

## THE EFFECT OF URETHANE ON THE NUCLEAR MORPHOLOGY OF CELLS OF THE GRANULOCYTE SERIES AS OBSERVED IN MARROW CULTURES AND LEUKEMIC BLOOD

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INTEREST in urethane ( $\text{NH}_2\cdot\text{CO}\cdot\text{OC}_2\text{H}_5$ ) and related carbamic acid esters in the treatment of metastatic malignant tumors and leukemias has been greatly stimulated by the report of Haddow and Sexton<sup>1</sup> on the effects of urethane on tumors in experimental animals, and of Paterson, Haddow, Thomas and Watkinson<sup>2</sup> on urethane therapy of human leukemias and malignant tumors. They demonstrated striking decreases in the leukocyte count and spleen size in certain patients with granulocytic or lymphocytic leukemias and in the size of metastatic nodules in a small proportion of patients with other malignant tumors. The observations of Paterson, Haddow, Thomas and Watkinson<sup>2</sup> have been confirmed by Goodman and Lewis<sup>3</sup> and by the authors at the University of Oregon Medical School.

Although urethane has long been in use as a narcotic and there is much literature on the action of the urethanes and related karyoklastic and karyokinetic poisons on the cells of lower forms of life,<sup>4</sup> we were unable to find any reference to morphologic changes produced by urethane in the nuclei of human cells.

The present study, using the technic of human marrow culture,<sup>5</sup> was undertaken as part of a long-term investigation of the factors influencing the fundamental phases of cell growth, namely, increase in size, mitotic and amitotic division, differentiation, and life span. This paper will be limited to the morphologic changes in cells of the granulocyte series produced by the action of urethane. Quantitative data on the comparative effects of urethane and methyl-bis (B-chloroethyl) amine hydrochloride obtained in the course of this study will appear later.

### METHOD

Marrow cultures were set up as previously described,<sup>6</sup> using sterile ascitic fluid as a source of protein instead of human cord serum. A marrow culture was prepared from each of 8 patients with miscellaneous diseases displaying essentially normal marrow pictures. In addition, the bloods of 4 patients with chronic granulocytic leukemia, 2 with acute lymphocytic leukemia, 1 with acute monocytic leukemia, and 1 with multiple myeloma with a plasmacytic-leukemia blood picture were cultured in the same way as marrow. Each culture was first thoroughly shaken in one vial and then equal parts were transferred to a series of vaccine vials, one of which was left as a control to which the solvent only was added and to the others equal volumes of varying concentrations of urethane or of methyl-bis (B-chloroethyl) amine hydrochloride in isotonic saline were added. Since no references were found giving the actual blood levels of urethane obtained in clinical therapy, a wide range of final concentrations was used, including 1:200, 1:1,000, 1:2,500, 1:5,000, 1:10,000, 1:20,000, and 1:40,000, although not all concentrations were used in each experiment. The morphologic data were based on the study of Wright's stained smears made at the same time from the control, urethane and nitrogen mustard cultures. The samples for the smears were removed three hours after the drugs were added and at approximately twenty-four hour

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intervals thereafter for five days. The criteria of cell identification and classification were those given and illustrated by Osgood and Ashworth.<sup>7</sup>

#### OBSERVATIONS

The morphologic changes in structure of the cells of the granulocyte series noted in the cultures containing urethane are illustrated in figure 1 and outlined in table 1.

The first change to appear was a 5 to 10-fold increase in the number of progranulocytes in process of mitotic division as compared to the control. This was noted as early as three hours and in concentrations as low as 1:40,000. The increase in mitoses was most marked at twenty-four hours and persisted for seventy-two hours. All phases of mitotic division were seen but most were normal appearing metaphases similar to those illustrated in figure 2 by Osgood.<sup>8</sup>

The most commonly observed change was the condensation of the basic chromatin in the nucleus of the progranulocytes and granulocytes into dense blocks separated by clear spaces and still surrounded by a nuclear membrane as shown in figure 1-a. This appeared as early as three hours, steadily increased as long as the cultures were observed, and was most marked in the higher concentrations, although it was noted with all concentrations.

The alteration in morphology shown in figure 1-b was frequently observed in the progranulocyte and granulocyte stage. There seemed to be a loss of the nuclear membrane and imperfectly square or rectangular projections from a mass of clumped chromatin. A somewhat similar picture is seen in the anaphase of normal mitotic division when the cell is so oriented that the plane of cell division is parallel to the plane of the slide, but the number of these cells was greater than would be expected from the number of cells seen in the metaphase of division and it seems probable that at least some of the cells showing this appearance represent an intermediate stage of karyorrhexis between figure 1-a and figure 1-c.

The most striking but least frequently observed change is shown in figure 1-c. It consisted of the appearance within the cell of numerous fragments of densely staining, structureless chromatin in round, ovoid or rectangular blocks with no nuclear membrane. This appearance was somewhat suggestive of the colchicine arrest of mitosis in the metaphase,<sup>9</sup> illustrated in figure 1 in Osgood,<sup>10</sup> but careful studies showed the points of difference outlined in table 2. It seems more probable, therefore, that the urethane effect illustrated in figure 1-c represents karyorrhexis or fragmentation of a nucleus similar to that in figure 1-a.

Double nuclei with no suggestion of fission of the cytoplasm were frequent in the progranulocyte and all subsequent stages of the granulocyte series in the urethane-containing cultures, yet the cells did not appear to be appreciably larger than the corresponding cell type with a single nucleus. They seemed to be most abundant in the granulocyte stage but appeared first in the progranulocyte stage as illustrated in figure 1-d.

Another change in morphology which is not illustrated was a marked decrease in size of some of the neutrophil lobocytes and rhabdocytes, both as compared to the control culture and to the average normal size of these cells. This change was even more noticeable in the blood of some of the patients treated with urethane over long periods of time.

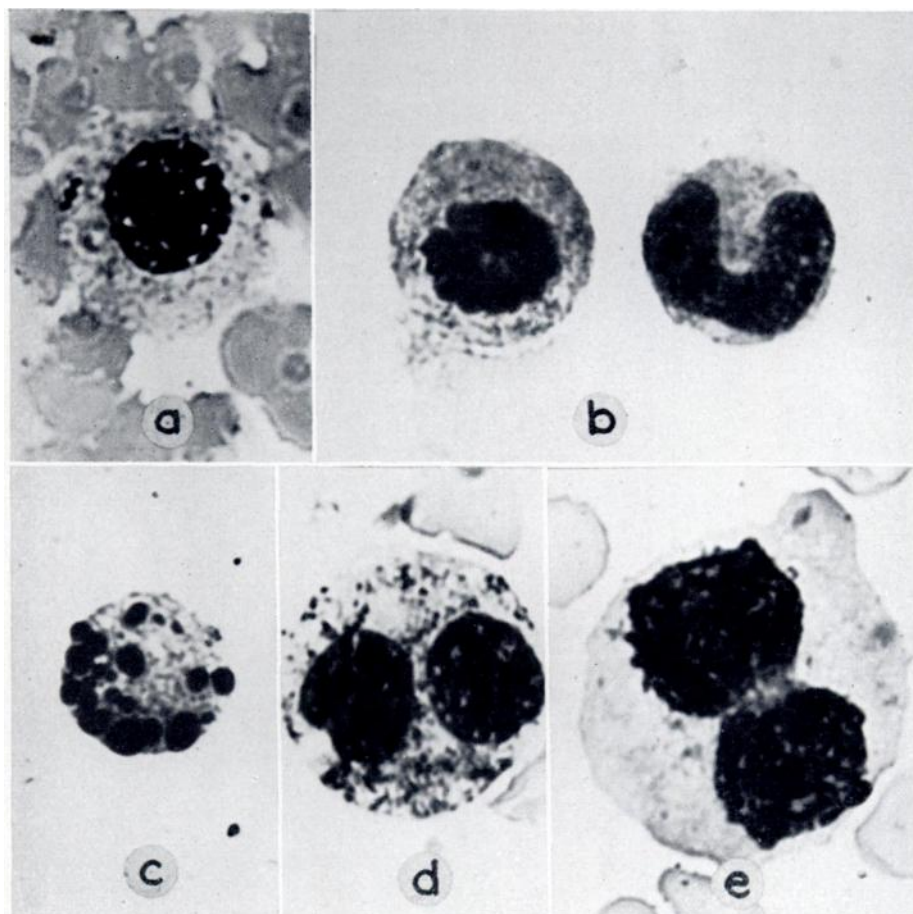


FIG. 1. Photomicrographs of cells of the granulocyte series from urethane-containing culture (a-d), and leukemic blood (e), Wright's stain  $\times 1800$ .

a. Neutrophil granulocyte showing chromatin clumps within the nucleus.

b. On the left is a cell which is either a neutrophil progranulocyte S in the anaphase of mitotic division viewed end-on or a neutrophil granulocyte showing an intermediate stage of karyorrhexis of the nucleus between figure 1-a and 1-c. On the right is a neutrophil rhabdocyte of perfectly normal morphology.

c. Neutrophil granulocytes showing fragmentation or karyorrhexis of the nucleus into numerous deeply-staining round or ovoid blocks of varying size.

d. Progranulocyte A with double nucleus.

e. This is a progranulocyte A from the blood of a patient with chronic granulocytic leukemia under treatment with urethane in process of amitotic division showing the mode of formation of the double nuclei. Note the rectangular projections from the nuclei with rounded corners. Amitotic division and double nuclei are almost never noted in the granulocyte series in either normal blood or marrow or blood or marrow from patients with leukemia who are not receiving urethane. Note that there is no tendency toward fission of the cytoplasm.

In addition, many of the cells of the granulocyte series developed dense, pyknotic, structureless nuclei and showed signs of loss of the cell membrane and disintegration.

None of these changes was noted in significant numbers in cells of the lymphocyte, monocyte, or plasmacyte series cultured or in the granulocyte series in the control and nitrogen mustard cultures. They have rarely if ever been observed by the authors in the course of an extensive study of the blood and marrow of patients with granulocytic leukemia or other diseases when urethane was not being given.

In each of the cultures and with each of the concentrations of urethane employed, many of the cells at each stage of development showed none of these changes and

TABLE 1.—Approximate Percentage of Progranulocytes and Granulocytes Showing Morphologic Changes

Changes observed	Control cultures 1 to 5 days	Cultures containing urethane	
		1 day	5 days
Normal	Over 99%	90%	Less than 20%
Mitoses	Less than 1%	7%	Less than 1%
Chromatin blocks (Figure 1-a)	Not seen	Less than 1%	60-70%
Separate chromatin blocks (Figure 1-c)	Not seen	Not seen	Less than 1%
Double nuclei (Figure 1-d)	Not seen or less than 0.1%	Less than 1%	15%

TABLE 2.—Effects on Morphology of Cells Produced by Colchicine and Urethane

	Colchicine	Urethane
Chromatin blocks	rectangular or square uniform in size number = or > normal no. of chromosomes	round, ovoid or with rounded corners unequal in size number < normal no. of chromosomes
Mitosis	arrested in metaphase	initial stimulation goes on to completion
Amitosis	not affected	greatly increased
Differentiation	prevented in higher concentration (1:100,000)	markedly abnormal but occurs in 1:200
Cell size	giant forms common	dwarf forms common
Nuclei	enlarged	pyknotic

appeared indistinguishable in morphology from cells of the corresponding stage of development in the control cultures or in the original marrow or blood of the patient. Such a normal appearing cell is illustrated by the neutrophil rhabdocyte in figure 1-b.

All of the changes noted in the cultures were also noted in blood or marrow of patients receiving 1 gram of urethane three times daily. The cell in figure 1-e is from the blood of a patient receiving urethane therapy. It shows the formation of double nuclei, apparently by amitotic division.

## COMMENT

The changes in morphology of the cells of the granulocyte series observed in blood and marrow cultures containing urethane suggest an effect of this drug on the state of aggregation or organization of the nucleoprotein within the cell nucleus, a tendency to disrupt the nuclear membrane and to interfere markedly with the normal process of cell division and cell growth. They resemble in many respects the changes described by Dustin,<sup>4</sup> Burt,<sup>11</sup> Piton,<sup>12</sup> and Chodkowski<sup>13</sup> and others whose work is cited in the references herein given,<sup>1,2,4,11-13</sup> as produced by a wide variety of unrelated "karyoklastic poisons," including many urethanes, many narcotics and arsenical compounds which seem to act by altering the permeability and integrity of cell membranes and nuclear membranes and the state of colloid aggregation of the nuclear and cytoplasmic proteins.

The morphologic effects produced by urethane were not seen in marrow cultures exposed to nitrogen mustard. While some of the changes superficially resemble those produced by colchicine,<sup>5,9-10</sup> there are important differences. Colchicine, in adequate concentration, seems to arrest mitotic division completely, whereas urethane apparently stimulates division at first and the majority of cells complete division and differentiation.

In previous studies, using the marrow culture technic, of the action of ionizing radiation including 200 KV and 1,000 KV roentgen rays, neutron rays and radioactive phosphorus, it was shown<sup>5,9,14-16</sup> that in the dosage employed in treatment of leukemias, each modality of ionizing radiation inhibited the onset of the next division, mitotic or amitotic, rather than actually killing cells. In none of these experiments were morphologic changes similar to those noted in the urethane cultures observed, nor was the initial increase in mitoses and in total cell count which occurred in the urethane cultures noted.

We have confirmed the clinical observations of Paterson and her co-workers<sup>2</sup> that there is a great difference in clinical response of patients with apparently identical forms of leukemia to similar doses of urethane. It so happened that bloods of 2 of these patients were selected for culture before urethane was administered. Both patients were middle-aged women with long-standing chronic granulocytic leukemia with typical blood pictures. Both had been treated until resistant with local x-ray and not with the preferred total body irradiation at regular intervals. Both had refused further x-ray treatment. Case 1, unit number 157378, who responded well to urethane, had had a splenectomy several years previously because of the huge size of the spleen. Case 2, unit number 157231, which failed to respond to urethane, had a huge spleen reaching to the left iliac fossa 25 cm. below the costal margin and extending across the midline. In other respects, they were as nearly alike as any two cases of chronic granulocytic leukemia could be. Both were given 1 gram of urethane three times a day.

In case 1, the leukocyte count had dropped from 33,800 to 7,700 after thirteen days of therapy, totalling 36 grams, and to 2,000 by the eighteenth day of therapy, totalling 51 grams, at which time the therapy was discontinued. In the course of the next twelve days, the leukocyte count dropped to 600 and, although the urethane had been stopped, it continued at this low level for many weeks, during

which time stomatitis from the agranulocytosis was controlled with difficulty by penicillin therapy. She then had a gradual reversion to a more normal leukocyte count.

In case 2, the initial leukocyte count before therapy was oscillating between 50,000 and 90,000 and was still about 50,000 after 141 grams of urethane were given in the course of thirty-nine days. The differential cell count pattern was not significantly altered and the size of the spleen was unchanged. Both patients had a good deal of nausea and some vomiting from the drug, and it is possible that in case 2 some of the drug was lost in the vomitus or was not taken, so during the last of the course the dose was increased to 4 grams daily, still without effect. This patient subsequently showed an excellent response to intravenous radioactive phosphorus therapy as far as leukocyte count and alteration in spleen size were concerned, although requiring somewhat higher dosage than the average patient who had not had x-ray therapy previously.

In the cultures of these two bloods to which urethane was added, however, the morphologic changes observed were, within the experimental limits of the method, indistinguishable in character and percentage of cells involved, suggesting that the clinical differences in response were due to differences in concentration of the active compound actually reaching the cell.

#### SUMMARY

In cultures by the marrow culture technic of human marrow and leukemic blood containing concentrations of urethane from 1:200 to 1:40,000, marked changes in the morphology of the cells of the granulocyte series were noted.

These changes were not noted in the control nor in duplicate cultures containing the methyl-bis (B-chloroethyl) amine hydrochloride form of nitrogen mustard in concentrations from 1:500,000 to 1:40,000,000, nor were they noted in previous studies of cultures containing colchicine or exposed to 200 kilovolt or million volt x-rays, neutron rays or radioactive phosphorus, nor in the bloods or marrows of patients with untreated chronic granulocytic leukemia, of healthy individuals or of persons with miscellaneous diseases.

The changes consisted of an early increase in number of normal mitoses in the progranulocytes; a steadily rising percentage of granulocytes and progranulocytes showing condensation of the chromatin in the nucleus into dense fragments separated by clear spaces; a progressive increase in the number of cells of the granulocyte series with double nuclei, affecting all cells from the progranulocytes to the neutrophil lobocytes but appearing to be most numerous in the granulocyte stage; and the appearance in the cultures by 4 to 5 days of cells containing separated fragments of structureless material staining like basichromatin, which probably represents a karyorrhexis of the nucleus.

*Note:* Nothing in this article is to be construed as a recommendation of urethane for the clinical treatment of leukemias. While many years must elapse before its place in therapy can be evaluated, it does seem worthwhile to give urethane a trial for metastatic malignant tumors. Our present impression is that either radioactive

phosphorus or total body irradiation with x-rays given in small regularly spaced doses is far superior to urethane in the treatment of leukemias.<sup>17</sup> When the cells become resistant to radiation therapy, urethane may be worthy of a trial.

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