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On the Pathogenesis of Preleukemic Myelodysplastic Syndromes

Development of a Dysplastic Hemopoietic Proliferation in the Rat After a Single Pulse Dose of Dimethylbenz(a)anthracene (DMBA)*

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Summary. After a single pulse dose of DMBA, rats develop bone-marrow hypoplasia, which is almost compensated for by regeneration after 16 weeks. Subsequently, dysplastic signs of hemopoiesis appear in all experimental animals as massive extrusion of normoblasts into the peripheral blood, red-cell aniso- and poikilocytosis, nuclear deformities, atypical mitoses, and PAS-positivity, as well as megaloblastoid maturation dissociation of erythroblasts and nuclear and granulation anomalies of neutrophilic granulocytes and monocytes, comparable to human "pseudo-Pelger cells" and "paraneutrophils". At the time of death (112–497 days after DMBA pulse) experimental animals showed hyperplastic bone marrow with increased granulopoietic/erythropoietic ratios and an augmented, mainly erythropoietic, hemopoiesis in the spleen, with splenomegaly in six rats. Splenic hemopoiesis is accompanied by white pulp atrophia. The cause of death was septicopyemia in three rats, anemia in three, and bleeding in one rat. None of the animals developed a leukemic blast phase. Myelodysplastic changes in this experiment are the same as have been shown to precede leukemia in rats treated with five DMBA pulses (Fohlmeister et al. 1981). Possible relations of myelodysplasia and leukemia are discussed.

Key words: Myelodysplasia – Hemopoietic proliferation – Chemical leukemogenesis – Animal model for "preleukemia"

Introduction

Human myelodysplastic syndromes preceding acute leukemia, summarized under the term "preleukemia," are characterized by hemopoietic insufficiency, variable probability, and widely differing time lag (from less than 6 months to more than 20 years) for transformation into acute leukemia (Catovsky et al. 1971; Heimpel et al. 1972; Linman and Bagby 1976). Since these characteristics may lead to death from infection, anemia, bleeding or unrelated causes without (or before) manifes-

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tation of leukemia (Catovsky at al. 1971; Lehrer et al 1972; Arakawa et al. 1965), it is difficult to clarify the pathogenetical mechanism for development of both myelodysplasia and leukemia and their mutual relationship. Causal factors are unknown though toxic agents are believed to play a role in at least some cases (Brauer and Dameshek 1967; Court-Brown and Doll 1957; Fohlmeister et al. 1979; Reimer et al. 1977; Renoux et al. 1978). Therefore supportive examination of pathogenetical factors in an animal model seemed desirable. Requirements for the animal model were a high yield of leukemia within a time phase considerably shorter than the normal life span of the animals used and development of a preceding myelodysplastic syndrome comparable to human "preleukemia" under common environmental conditions. The rat leukemia model of Huggins and Sugiyama (1966) and Huggins et al. (1970), in which a blast-cell leukemia is elicited in more than 80% of rats within 100 days by five successive pulse doses of DMBA, was found suitable since hematological examination of the preleukemic phase revealed the existence of a myelodysplastic syndrome (Fohlmeister et al. 1981).

Furthermore, modification of the application schedule highly affects the yield of leukemia (Huggins and Sugiyama 1966; Huggins et al. 1970; Zeller et al. 1980). While changes in the time interval or dose are less effective, reduction of the number of injections to only one results in a decline of leukemia incidence to less than 10% (Huggins and Sugiyama 1966; Huggins et al. 1970). With this investigation, we show that the same myelodysplastic changes as seen in the preleukemic phase of rats treated with five DMBA pulses can be elicited by a single DMBA injection in rats that do not develop leukemic blast-cell proliferation. In parallel to the situation in human "preleukemia," these rats die from causes related to hemopoietic insufficiency.

Materials and Methods

Animal Experiments

A single dose of DMBA was injected into the tail vein of ten male Wistar rats (body weight 50–70 g) aged 25 days (group I). Ten control animals received only the equivalent dose of the solvent (group II). In most of the animals of group I, we waited for spontaneous death and only two animals (5 and 10) were killed by decapitation in ether anesthesia.

At the time of death of each of the experimental animals, one of the control animals was killed simultaneously. Survival times are given in Table 1. One animal in group I died during the injection and was not included in the study.

7.12-Dimethylbenz(a)anthracene (DMBA)

DMBA was dissolved in an oil-in-water emulsion (concentration: 0.5%. dose: 35 mg/kg body weight; for details see Fohlmeister et al. 1981).

Intravital and Postmortem Examinations

For counting erythrocytes and leukocytes and for performance of smears, blood was obtained from the tail vein at 2, 16, 38, 46, 63 und 71 weeks after injection from all animals still alive. Two and 16 weeks from the start of the experiment, iliac crest biopsies were obtained from one half of the experimental and control animals. Liver and spleen were weighed and examined histologically postmortem, as was the bone marrow from femur, sternum, spine, and iliac crest. From femoral marrow, imprints were made in addition for cytologic examination. For technical details of iliac crest biopsy, tissue preparation and standard stains see Fohlmeister et al. (1981).

Cyto(histo)chemical Reactions for Specific Demonstration of Granulopoiesis

In smears and imprints we used the peroxidase reaction and in tissue sections the naphthol-AS-D-chloracetate-esterase reaction (see Schaefer et al. 1970) was used for specific demonstration of granulocytopoiesis. In the rat. neutrophilic and eosinophilic granules exhibit peroxidase activity with a slightly different color of the reaction product, which is strongest in the myelocytic stage. Naphtol-AS-D-chloracetate-esterase activity is confined to neutrophils and mast cells in tissue sections. The finely granular reaction of neutrophils, which is again strongest in the myelocytic stage, is easily discernible from the coarse granulation of mast cells. For further details of enzymatic reactions of granulopoiesis in rats see Schaefer et al. (1970).

Cell Counts and Morphomerrir Analysis

Erythrocyte counts were performed with the "Cellcounter 2041" (Labora Mannheim). Leukocytes were counted in the Bürker chamber (capacity: 0.9 mm^3) after 20-fold dilution with Turk's solution. Two hundred cells were evaluated for differentiation of blood smears and 400 cells from bone-marrow imprints. Morphometric analysis of bone-marrow sections was undertaken with the Zeiss integration plate II at 250-fold magnification; 500 points were counted per section. Results express the percentage volume density of hemopoietic bone-marrow constituents.

Statistical Analysis

Blood counts of control animals (group II) were tested by variance analysis for differences between the various times. It turned out that for the erythrocyte and normoblast count, significant ($p < 0.01$) differences exist between the 2-week values and later values, which must be attributed to the completion of the physiological maturation process. In contrast, the granulocyte count of control animals rises after the 46th week, which seems to be the result of local inflammation after repeated bleeding. Means and standard deviations were calculated together for control values of all times between which statistical differences could not be shown. Values of experimental animals were tested with the Pearson-Stephens Criterion and those with less than 10% probability of belonging to the same statistical collective as controls were considered significantly different (see Koller 1969). This was the case for values outside the double standard deviation of controls. Bone-marrow values and organ weights of control and experimental animals were compared by Wilcoxon rank test.

Results

Hypoplasia

Iliac crest biopsy performed 14 days after commencement of the experiment in five animals of groups I and II shows hypoplasia in the experimental animals (see Fig. 3) which is nearly compensated for by regeneration 16 weeks after DMBA injection.

Hemopoietic Proliferation

At time of death (1 1 2 4 9 7 days after DMBA injection), an altered hemopoietic activity in experimental animals as compared with controls is seen in bone marrow and spleen. In the bone marrow the percentage volume density of hemopoietic cells is increased or within the upper normal range (see Fig. 3).

In five experimental animals, the granulopoietic/erytropoetic ratio ist shifted toward granulopoiesis and in one it is shifted toward erythropoiesis (see Fig. 4). In some animals there is also a distinct rise in the number of megakaryocytes, which are arranged in small groups. Granulopoiesis and erythropoiesis are slightly to moderately shifted to the left. Splenic hemopoietic activity in particular attains a fairly striking level in all but one experimental animal (no. 1). This is best reflected

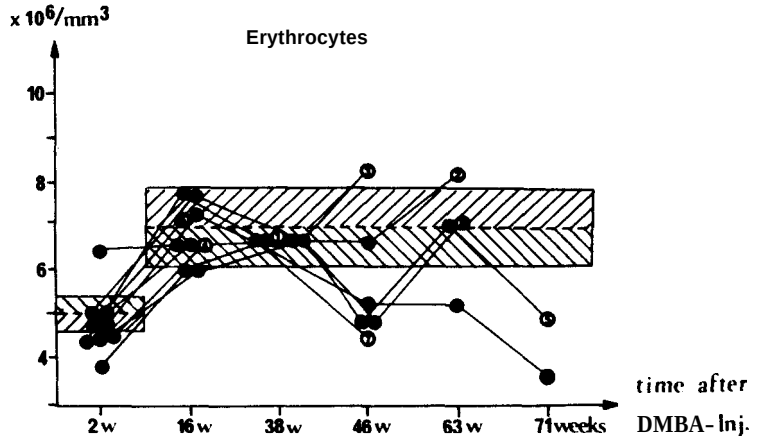

Fig. 1. Development of erythrocyte count after a single pulse of DMBA. Hatched bars: means and standard deviations of controls

in the spleen weight (see Fig. 4). The spleen architecture is grossly changed in experimental animals as compared with controls. Control animals show a well developed white pulp with broad periarteriolar lymphocyte cuffs and large follicles, which often show germinal centers. In the perifollicular zone a few maturing and degenerating granulocytes can usually be seen though no granulopoietic proliferation nests (see Fig. 6). In the younger control animals, the red pulp in addition contains a few small aggregates of normoblasts, whereas in the older animals no signs of erythropoiesis can be detected (see Fig. 6). In contrast, an atrophic white pulp is found in experimental animals. The periarteriolar lymphocyte cuffs are absent and the follicles are small; germinal centers are missing. Adjacent to the narrowed outer follicular zone there is a perifollicular ring of granulopoietic cell nests mainly at early stages as judged by their notched or early ring form nuclei, often found in mitosis, and the presence of specific cellular granules, which can be demonstrated with the naphthol-AS-D-chloracetate-esterase reaction (see Fig. 5 a, b). Smaller granulopoietic proliferation nests are distributed around the arterioles. Most of the splenic hemopoietic activity, however, is located in the red pulp and shows erythropoietic differentiation. Large confluent erythropoietic proliferation nests with immature forms surrounding the more differentiated cells occupy the sinuses and the reticular network, which can no longer be identified in histological section. Scattered throughout the erythropoietic nests there are partly immature megakaryocytes lying singly or in small groups (see Fig. 5 a, c). Four experimental animals (nos. 2, 7, 8, 10) in addition exhibit within the liver sinusoids small erythropoietic nests, which are found in none of the control animals.

Dysplasia

Erythropoiesis. Four animals from group I (nos. 5, 7, 8, 10) developed transient or permanent anemia after the 38th week, i. e., after completion of regenerative processes (Fig. 1). Besides a decrease in number, there is marked aniso- and poikilocytosis as well as polychromatophilia of erythrocytes. Three of these animals

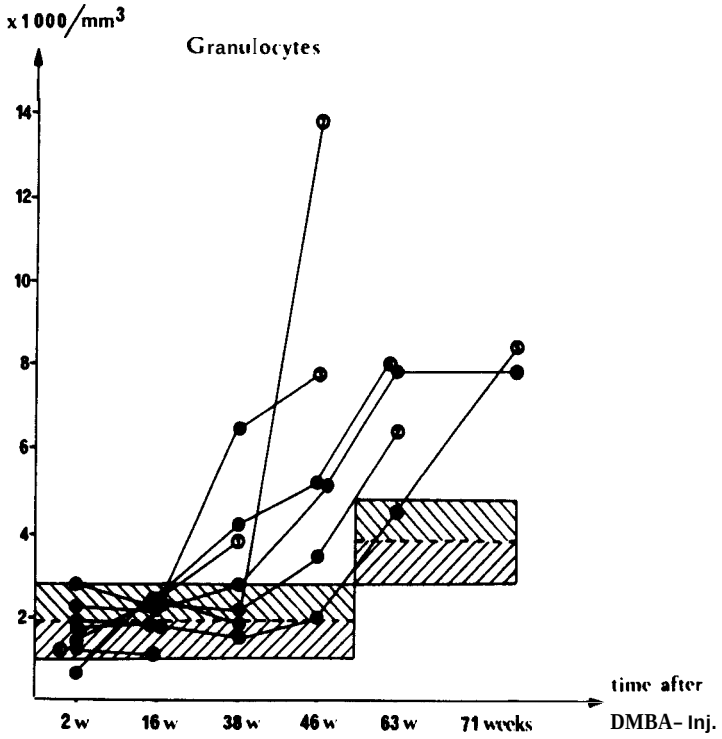

Fig. 2. Development of granulocyte count after a single pulse of DMBA. Hatched bars: means and standard deviations of controls

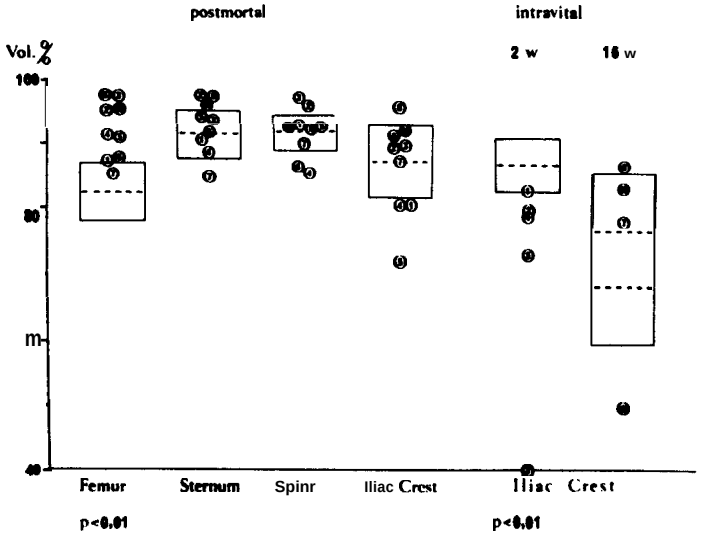

Fig. 3. Volume-share of hemopoiesis to whole marrow in bone specimens of different skeletal regions determined from histological sections. Bars: means and standard deviations of controls. Numbers in circles: number of experimental animals

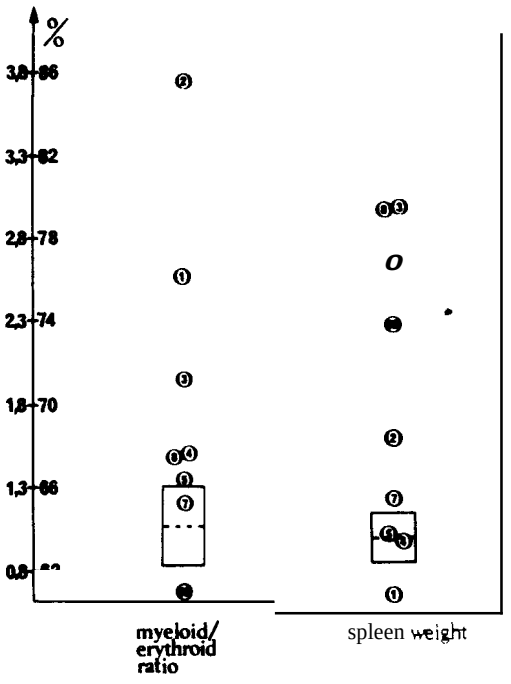

Fig. 4. Granulopoieticierythropoietic ratio in postmortem bone-marrow imprints (values of animal no. 6 discarded because of autolysis). Spleen weight. Bars: means and standard deviations of controls. Numbers in circles: number of experimental animals

(nos. 5, 8, 10) also present a slight to massive extrusion of normoblasts into the peripheral blood with values between 100 and 8,900/mm^3. Similar alterations of red blood cells with 400 normoblasts/mm^3 in the peripheral blood but a normal erythrocyte count are seen in another experimental animal (no. 2). Erythropoiesis of these five animals is characterized by budlike nuclear deformities, atypical mitoses, increase of nuclear doubling, PAS-positive erythroblasts, and a megaloblastoid dissociation of maturation. The amount of hemosiderin in the marrow reticulum cells is found to be the same in experimental and control animals. Though intervals between examination are too great for an exact determination of the duration of dysplastic changes, it is possible to state that in two cases they were present for more than 6 months.

Granulopoiesis. Five DMBA-injected animals developed granulocytosis (nos. 3, 5, 7, 8, 10; Fig. 2) and two of these were septicopyemic (nos. 3 and 8). One septicopyemic (no. 8) and two other experimental animals (nos. 5 and 7) show in addition

Fig. 5 a–c. Massive extramedullary hemopoiesis in the spleen of experimental animal no. 10. AS-D chloracetatesterase reaction with hematoxylin counterstaining of nuclei. (a) Specific histochemical presentation of granulopoietic cell nests, mainly in the perifollicular zone (*single arrows*), by positive AS-D-chloracetatesterase reaction. Red pulp consists of erythropoietic proliferation nests (*triple arrows*) with dark-staining nuclei, and scattered megakaryocytes (double *arrows*) with large unstained cell body; X 750 magnification. (b) Enlarged granulopoietic cell nest from perifollicular zone in (a). Mostly early stages with notched or early ring-form nuclei. and AS-D-chloracetatesterase positive specific granules crowded within the nuclear hole; X 3,200 magnification. (c) Two megakaryocytes (double arrows) surrounded by erythropoietic cells, enlarged from (a), left upper field: X 3.200 magnification

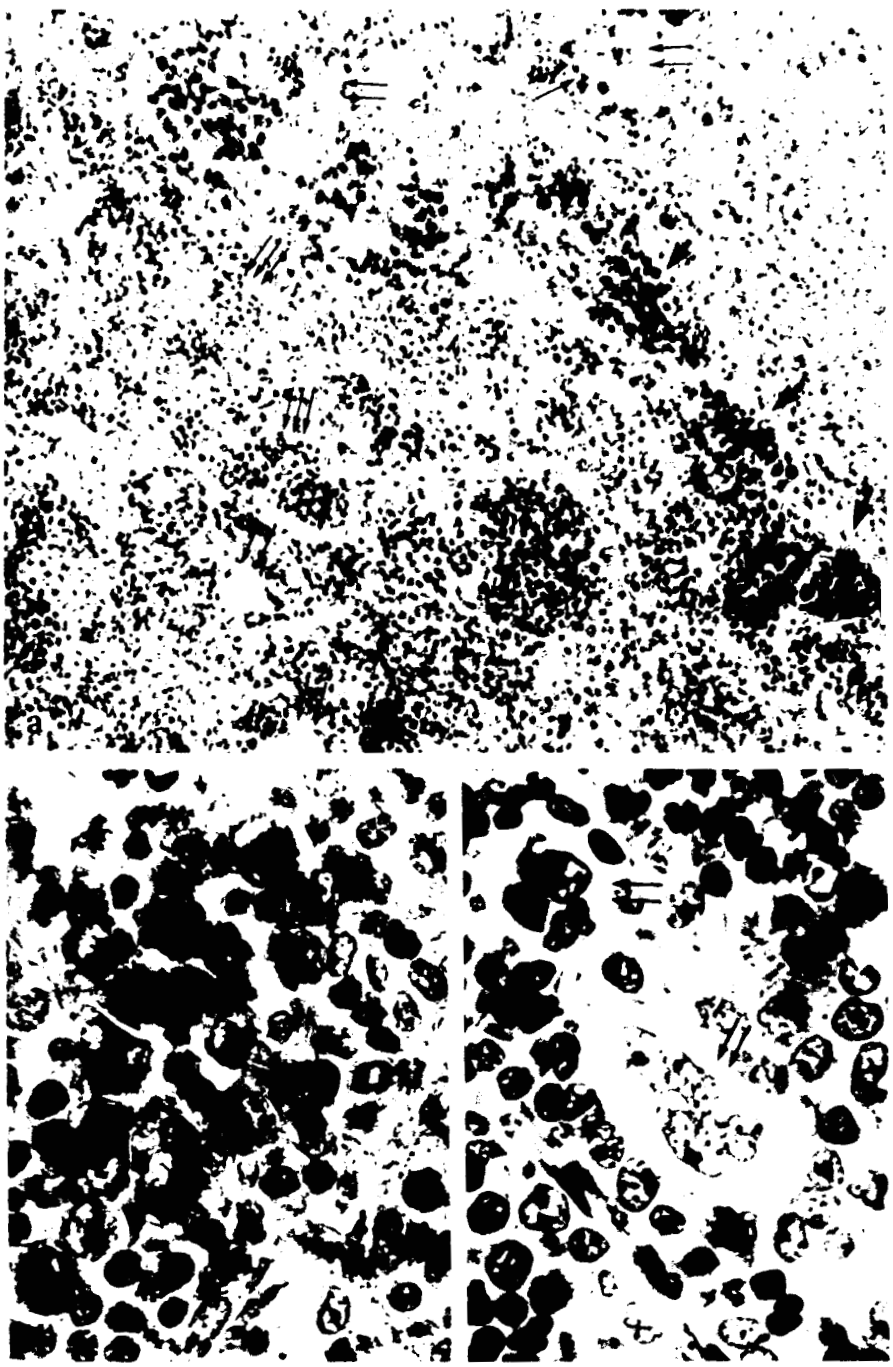

Fig. 5a–c

Fig.6. No signs of extramedullary hemopoiesis in spleen of control animal no. 10. Empty perifollicular zone *(arrows)* and red pulp (compare with Fig. 5 a). AS-D-chloracetatesterase reaction with hematoxylin counterstaining of nuclei; X 750 magnification

nuclear and granulation anomalies of neutrophilic granulocytes and monocytes. Animal no. **5** shows a coarsening of neutrophilic granules, both after performance of the peroxidase reaction and in Pappenheim stained slides. Additional atypias of granulocytic cells reminiscent of human pseudo-Pelger cells and partial peroxidase-deficient so-called "paraneutrophils" are seen in animals 7 and 8. In blood smears lymphocyte-sized cells with chromatine-dense, round to oval nuclei and specific, peroxidase-positive, neutrophilic granules are found in addition to larger neutrophilic cells with lobulated nuclei and an intensity of the peroxidase reaction that is intermediate between that of a normal monocyte and a normal myelocyte. These phenomena are seen in none of the control animals. In experimental animals they may be observed over a period of 2 to 6 months.

Cause of Death and Other Findings

Three experimental animals died from septicopyemia (nos. 3, 6, 8) and one from anemia (no. 7). Two rats were killed in a severely anemic state (nos. **5,** 10), one experimental animal had bleeding in a large subcutaneous fibroma (no. 2), and in two animals no explanation for death could be found in the autopsy. One of these animals differed from the others by a more pronounced atrophy of lymphatic tissues

Table 1. Survival times and pathologic-anatomical findings in experimental animals at time of death

No. of animals	Survival time after injection (days)	Findings at time of death
1	319	Increased hemopoietic proliferation in bone marrow, extreme atrophy of lymphatic tissues
2	439	Apple-sized subcutaneous fibroma, dysplastic hemopoietic proliferation in bone marrow and spleen with splenomegaly, small erythropoietic nests in liver sinusoids
3	438	Apple-sized subcutaneous fibroma, septicopyemia, dysplastic hemopoietic proliferation in bone marrow and spleen with splenomegaly
4	112	Cherry-sized subcutaneous fibroma, increased hemopoietic proliferation in bone marrow and spleen without enlargement of spleen
5	491	Apple-sized subcutaneous fibroma, dysplastic hemopoietic proliferation in bone marrow and spleen without enlargement of spleen
6	208	Septicopyemia, increased hemopoietic proliferation in bone marrow and spleen with splenomegaly
7	323	Dysplastic hemopoietic proliferation in bone marrow and spleen with moderate enlargement of spleen, small erythropoietic nests in liver sinusoids
8	439	Septicopyemia, dysplastic hemopoietic proliferation in bone marrow and spleen with splenomegaly, small erythropoietic nests in liver sinusoids
10	497	Dysplastic hemopoietic proliferation in bone marrow and spleen with splenomegaly, small erythropoietic nests in liver sinusoids

with barely detectable remnants of thymic tissue, lymph nodes, which were difficult to find even in the mesenterium, and a spleen virtually devoid of white pulp in the histologic section (no. 1). Pathologic-anatomical findings at the time of death are summarized in Table 1. Furthermore, it seems noteworthy that one of the control animals was also seen to have septicopyemia when killed on day 497. Surprisingly, in this animal there is no substantial shift of granulopoietic/erythropoietic ratio and no extramedullary myelopoiesis in the spleen. Spleen weight (0.8 g) is below the mean of control animals. Atypias in blood or bone marrow are not found. Peripheral granulocyte count is only slightly raised with 4,000/mm^3.

Discussion

It was the aim of this investigation to compare the morphological changes of hemopoiesis in rats after intravenous application of DMBA in a regimen with low leukemia incidence with those seen in the preleukemic phase of rats after the highly leukemogenic treatment with five DMBA pulse doses (see Fohlmeister et al. 1981).

Reduction of DMBA pulses to one has been shown to lower leukemia incidence to less than 10% (Huggins and Sugiyama 1966; Huggins et al. 1970) within an observation time of 100 days. None of the rats in our experimental series developed leukemic blast-cell proliferation during a prolonged observation time of up to 15 months, which is compatible with the results in the literature. However, after a transitory phase of severe bone-marrow hypoplasia, the animals died after a sur-

vival time of roughly 100–500 days in a state of hemopoietic hyperplasia. All animals showed hyperplastic bone marrow shifted to the left with increased granulopoietic/erythropoietic ratios and all but one also had an augmented, mainly erythropoietic, extramedullary hemopoiesis in the spleen with splenomegaly in six rats. In addition there were such dysplastic signs of hemopoiesis as appearance of normoblasts in the peripheral blood, red cell aniso- and poikilocytosis, nuclear deformities, atypical mitoses, and PAS-positivity, as well as megaloblastoid maturation dissociation of erythroblasts and nuclear and granulation anomalies of neutrophilic granulocytes and monocytes, comparable to human "pseudo-Pelger cells and "paraneutrophils." Hemopoietic proliferation in the spleen was accompanied by depletion of the white pulp. This lymphatic atrophy probably precedes hemopoietic proliferation, since the only experimental animal without evidence of splenic hemopoiesis nevertheless had developed atrophy of the white pulp as well as of all other lymphatic tissues. Seven of the experimental animals died from infection, bleeding or anemia, while the cause of death was unexplained in two rats.

It could be argued that the observed hemopoietic changes are merely the consequence of infection, which surely is promoted by hemopoietic and lymphatic depletion, and indeed has been proved by autopsy in three animals. However, the control animal, which showed septicopyemia when killed on the 497th day of experiment did not show bone-marrow hyperplasia or splenic hemopoiesis, and signs of dysplasia were not found. The reported dysplastic hemopoietic proliferation of experimental animals also seems quite out of line with the results of Fliedner and Heit (1969), who studied the influence of infection on regenerating bone marrow in mice after sublethal irradiation. These authors only found a delay in regeneration and a shift towards granulopoiesis compared with germ-free mice. Furthermore, the fact that in our experimental animals anemia was not seen and death from septicopyemia and bleeding did not occur during the phase of acute severe hemopoietic destruction, but long after complete restoration in a state of strongly increased medullary and extramedullary hemopoiesis, casts doubt on the interpretation of the expenmental results as reactive changes after infection. The character of the observed hemopoietic changes rather points to the possibility of primary hemopoietic disturbances with hemopoietic insufficiency and subsequent increased inclination to infection. The marked lymphatic atrophy, which already has been observed in the previous experiment (not reported), may well be an integral part of hemopoietic disturbances, since the lymphatic tissues seem to influence stem-cell proliferation and differentiation (Barret al. 1977; Petrovet al. 1977; Shinpock and Goodman 1978). and since the pluripotent hemopoietic stem cell is also the ancestor of lymphatic cells (Celada and Wigzell 1966; Wu et al. 1968).

The morphological hemopoietic changes in our experiment are principally comparable to those seen in human "preleukemia." With regard to the cause-effect sequence between morphological and functional hemopoietic changes and infection and vice versa, the situation ist much the same as in patients with longterm myelodysplasia, who – under the same common environmental conditions as in our experiment – eventually die from infection without (or before) developing leukemia (Arakawa et al. 1965; Catovsky et al. 1971; Lehrer et al. 1972). The relation of the syndrome to leukemia is still subject of discussion (see Fischer and Schaefer 1979; Heimpel et al. 1972; Linman and Bagby 1976). Myelodysplasia could merely

represent a state of risk for leukemic transformation, but most authors favor the theory of a true latency period with increasing expression of leukemic potential of an already transformed cell clone. The target cell seems to be the pluripotent stem cell.

In order to explain altered cell kinetics and increasing dedifferentiation during leukemia development several animal models for leukemogenesis are now under study (see Seidel **1980).** Considerable efforts have already been made to define the nature of the unterlying stem-cell defect and to examine the resulting functional changes, among which the immunological defects recently reported by Seidel **(1980)** seem to fit well our observation of a lymphatic atrophy preceding hemopoietic proliferation in the spleen. But correlation of these results from experimental leukemogenesis to observations made in patients with preleukemic syndrome are difficult, since detailed morphological studies in animal models are scarce.

Morley and Blake (1974) reported on chronic marrow aplasia as a late effect of prolonged Busulphan administration occurring about 8 months after restoration of initial damage in mice, 9% of which develeoped lymphoblastic leukemia. Different patterns of hemopoietic reactions were observed in dogs exposed continously to a d o x of ^{60} Co gamma irradiation of 10^{rad}/day by Seed et al. (1980). After 200 to 300 days most of the dogs died from anemia or septicopyemia in a state of hemopoietic aplasia. In dogs surviving this phase, marrow biopsies became hypercellular with increased myeloid/erythroid ratios and shift to the left of granulopoiesis. In **addition they showed partly the same cellular atypias as seen in our experiment and as reported from human "preleukemia." These dogs had a 45% incidence of nonlymphocytic leukemia.**

From these experimental results it is apparent that myelodysplastic changes are more readily elicited by chemical and physical agents than leukemia. In our experimental model myelodysplastic changes could be demonstrated in all rats both after a single DMBA pulse and after repeated applications, whereas repeated DMBA injections were required for leukemia development (see also Fohlmeister et al. **1981).** Obviously at least one additional event has to occur to cause a change in biological behavior and morphology in the sequence of leukemogenesis. The nature of this event may be related to the toxic or the oncogenic action of the drug. In general, oncogenic activity of drugs has been attributed to their mutagenic potential, for which covalent binding of the compound to nucleic acids is considered to be an essential part (Miller and Miller **1971).** Since covalent binding to DNA (Blobstein et al. **1975;** Bowden et al. **1974)** and replication of DNA after binding (Bates et al. **1970)** has been observed for DMBA, leukemic transformation may be regarded as the result of mutational changes of the pluripotent stem cell. In principal, this may take place already after a single injection. Additional DMBA Applications may just play a promoting role by eradicating competing normal hemopoiesis and by destroying immunological control. However, in our animal model as well as in those mentioned above myelodysplastic changes were a late effect of the causing agent, preceded by severe hemopoietic and (in our experiment) lymphoooietic damage. This seems to be the prerequisite for myelodysplasia development. Interestingly this view finds a counterpart in human "preleukemia" in the observation of a preceding aplastic phase in at least some of the patients (Fohlmeister et al. **1979).** On the other hand, after repeated DMBA injections the mutation rate will be increased. It seems likely, therefore, that myelodysplasia needs a lower mutation rate than leukemia. Alternatively, leukemia may be the result of a special. rare mutation or mutational sequence. whereas myelodysplasia may be related to

various genetic changes. This would mean that myelodysplasia is principally different from leukemia. In contrast to ordinary hyperplastic states of hemopoiesis myelodysplasia would represent a state of risk for leukemic transformation due to irreversible genotypic alterations of stem cells. In this sense myelodysplasia could be defined as a true prestage of leukemia.

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