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Secondary Tumors after High-dose Cyclophosphamide and Total-body Irradiation Followed by Bone Marrow Transplantation in a Rat Model for Human Acute Myelocytic Leukemia (BNML)*

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Abstract—*Brown Norway (BN) rats carrying a transplantable acute myelocytic leukemia (BNML) were given a supralethal combination of cyclophosphamide* (80–100 mg/kg *i.p.*) *and total-body irradiation* (9.0 Gy *gamma rays or* 8.5 Gy *X-rays) followed by isologous bone marrow transplantation. Of* 110 *long-term survivors*(>95 days), 40 (45%) *died of a secondary malignancy at a median post-treatment age of* 450 days. *At a comparable age, non-treated control BN rats show a spontaneous tumor incidence of* 5% *only, which increased to* 83% *during the aging process. Thus the latency period for the appearance of tumors was impressively shortened. Tumors of neurogenic origin and acute leukemias were the most prominent types, in contrast with non-treated control rats.*

INTRODUCTION

HIGH-DOSE chemo-radiotherapy followed by bone marrow transplantation (BMT) has become standard treatment for (hematological) malignancies and aplastic anemia in various centers [1, 2]. Since the number of long-term disease-free survivors steadily increases, late effects due to initial high-dose treatment become increasingly important. The pretransplant conditioning agents cyclophosphamide (Cy) and total-body irradiation (TBI) are both carcinogenic [3–10]. The first cases of secondary tumors after BMT in leukemic patients have been reported [11, 12]. In the next decade the appearance of secondary malignancies in patients, who have been treated and, indeed, cured with this therapy, might prove to be a serious problem.

As the latency period of tumors in man may be very long [13], the long-term effects of this treatment modality were studied in a rat model for human acute myeloid leukemia (AML), which has been described previously [14, 15].

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MATERIALS AND METHODS

Experimental animals

The experiments were performed with barrier-derived inbred Brown Norway (BN/BiRij) rats. Male rats of between 12 and 14 weeks of age were used.

Rat leukemia model (BNML)

The rat leukemia model has been described in detail elsewhere [14, 15]. The leukemia was induced in a female BN rat by 9,10-dimethyl-1,2-benzanthracene. It shows a reproducible growth pattern upon intravenous cellular transfer within the BN rat strain. Cytologically and cytochemically, it is similar to human acute promyelocytic leukemia. Additional analogies with the human disease are: (a) a slow growth rate; (b) a severe suppression of normal hemopoiesis due to an absolute numerical decrease in the number of normal hemopoietic stem cells; (c) diffuse intravascular coagulation; (d) prolonged

blood transit time of leukemic cells (34–36 hr); (e) response to chemotherapy as in human AML; (f) presence of clonogenic leukemic cells; (g) low antigenicity; and (h) no evidence for a virus as the etiologic agent.

Drugs

Cyclophosphamide (Cy; ASTA, Weesp, The Netherlands) was dissolved in 0.9% NaCl and injected i.p. in a volume of 1.0 ml.

Total-body irradiation (TBI)

TBI was carried out with (1) a gamma radiation source (gamma cell 20, Caesium-137, Atomic Energy of Canada, Ltd.) at a dose rate of 0.9 Gy/min; (2) X-radiation (X-ray machine, Philips–Müller 300), physical parameters: 300 kV, 10 mA, HVL of the beam 3.0 mm^3, distance from the focus to the bottom of the cup 14–16 cm; dose-rate: 0.35 Gy/min.

Experimental design

At day 13 after i.v. inoculation of 10^7 BNML cells, cyclophosphamide in a dosage of 80 or 100 mg/kg was injected i.p. In some cases (±10%) additional treatment with 20–50 mg/kg Cy was later given for maintenance therapy. The total dose of cyclophosphamide varied from 80 to 200 mg/kg. At day 14 a total of 230 rats were irradiated (TBI) with either 9.0 Gy gamma rays or 8.5 Gy X-rays (TBI) ('flash' or fractionated in 2–3 fractions). This was followed by transplantation of 1×10^8 isologous bone marrow cells i.v. on the same day. Following this, each rat was administered a transfusion of 1 ml of packed cells each day for 4 days as supportive care during the period of leukopenia and thrombocytopenia. Blood was obtained from normal BN rats by puncture of the abdominal aorta. The rats were kept under conventional conditions with food pellets and water *ad libitum* as described previously [16]. Rats with abnormal growth of the teeth causing difficulties in food-intake received powdered food pellets. All animals were allowed to complete their life spans. A complete necropsy was performed on those found dead. Tissues were routinely processed on 5-μm slides and stained with hematoxylin-phloxine safran (tips). Extra stains were prepared when required.

RESULTS

Of 230 male BN/Bi rats, 110 (47%) lived longer than 95 days after treatment. Death before this time was due to leukemia relapse, sepsis, aplasia or drug-related toxicity. Only those rats surviving past that time were followed for the occurrence of secondary tumors, as a leukemia relapse generally occurs prior to day 95 [15]. At death, histological examination was performed in 88 of the 110 long-term survivors (80%).

The incidence of secondary tumors in this group was 40 of 88 (45%); 44 of 88 (50%) of the rats died from generalized cachexia. Histological examination revealed no other possible cause for this emaciation than a severe periodontitis and loosening of front teeth, probably due to growth abnormalities at the roots of the incisors. In four cases (5%) death was due to lung problems (radiation pneumonitis or pneumocystis carinii pneumonia).

As can be seen in Fig. 1, the median post-treatment age of rats that developed neoplasia was 450 days (range, 93–744 days). At a comparable age the tumor incidence in untreated male BN/Bi rats is less than 10 (Figs 1 and 2; [17]). The median age of rats that died from cachexia was 210 days (range, 135–624 days). The four rats dying with lung problems died at a median post-treatment age of 570 days (range, 228–618 days). The age distribution of male BN/Bi rats dying with a secondary malignancy is listed in Table 1. The majority of tumors appeared after day 200. The types of tumors found are listed in Table 2. Tumors of neurogenic origin, e.g. malignant Schwannoma, were relatively frequent compared to the incidence of such tumors in untreated aging rats of this strain and sex (malignant Schwannoma 0% [17]). Lymphohemopoietic malignancies were observed in a frequency comparable to that in untreated aging rats (15% [17]), but at a much younger age. Urothelial carcinomas, which are common (35% [17]) in untreated male BN/BiRij rats, had a low incidence in the treated rats.

The spontaneous incidence of cortical adenomas is 12% and of cortical carcinomas 1%.

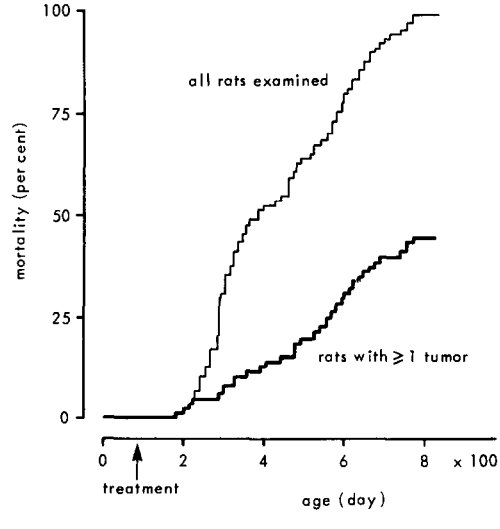

Fig. 1. Cumulative mortality in 88 BN/Bi rats treated with high-dose cyclophosphamide and total-body irradiation.

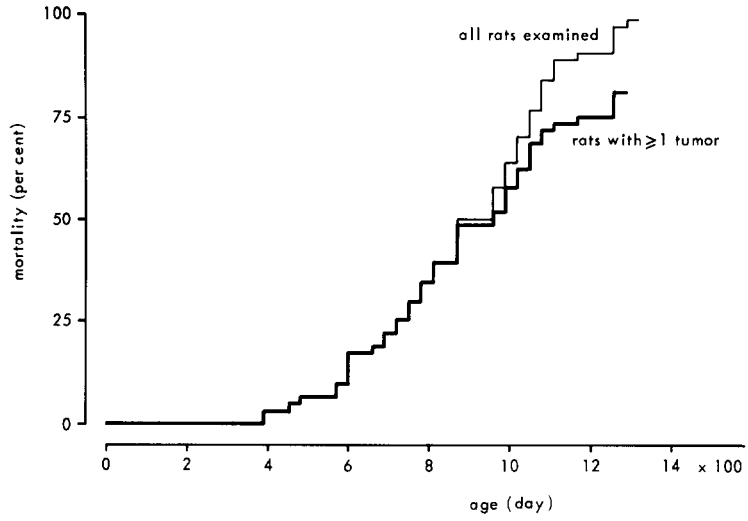

Fig. 2. Cumulative mortality in 64 untreated BN/Bi rats.

Table 1. Age distribution of male BN/Bi rats dying with secondary tumors after treatment with high-dose cyclophosphamide-total-body irradiation

Post-treatment age (days)	No. of rats examined histologically	No. of rats with histologically verified neoplasms
95–99	1	1
100–199	17	3
200–299	27	7
300–399	11	6
400–499	10	8
500–599	15	10
600–699	6	4
700–799	1	1
Total	88	40

Table 2. Histological classification of secondary tumors in 88 male BN/Bi rats treated with high-dose cyclophosphamide and total-body irradiation

	No.
Malignant Schwannoma/neurofibrosarcoma	8
Pheochromocytoma:	
benign	3
malignant	2
Adrenal cortical adenoma	3
Adrenal cortical carcinoma	1
Urothelial carcinoma	3
Myelomonocytic leukemia	10
Melanoma	2
Other tumors in various tissues	8

DISCUSSION

The incidence of secondary tumors in rats treated with high-dose CY and TBI was 45%, compared with 83% in control groups. However, the tumors in the treated rats appeared at a much younger age (Figs 1 and 2). Thus the latency period for the appearance of tumors is impressively shorter than in control rats, i.e. 450 vs 870 days (median).

Of the tumor types observed, malignant Schwannomas do not occur in untreated controls from the same strain and sex. Myelomonocytic tumors and pheochromocytomas do occur in about the same frequency, while urothelial tumors are somewhat less frequent than in the controls, although all occur at a much younger age. The other malignant and benign tumor types encountered in the treated rats were not exceptional for this strain. The secondary myelomonocytic leukemias are completely different from the original BN leukemia, as was proven by cytology and cytochemistry.

Periodontitis and abnormal growth of teeth were seen in at least 50% of the rats. This seems to be the cause of breaking, loosening and subsequent loss of teeth, thus interfering with the intake of food so that many animals died from cachexia. This is a well-known side-effect of Cy in rodents [18–21]. Because wearing down of the incisors by gnawing action was lessened, continuous growth resulted in very long incisors in some cases, and these had to be artificially cut. Powdered food instead of hard food pellets seemed effective for a while as a means of facilitating chewing and increasing the food intake. However, this did not prevent the early death of the majority of animals. As they died relatively early they were not yet at risk for the development of secondary tumors. Therefore the incidence of early secondary tumors mentioned above (45%) is probably an underestimation. Pulmonary complications also contributed to death in a few cases.

High-dose chemo-radiotherapy is clearly carcinogenic in the BN rat, inducing tumors at various sites. Cyclophosphamide in combination with total-body irradiation is a mode of treatment

widely used in leukemia and aplastic anemia patients as a preparation for bone marrow transplantation.

The survival time of an increasing number of these patients extends to a 5-yr period. A review of the carcinogenicity of antineoplastic agents in man indicates that the shortest period of time from the initiation of therapy to the development of malignancies is at least 4 yr [13]. Secondary tumors due to this therapy in man are to be expected in the next decade. Although our data, particularly as regards the incidence percentage, might not be directly comparable to the clinical situation, the first case reports of patients who developed tumors after treatment with high-dose Cy and TBI [11, 12] support this view.

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