PLAINTIFF'S EXHIBIT 1001

Blut Vol. 25 (1972) p 97-103

Blut, Band XXV, Seite 97–103 (1972)

From the Department of Clinical Hematology and the Unit for Protective Isolation of the Institute of Radiopathology and Radiation Protection – University Medical Center – Leiden, Holland

Further Studies on experimental Benzene induced Aplastic Anemia

By Margot Kissling and Bruno Speck

Summary

Autoradiographic studies on bone marrow cells of rabbits with benzene induced pancytopenia revealed a severely disturbed DNA and RNA synthesis. Also a high incidence of chromosome aberrations were demonstrated. It is concluded this type of pancytopenia is primarly due to interference of benzene with DNA and RNA synthesis and not to a failure of the hemopoietic stem cell.

Zusammenfassung

Autoradiographische Untenuchuagen an Knochenmarkzellen von Kaninchen mit benzolinduzierter Panzytopenie zeigten eine schwer gestörte DNS- und RNS-Synthese. Ebenfalls wurde eine hohe Inzidenz von Chromosomenaberrationen gefunden. Es wird der Schluß gezogen, daß diese Art von Panzytopenie, verursacht durch Benzol, primär eine Störung der DNS- und RNS-Synthese und nicht ein Versagen der hämopoietischen Stammzellen ist.

Despite the fact that benzene has been largely replaced as a solvent in the industry, benzene induced blood dyscrasias have still frequently been reported, in particular aplastic anemia and leukemia [12,26]. For several years we have been interested in clarifying the mechanism of action of benzene on bone marrow cells. In previous experiments with rabbits that were subjected to subcutaneous injections with benzene we demonstrated a severely disturbed DNA synthesis [20,31]. In the present study, the interference of benzene with RNA-synthesis of bone marrow cells is examined with tritiated cytidine. Cytogenetic studies are performed before and after exposure to benzene.

Material and Methods

30 rabbits of a cross between Vienna white and Alaska with a body weight of 1.0 to 2.0 kg were studied. A group of 10 animals served as normal controls and 20 rabbits were subjected to subcutaneous injections of pure benzene three times weekly. Severe pancytopenia was x c n with an average dose of 0.2 ml/kg/day within 6 to 12 weeks. As soon as the leukocyte count of the peripheral blood dropped below 1000/mm³, 0.5 µC/kg of ^{3}H-thymidine sa. 5.9 µC/mM was injected intravenously to one group of 10 animals. 0.5 µC/kg of ^{3}H-cytidine was injected intravenously to the second group of ten animals. After an incubation period of exactly 1 h bone marrow was aspirated under general anaesthesia. Slides for radio-

Eingegangen am 10.4. 1972.

autography (RAG) were made. These slides were air dried and fixed for 20 min in methanol, then coated with Ilford-K$_5$ liquid nuclear track emulsion. After 7 drys csposucc at 4 °C *^{3}H-thymidine preparations were developed for quantitation of DNA synthesis.* The ^{3}*H-thymidine preparations for estimation of RNA-synthesis* were developed serially on day 7, 14 and 21. Kodak D-19 devclopper was uscd. The slides were stained with *May-Grunewald-Giemsa* solution through the film. 100 cells of each cell type were enumerated and the percentage of labeled cells calculated. Cells were considered to be labeled if the number of grains over the nucleus was more than five times the background grain count over an equal area.

Cytogenetic studies were performed with a modified short term culture method described by *Moorhead* et al. [*21*]. 0.5 ml of bone marrow were added to 5 ml *TC* 199 Eagles Medium. 0.05 ml of phytohaemagglutinine (Burroughs Wellcome, England) and 0.05 µg/ml of colchicine were then pipetted to the suspension. After washing with *Hanks* balanced salt solution, the cells were suspended for 9 min at 37° C in 0.075 M KCL [*15*]. Following centrifugation the cells were fixed in glacial acetic acid and methanol (1 : 3) for 30 min. Cleaned slides were dipped into chilled distilled water, then 2 to 3 drops of the cell suspension were pipetted on each slide. Staining was performed with a 1.5% orcein-sol. for 60 min. From each rabbit 100 mitoses were examined microscopically.

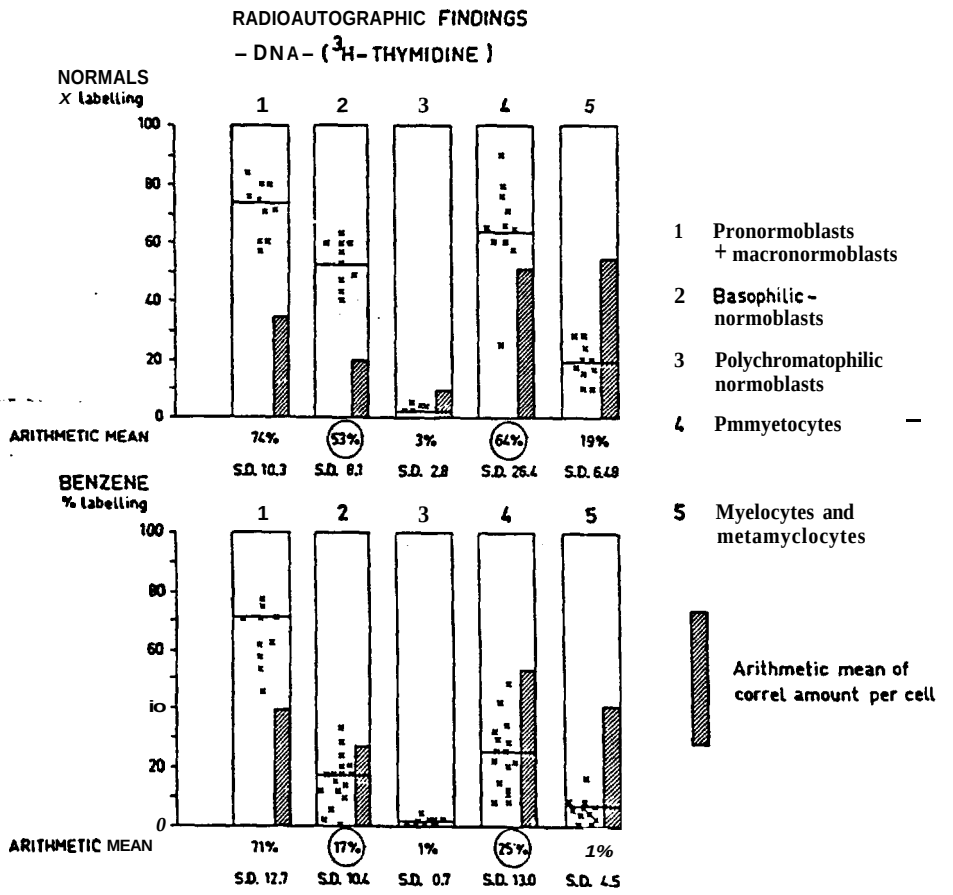


Table 1 illustrates the findings with ^{3}H-thymidine which was uscd for quantitation of DNA synthesis.

Results

Peripheral blood

With a dose of 0.2 ml/kg/d of pure b
6 to 12 weeks. A marked drop of 1
was seen within three weeks. Later t
of the haemoglobinconcentration. I
mals there was granulocytopenia. T
animals presented with a marked b.

Bone marrow

Two weeks after starting benzene i
poiesis was found. In addition th
structure of the nuclei. Not all the
were hypocellular. Of 16 specimen:
to be hypocellular, 4 normocellula

Radioautographic studies

Quantitation of labeling did n
series. In the myeloid line, howe
only young myeloid precursors .
myeloid elements which we calle
distinguished. There was practical
blasts of normal animals and the
decrease of labeling indices were
in the erythroid series and at the
series and at the stage of the pr
the amount of correls per cell y
and in animals exposed to benz

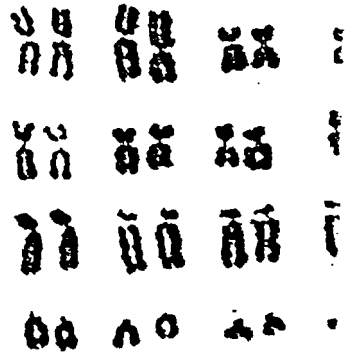


Fig. 1: shows a mitosis and kar

Results

Peripheral blood

With a dose of 0.2 ml/kg/d of pure benzene peripheral pancytopenia was seen within 6 to 12 weeks. A marked drop of leukocyte-thrombocyte-and reticulocyte counts was Seen within three weeks. Later there was also a fall of the erythrocyte count and of the haemoglobinconcentration. In the differential counts of the leukopenic animals there was granulocytopenia. The lymphocyte counts were less affected. Most animals presented with a marked basophilia after a few weeks of injection.

Bone marrow

Two weeks after starting benzene injections a left shift in erythropoiesis and myelopoiesis was found. In addition there were severe aberrations in the chromatine structure of the nuclei. Not all the marrows of animals with peripheral pancytopenia were hypocellular. Of 16 speamens where this could be judged adequately 10proved to be hypocellular, 4 normocellular and 2 hypercellular.

Radioautographic studies

Quantitation of labeling did not present any major difficulties in the erythroid series. In the myeloid line, however, qualitative aberrations were considerable and only young myeloid precursors which we called promyelocytes and mor: mature myeloid elements which we called myelocytes and metamyelocytes could be clearly distinguished. There was practically no difference of labeling indices in the pronormoblasts of normal animals and the ones exposed to benzene. The most pronounced decrease of labeling indices were found at the stages of the basophilic normoblast in the erythroid series and at the stage of the basophilic normoblast in the erythroid series and at the stage of the promyelocyte in the myeloid series. Quantitation of the amount of correls per cell yielded virtually identical results in normal controls and in animals exposed to benzene.

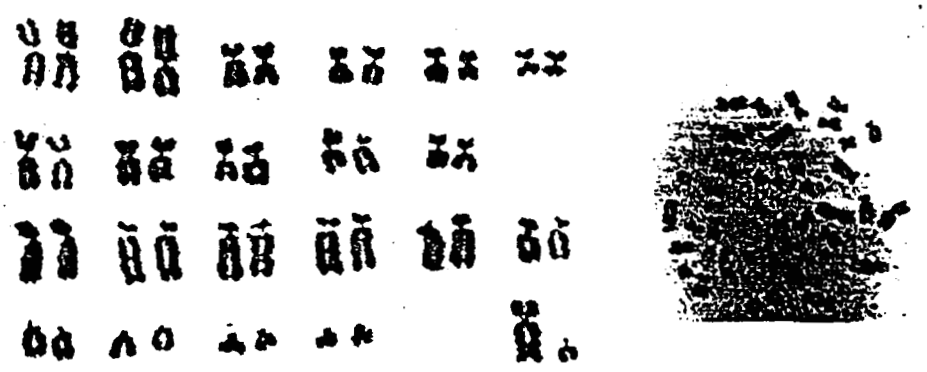


Fig. 1 : shows a mitosis and karyogram of a normal control animal.

Cytogenetic studies of the bone mar

Cytogenetics could be adequat
was 2 N 44/XY, XX.

Before exposure to benzene
was 5.9%. After 18 weeks of
increased to 57.8%.

Fig. 2 illustrates a mitosis and
after exposure to benzene. 47.50.
tid gaps. 8.8% were chromatic
and dicentric chromosomes we
Hyperploid mitoses were ob

Table 2: Here the studies on RNA synthesis quantitated with ^{3}H-cytidine
of exposure, column b at 14 days and column c at 21 days. A linear
ing is most markedly impaired
the stage of the basophilic normoblast
e depicted. Column a illust
of labeling was found after
and the promyelocyte.
the la
eling percentage at 7
longed exposure. Here again

Discussion

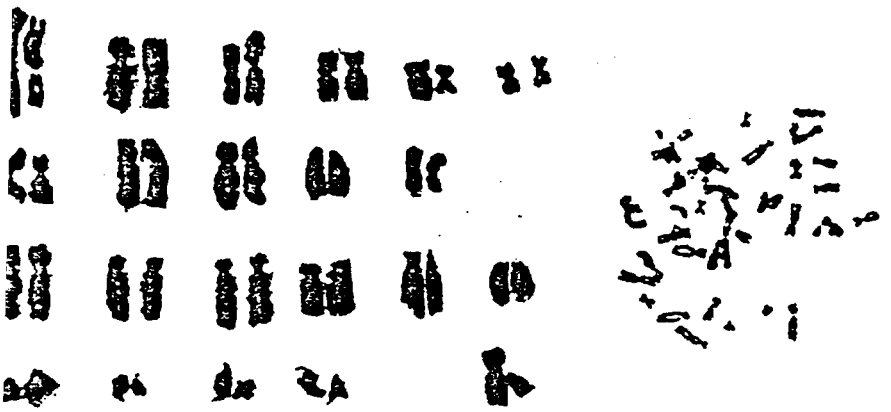
The pancytopenia which fol
DNA- and RNA-synthesis
maturation arrest between t
myeloid series a depression
recognizable stage.

Our data in mice expose
assayed with the in vivo c
conclude that the myelotoxi
synthesis of bone marrow
fact that ^{3}H-cytidine is no
evidence for a disturbed R
some aberrations in bone
to those found in some
glutathion reductase defi
drome [2,9], *Kostmann's* a
nicious anemia [13,29,36]
these patients will devel

genetic studies of the bone marrow cells

genetics could be adequately documented in 16 animals. In 15 the karyotype 2 N 44/XY, XX.

re exposure to benzene the amount of mitosis with chromosomal aberrations 5.9%. After 18 weeks of benzene injections chromosomal aberrations had eased to 57.8%.



g. 2 illustrates a mitosis and karyogram with the most frequent chromosomal aberrations ter exposure to benzene. 47.5% of the aberrations proved to be chromatid- and isochromad gaps. 8.8% were chromatid-and isochromatid breaks. Chromosome exchange figures nd dicentric chromosomes were found in 1.5% of the analysed metaphases.
Hyperploid mitoses were observed in one animal.

Discussion

The pancytopenia which follows benzene exposure seems to be due to a disturbed DNA- and RNA-synthesis of bone marrow cells. In the erythroid line there is a maturation arrest between the pronormoblast and the basophilic normoblast. In the myeloid series a depression of the labeling index is found already at the earliest recognizable stage.

Our data in mice exposed to benzene indicate that the hemopoietic stem cell, assayed with the in vivo colony forming technique are intact [*30*]. Therefore we conclude that the myelotoxicity of benzene is due to interference with DNA and RNA synthesis of bone marrow cell and possibly also with protein synthesis. Despite the fact that ^{3}H-cytidine is not an entirely specific precursor of RNA, we consider the evidence for a disturbed RNA synthesis from our data as conclusive. The chromosome aberrations in bone marrow cells after exposure to benzene are comparable to those found in some congenital syndromes such as *Fanconi*s anemia [*7,32*] glutathion reductase deficiency [*28*], ataxia teleangiectasia [*14,22*], *Bloom*s syndrome [*2,9*], *Kostmann's* agranulocytosis [*16,19*] and also with the findings in pernicious anemia [*13,29,36*], chemotherapy [*4,25*] and ionizing irradiation. Many of these patients will develop acute myeloid leukemia. It is difficult to answer the

question of the causal relationship between chc chromosome aberrations and [illegible] velopment of leukemia. The most likely explanation \ ~ ~lie that these ci. somal aberrations cause a high susceptibility for oncogenic viruses. or that in these patients the normal homeostatic immunologic mechanism which normally eliminates malignant cell clones fails more frequently than in normals.

References: 1 Biscaldi, G. P., L. Carosi and P. Invernizzi: Aspetti clinici rd anatomo patologici di due casi di panmieloftisi acuta benzolica. Lavoro Umano 3, 111 (1969). — 2 Bloom, D.: The syndrome of congenital teleangietatic erythema and stunted growth. J. Pediat. *68*, 103 (1966). — 3 Coscia, G. C., G. Perrelli and C. Meo: Aspetti clinici dell'emopathia da benzolo. Fol. med. (Nap.) *46*, 791 (1963). — 4 Catovsky, D. and D. A. G. Galton: M[illegible] monocytic leukemia supervening on chronic lymphocytic leukemia. Lancet *1*, 478 (19[illegible] — 5 Downey, H.: Handbook of hematology-Section XXXIX, 2693 (1966). — 6 Doi[illegible], G. W.: Methods of biological dosimetry with particular reference to chromosome aberration studies. Proc. Symp. handling of radiation accidents I.A.E.A. Vienna 1969. — 7 Forni, A. L. nnd C. Morea: Cytogenetic studies in a case of benzene leukemia. Europ. J. Cancer *3*, 251 (1967). — 8 Gallinelli, R. and A. Traidi: L'emopatia benzenica. Tre casi di benzolismo cronico di cui due mortali. Med. Lav. 54,169 (1963). — 9 German, J.: Bloom's syndrome. 1. Genetical and clinical observations in the first twenty-seven patients. Amer. J. Hum. Genet. 21,196 (1969). — 10 Goguel, A., L. Cavigneaux and J. Bernard: Les leucémies benzéniques de la région Parisienne. Mouv. Rev. Franç. Hémat. 7, *465* (1967). — 11 De Gowin, R. L.: Benzene exposure and aplastic anemia. J. Amer. Ned. Ass. 185,748 (1963). — 12 Hartwich, G. and K. Th. Schricker: Chromosomenuntersuchungen nach Benzol-Exposition. Fortschr. Med. *13*, 563 (1963). — 13 Heath, C. W.: Cytogenetics in Vitamin B_{12} and folate deficiency. Blood 27, 800 (1966). — 14 Hecht, F., R. D. Ka[illegible] D. A. Rigas, G. S. Dahnke, M. P. Gase, V. Tisdalc and R. W. Miller: Leukemia and lymp[illegible] cytes in ataxia teleangiectasia. Lancet 2,1093 (1966). — 15 Hungerford, D. A.: Leukocytes cultured form small inocula of whole blood and the preparation of metaphase chromosomes by treatment with hypotonic KCl. Stain Technol. *40*, 333 (1965). — 16 Kostmann, R.: Infantile genetic agranulocytosis (Agranulocytosis infantilis hereditaria). Acta Paediat. (Stockholm) 45, Suppl. 105 (1965). — 17 Loge, J. P.: Aplastic anemia following exposure to benzene hexachloride. J. Amer. Med. Ass. *193*, 110 (1965). — 18 Ludwig, H. and A. Wethemann: Benzolmyelopathien. Schweiz. mcd. Wschr. *92*, 384 (1962). — 19 Matsaniotis, N., K. A. Kiossoglou, J. Karponzas and K. Anatasia-Vlachou: Chromosomes in Kostmann's disease. Lancet *2*, 14 (1966). — 20 Xlocschlin. S. and B. Speck: Experimental studies on the mechanism of action of benzene on the bone marrow (radioautographic studies using ^{3}H-thymidine). Acta hacmat. 38, 104 (1967). — 21 Moorhead, P. S., P. C. Nowell, W. J. Mellman, D. W. Bactips and D. A. Hungerford: Chromosome preparations of leukocytes cultured from human peripheral blood. Exp. Cell Res. 20,613 (1960). — 22 Peterson, R. *3*., W. D. Kelly and R. A. Good: Ataxia teleangiectasia: Its association with dcfcctire thymic. immunological deficiency disease and malignancy. Lancet I, 1189 (1964). — 23 Pollini, G. and R. Colombi: Ricerche citogenetiche nell'aplasia midollare dall'intossicazione cronica da benzolo. Med. Lav. *54*, 837 (1963). — 24 Pollini, G. and R. Colombi: Il danno chromosomico medollare nell'anemia aplastica benzolica. Xlcd. Lav. 55, 241 (1964). — 25 Rccs, R. B, J. H. Bennet, E. M. Hamlin and H. I. Maibach: Aminopterin for psoiasis. Arch. Derm. (Chicago) 90,544 (1964). — 26 Rondanelli, E. G., P. Gorni, D. Pecorari, N. Trotta and R. Colombi: Effects du benzene sur la mitose érythroblastique. Acta hacmat. *26*, 281 (1961). — 27 Schoenenberger, E. M.: Erncutc Benzolvergiftungen in der schweizerischen Uhrenindustrie. Schweiz. mcd. Wschr. 93, 1469 (1963). — 28 Schrocdcr, T. M.: Cytogcnctische und cytologischc Bcfundc bci cnzpmopenischcn Panmyelopathien und Pancytopcnicn (Familiäre Panmyclopathie Fanconi. Glutathionreduktasemangel-Anämie). Humangenetik 2,287 (1966). — 29 Schroeder, T. M. and R. Kurth: Spontaneous chromosom[illegible] breakage and high incidence of lcukcmia in inhcrircd discasc. Blood 37, 96–112 (1971).

30 Speck, B. and M. Kissling: XII[illegible] p. 134. — 31 Speck, B., Th. Schnid[illegible] suchungen über den Wirkungsmech[illegible] graphische Studien mit ^{3}H-Thymid[illegible] M. R. and K. Hirschhorn: Fanc[illegible] breakage in various tissues. Ann. Kontchalovskaya and L. A. Zori[illegible] (1963). — 34 Vannuchi. V. and R. [illegible] iniziale die aplasia c aspetto succes[illegible] 57, 51 (1967). — 35 Vigliani, E. C[illegible] Med. *271*, 872 (1963). — 36 Winte[illegible] finaler Leukose. Bericht iiber zwe[illegible]

Author's Address: M. Kissling a[illegible] Protective Iso[illegible] Univ. Xkd. C[illegible]

30 Speck, B. and M. Kissling: XIII. International congress of hematology, Munich 1970 p. 134. — 31 Speck, B., Th. Schnider, U. Gerber and S. Moeschlin: Experimentelle Untersuchungen über den Wirkungsmechanismus des Benzols auf das Knochenmark (Autoradiographische Studien mit ^{3}H-Thymidin). Schweiz. med. Wschr. *96*, 1274 (1966). — 32 Swift, M. R. and K. Hirschhorn: Fanconi's anemia. Inherited susceptibility to chromosome breakage in various tissues. Ann. Int. Med. *65*, 496 (1966). — 33 Tareeff, E. M., N. M. Kontchalovskaya and L. A. Zorina: Benzene leukemias. Acta Un. int. Cancer *19*, 751 (1963). — 34 Vannuchi, V. and R. Mielosi: Mielosi acuti de benzolismo cronico con aspetto iniziale die aplasia e aspetto successivo de leucemia o di eritroleucemia. Riv. crit. clin. med. *57*, 51 (1967). — 35 Vigliani, E. C. and G. Saita: Benzene and leukemia. New England J. Med. *271*, 872 (1963). — 36 Winter, H., G. Huels and M. Faubert: Perniciöse Anämie mit finaler Leukose. Bericht über zwei Beobachtungen. Folia Haemat. (Leipzig) *3*, 91 (1958).

Author's Address: M. Kissling and B. Speck, Dep. Clinical Hematology and Unit for Prorective Isolat., Inst. of Radiopathology and Radiation Protection. Univ. Med. Center Leiden, Holland.