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Medical Department and Isotope Laboratory of the Biirgerspital Solothurn
(Physician-in-chief: Prof. S. MOESCHLIN)

Experimental Studies on the Mechanism of Action of Benzene on the Bone Marrow (Radioautographic Studies Using ^{3}H-Thymidine)*

S. MOESCHLIN and B. SPECK

Despite the fact that the use of benzene as a solvent is prohibited in most countries there are still many reports on chronic benzene intoxications (1, 2, 3, 4, 5). The most frequently quoted sign is aplastic anemia, but also there has been a high incidence of leukemia which generally has been of the acute myeloblastic type. The elective toxic effects of benzene on the bone marrow and the persisting inhibition of the proliferation of the marrow cells even after stopping benzene exposure still remain unanswered questions (6a).

In the present study, the mechanism of action of this substance on the bone marrow cells is examined with tritiated thymidine.

Methods

Sixty rabbits with a body weight of 1.1 to 3.7 kg and of both sexes were examined. In preliminary studies 28 animals received benzene in oily solutions subcutaneously. This group of animals could not be evaluated statistically since there was a wide variation of resorption and of the resulting toxic effects. Another group of 5 animals was given a single injection of 2.0 ml/kg of pure benzene subcutaneously.

After the initial technical difficulties had been solved, a main experiment was started with 27 animals. This group was intoxicated with subcutaneous injections of pure benzene three times weekly. It should be mentionned at this point, that the difference between the lethal dose and the one inducing a pancytopenia was small. In our animals, the optimal dose of pure benzene proved to be in the order of 0.3 ml/kg/day. With this dose a severe pancytopenia could be induced in all the animals within one to nine weeks, except for those dying from early anaphylactic or infectious complications. As soon as the leukocyte count in the peripheral blood dropped below 3000/mm^{3}, 0.5 μc of ^{3}H-methyl-thymidine (New England Nuclear, Boston, Mass.) were given intravenously. The specific activity of this pyrimidine nucleoside labelled with tritium was

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6.7 C/mM throughout. After an incubation period of exactly 60 min, the animals were anestetized. Bone marrow was aspirated from the epiphysis of the femur. Thin slides were then prepared from solid marrow particles. After air drying, they were fixed in absolute ethanol for 10 min and then covered with Kodak AR-10 stripping film-in the darkroom. The preparations were then exposed for 20 days at 4°C. Subsequently they were developped with Kodak D-19 developper and Kodak fixator at 18°C. The staining was performed through the film with a Giemsa solution buffered to a pH of 6.5. A total number of 19 animals was studied radioautographically. In all these animals 500 basophilic and 500 polychromatophilic normoblasts were enumerated and the percentage of labelled cells was calculated. The pronormoblasts [= K_2 stage according to WEICKER (7)] and the macronormoblasts (= K_1) could be definitely quantitated only in three intoxicated animals, and the myelocytes only in 2 animals. Cells were considered to be labelled if the number of grains over their nucleus was more than three times the background graincount over an equal area.

Results

Peripheral blood. With a dose of 0.3 ml/kg/day of pure benzene, the time span until a peripheral pancytopenia was induced varied from 1 to 9 weeks. A single injection of 2.0 ml/kg did not cause any remarkable changes of the peripheral blood counts during a 6 weeks control period.

The differential counts of the leukocytes showed a slight drop of the lymphocytes and a slight increase of basophils and eosinophils. If the relative percents of the different cell lines were cal-

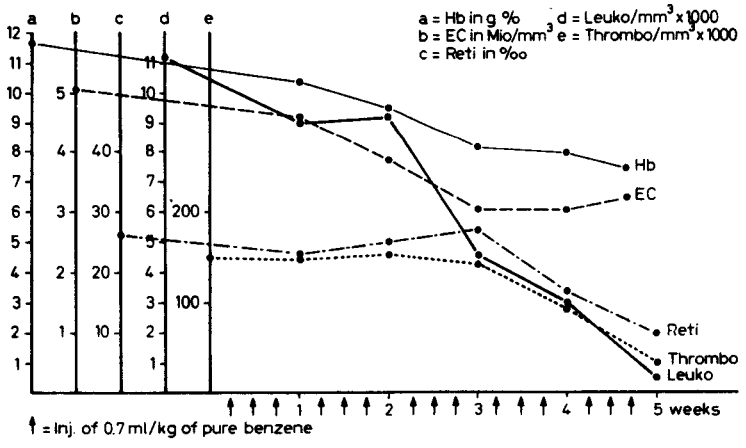

Fig. 1. Representative animal from the main experiment. 0.3 ml/kg/day of pure benzene was given subcutaneously in three weekly injections. Note the drop of the peripheral leukocyte, thrombocyte and reticulocyte counts: they generally occurred in the second to the fourth week. There were considerable difference of sensitivity though. The drop of the erythrocyte count and of the hemoglobin is relatively small.

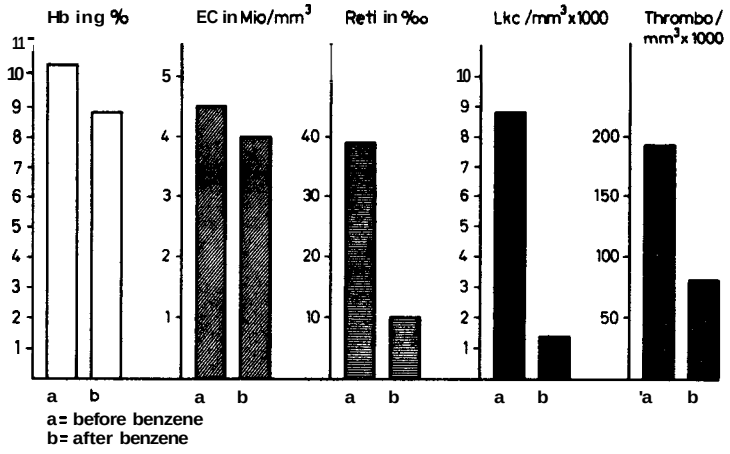

Fig. 2. Mean peripheral blood counts of 19 animals of the main experiment which came to radioautography. The bars at the left represent the values before benzene was injected, the ones on the right the values at the time radioautography was performed. Note again the striking drop of the leukocyte, thrombocyte and the reticulocyte counts with an only slight drop of the erythrocytes and the hemoglobin levels.

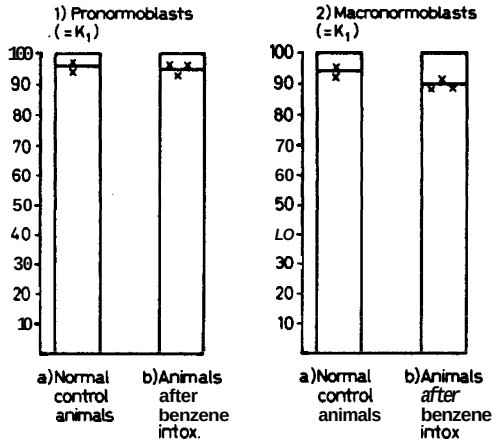

Fig. 3. Labelling of the young erythroid precurors. Note that there is only a drop of 95.5% to 94.5% labelling at the pronormoblast (= K,) stage and of 94% to 90% at the macronormoblast (= K,) stage in normal animals compared to animals intoxicated with benzene.

culated in absolute numbers of cells per mm^3, a decrease of all elements was found, which was most striking in the lymphocytes.

Bone marrow. Despite the fact that all the animals had a peripheral pancytopenia after exposure to benzene, there was a considerable difference in the morphologic marrow picture. Of the examined 19 marrows 4 were very hypoplastic, 6 hypoplastic, 5 of average

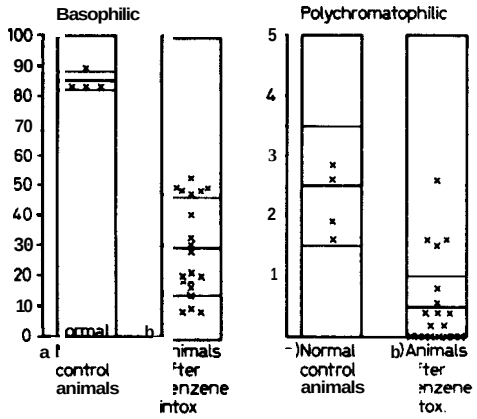

Fig. 4. The radioautographic findings of 4 normal animals are opposed to the findings in 19 animals intoxicated with benzene. Note the significant drop of labelling of the *basophilic normoblasts* (= $K_{1/4}$) from 84.5% (s.D. 2.6%) in normal animals to 30.2% (s.D. 16.5%) in the intoxicated animals. This decrease of labelling indicates clearly the diminution of the proliferative potential of the bone marrow cells at this stage of maturation. At the stage of the *polychromatophilic normoblast* (= $K_{1/4}$) the labelling dropped to 0.52% (s.D. 0.49%) in the intoxicated animals as compared to 2.5% (s.D. 1.05%) labelling in the normal controls.

cellularity and 4 were very cellular. There was no correlation between the cellularity and the duration of exposure to benzene. The diminution of the *myeloid precursors* was remarkable. The *erythroid line* was generally quantitatively well preserved, but there were a number of qualitative aberrations. Numerous pathologic changes of the chromatin structure were found. A shift to the left in erythropoesis with the appearance of very immature cells (=erythrogones), with giant nuclei was another characteristic feature. The interpretation of paraffin sections of the marrow of an intoxicated animal by Institute of Pathology of the University of Zurich (Prof. UEHLINGER), was in agreement with our findings: 'Predominant reduction of myelopoesis with maintained erythropoesis and appearance of erythrogones'.

Radioautographic findings. The young erythroid precursors (pronormoblasts = K_2) and the macronormoblasts (= K_1) could be quantitated only in a few of the cellular preparations. At this stage of the development there was no significant difference between the amount of labelled cells in the normal animals and in the intoxicated ones. At the stage of the *basophilic normoblast* (= $K_{1/2}$) though, there was a very pronounced drop of labelling in the in-

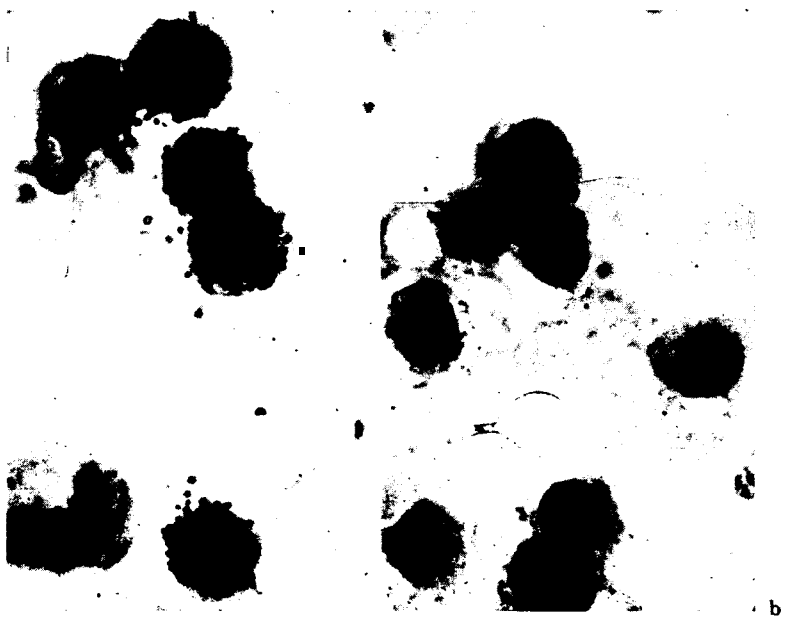

Fig. 5 (a) Radioautographic plate from the marrow of a normal animal. Note the distinct labelling of the basophilic normoblasts.
(b) Radioautographic plate from an intoxicated animal made under exactly the same conditions. Note the lack of labelling of most of the erythroid precursors. Below, there is a late myelocyte which is not labelled either. The background grain count is low in both preparations.

toxicated animals, which persisted at the stage of the polychromatophilic normoblast.

The myeloid line was so markedly reduced after exposure to benzene, that only in two animals 500 cells could be quantitated. At stage of the myelocyte the labelling was decreased from 67% in normal animals to 7.3% (s. D. 2.1%) in the intoxicated ones.

Discussion

Our radioautographic studies prove that the ***myelotoxicity of benzene and the resulting pancytopenia are caused by a very pronounced inhibition of DNA-synthesis.*** The ^{3}H-thymidine which was used for *in vivo* incubation is a direct precursor of DNA. During an incubation period of 60 min, all the cells which are in the process of active DNA-synthesis are labelled (8–10). Therefore ***the amount of labelling of the different cell types is a direct index for their proliferative potential.*** In the ***erythroid line,*** the proliferation of the pronormoblasts

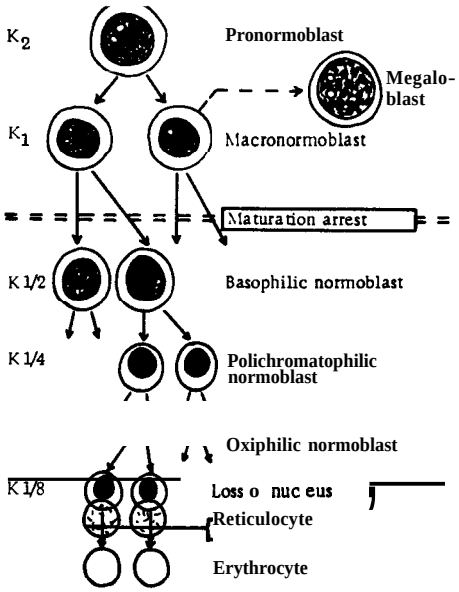

Fig. **6. Model of erythropoesis (WEICKER). The maturation arrest in erythropoiesis during chronic benzene intoxication must be between the stages K_1 and $K_{1/2}$.**

(= K,) and of the macronormoblasts (= K,) was in the normal range as far as this could be quantitated. On the other hand there was a very pronounced ***lack of proliferative potential at the stage of the basophilic normoblast (= $K_{1/2}$) in all the animals.*** This finding indicates a ***maturation arrest*** between the macronormoblast (= K,) and the basophilic normoblast (= $K_{1/2}$). Similar observations have been made in other intoxications, e.g. lead (6b).

In the ***myeloid line*** also, there was a marked decrease of the proliferative potential during benzene intoxication. It was quantitated at the stage of the myelocyte. The short survival time of the leukocytes and of the thrombocytes in the peripheral blood and the rapid maturation of the reticulocytes explain the early drop of these blood counts following severe inhibition of DNA-synthesis. On the other hand the erythrocyte counts and the hemoglobin levels remained relatively constant during benzene intoxication. This is due to the longer survival of the red cells in the peripheral blood. The fact that the human bone marrow may become aplastic as well as hyperplastic during chronic benzene poisoning, is well known (6a, 11).

The appearance of erythrogones as we found them in the intoxicated animals, has previously been observed during treatment with antimetabolites (12, 13), aplastic crises of hemolytic anemias and in pernicious anemia (14).

Addendum. Since this work has been completed, additional radioautographic studies have been performed in our laboratory, using ^{3}H-cytidine and ^{3}H-uridine. Our preliminary data indicate that in addition to the inhibition of DNA-synthesis, then may be an inhibition of RNA-synthesis of the bone marrow cells during chronic benzene intoxication.

Summary

In rabbits intoxicated by means of subcutaneous benzene injections, numeric studies of the labelled bone marrow cells after intravenous incubation with ^{3}H-thymidine were performed. There was a marked individual difference of sensitivity to the same dosis of benzene. A severe peripheral pancytopenia resulted in all the animals within one to nine weeks. The bone marrow became hypoplastic in about one half of the animals and it remained cellular in the other half. This is in agreement with the findings in chronic human benzene intoxication. For the first time it was shown radioautographically with ^{3}H-thymidine that the pancytopenia of chronic benzene poisoning is due to a severe inhibition of DNA-synthesis in the bone marrow cells. The severe disturbance of DNA-synthesis may also be one of the factors which could give rise to the development of leukemia.

Zusammenfassung

Bei Kaninchen mit experimenteller Benzolvergiftung wurde das Knochenmark autoradiographisch untersucht. Die individuelle Empfindlichkeit auf eine gleiche Benzoldosis schwankt erheblich. Bei dreimaligen s.c. Benzolinjektionen pro Woche kommt es nach 1 bis 9 Wochen zu einer schweren Panzytopenie. Das Knochenmark wurde bei der Halfte der Tiere hypoplastisch, bei den iibrigen war es noch zellreich. Dieses Ergebnis stimmt mit den Beobachtungen bei der menschlichen Benzolvergiftung iiberein. Erstmals konnte durch autoradiographische Untersuchung mit ^{3}-HThymidin eine schwere Hemmung der DNS-Synthese als Ursache fur die gehemmte Blutzellbildung bei der Benzolvergiftung nachgewiesen werden. Die schwerwiegende Störung der DNS-Synthese konnte ein Faktor sein, der die leukamixhe Entartung des Knochenmarkes bei der chronischen Benzolvergiftung begiinstigt.

Résumé

La moelle osseuse de lapins intoxiqués par des injections souscutanées de benzol a été étudiée par autoradiographie aprb l'injection intraveineuse de thymidine tritiée. La sensibilitt individuelle à la même dose de benzol varie considérablement. Une pancytoptnie périphérique sévère apparut chez tous les animaux au bout de une à neuf semaines, les injections étant administrées à raison de trois par semaine. La moelle osseuse devint hypoplasique chez à peu près la moitié des animaux; chez les autres, elle demeura riche en cellules. Ces résultats concordent bien avec les constatations faites dans les cas d'intoxication au benzol chez l'homme. Pour la première fois, il a été démontré par autoradiographie à l'aide de thymidine tritiée que la pancytopénie de l'intoxication chronique au benzol est due à une inhibition sévère de la synthèse de l'ADN. Cette inhibition est peut-être aussi l'un des facteurs pouvant favoriser l'apparition d'une leucémie lors d'une intoxication chronique au benzol.

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Authors' address: **Prof. Sven Moeschlin and Dr. Bruno Speck, Medizinische Abteilung, Bürgerspital, *4500 Solothurn* (Switzerland).**

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