominal aorta and inferior vena cava during and for 2–15 minutes after the administration of HN2, the femoral marrow was spared the usual nitrogen mustard effects.

It is readily understood why the action of HN2 has been confused in the past with the action of irradiation, since, with either, the segmented neutrophil count in the blood decreases arithmetically; and it would be easy to miss the greater time lag after irradiation before this decrease begins, because of the large variations in neutrophil count which normally occur diurnally, as well as many other variables present in the human body, most of which are controlled by the marrow culture technic. The early decrease of the leukocytes in the peripheral blood after HN2 therapy is well shown in Figure 11 of the report (7) by Dameshek, Weisfuse, and Stein.

SUMMARY

Studies according to the marrow culture technic indicate that the action of the nitrogen mustard methyl-bis (β-chloroethyl) amine hydrochloride is different from that of ionizing radiation, colchicine, or urethane on cells of the granulocytic series.

The nitrogen mustard stops differentiation and kills all cells less differentiated than the segmented neutrophil but has little or no effect on the lifespan of the segmented neutrophil. Cell death occurs exponentially, and equal percentages of the surviving cells are killed in equal time-intervals. In the concentrations investigated, HN2 does not appreciably affect the rate of cell death of the segmented neutrophils.
REFERENCES

The Effect of Nitrogen Mustard on Granulocytic Cells as Observed by the Marrow Culture Technic

Edwin E. Osgood and I. T. Chu


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/10/2/98

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.