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AMSA: *in Vivo* Log Cell Kill for Leukemic Clonogenic Cells versus Toxicity for Normal Hemopoietic Stem Cells in a Rat Model for Human Acute Myelocytic Leukemia (BNML)

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Abstract—*The efficacy of AMSA was evaluated quantitatively in a rat model (BNML) relevant for human acute myelocytic leukemia. The* LD_{50} *values observed in normal and leukemic Brown-Norway rats were* 26.4 *and* 28.3 mg/kg *respectively. In the higher dose ranges, the major cause of death was acute cardio-pulmonary toxicity. After single dose treatment,* 20 mg AMSA/kg *resulted in a surviving fraction of* 5.5×10^{-2} *for normal pluripotent hemopoietic stem cells and* 4.1×10^{-5} *for* in vivo *clonogenic leukemic cells. With repeated administration of the drug amounting to the same total dose, even a* 4 *log difference in cell kill was observed between both cell populations. These studies provide quantitative information on the therapeutic index of AMSA and support the inclusion of this drug in first-line treatment regimens for acute myelocytic leukemia.*

INTRODUCTION

AMSA (acridinyl anisidide) is an effective drug in the treatment of patients with refractory acute leukemia. As a single agent it has induced 20% complete remissions in this category of poor prognosis patients [1–5]. This has lead to the introduction of the drug in first line remission-induction chemotherapy regimens [6]. As with most effective anti-leukemic cytostatic agents, the dose-limiting tissue is the normal bone marrow.

In the present study, the efficacy of AMSA against leukemia was compared in terms of log cell kill with its toxicity for normal pluripotent hemopoietic stem cells. Both single injections and repetitive treatments were evaluated, as well as the role of bone marrow transplantation after high-dose AMSA. As a relevant model for human acute myelocytic leukemia (AML), a transplantable leukemia in the rat was employed (BNML). The BNML is widely used as a preclinical model for *in vitro* culture-, chemoradiotherapy- and bone marrow transplantation studies [7, 8].

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MATERIALS AND METHODS

Experimental animals

The experiments were performed with the inbred Brown–Norway (BN/Bi) rat strain produced in the Rijswijk colony. Male rats between 13 and 16 weeks of age were used (mean body wt: 240 g).

For the spleen colony assays of normal stem cells F1 mice from C57BL/Rij × C3H/Law were used.

The rat leukemia model (BNML)

The rat leukemia model (BNML) has been described in detail elsewhere (origin, classification, transplantation procedure, growth characteristics, etc.; [7, 8]). 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. Further analogies with the human disease are: (a) a slow growth rate (10^7 BNML cell kill after 18–23 days; growth fraction, 0.60–0.40); (b) a severe suppression of normal hemopoiesis due to an absolute numerical decrease in the number of normal hemopoietic stem cells; (c) diffuse intravascular coagu-

lation; (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 (including *in vitro* colony formation; TD50, 25 cells; (g) low antigenicity; and (h) no evidence for a virus as an etiologic agent.

Spleen colony assays

Hemopoietic stem cell assay (CFU-S). As a measure for the number of pluripotent hemopoietic stem cells, a modified spleen colony assay was used. Lethally irradiated (9.5 Gy of γ rays) mice (F1 from C57BL/Rij × C3H/Law) were injected with graded numbers of bone marrow cells. After 9 days the mice were sacrificed and the number of macroscopically visible spleen colonies was determined. The method, which yields a linear relationship between the number of injected rat bone marrow cells and the number of colonies counted at the surface of the mouse spleen has been described in detail by Van Bekkum [9].

Leukemic clonogenic cell assay (LCFU-S). Injection of graded low numbers of leukemic cells into normal BN rats results in the development of macroscopically visible colonies on the surface of the spleen 19–20 days later. Each colony is assumed to have been derived from one leukemic clonogenic cell. Details of this method have been described before [10].

AMSA

AMSA (acridinyl anisidide) was obtained from the Bristol-Myers Company International Division (New York, U.S.A.). The drug was solved in anhydrous *N,N*-dimethyl-acetamide and further diluted with 0.0353 M L-lactic acid and—if necessary—saline. The drug was administered intravenously.

Experimental designs

a. The LD_{50} *in normal- and leukemic rats.* The dose of AMSA which causes death in 50% of the recipient rats (i.e., the LD_{50}) was determined both in normal BN rats and in rats injected with AMSA at day 13 after inoculation with 10^7 BNML cells (8–16 rats per group). AMSA dosages varied from 5 to 40 mg/kg i.v. The surviving rats were observed at least for 150 days.

In a similar experimental setting it was evaluated whether infusion of 10^8 isologous bone marrow cells 24 hr after the injection of AMSA could prevent toxicity-induced deaths. This technique has been described extensively before [14].

b. Toxicity for normal hemopoietic stem cells and clonogenic leukemic cells. AMSA was injected i.v. into normal BN rats (5–30 mg/kg). Twenty four hours later the rats were killed, and a CFU-S assay was performed on the femoral bone marrow. The reduction in the number of CFU-S in the marrow was calculated compared to untreated controls.

Toxicity for leukemic cells was determined by injecting AMSA (5–30 mg/kg) into BNML rats at day 13 after inoculation with 10^7 leukemic cells. An LCFU-S assay was performed 24 hr later on femoral bone marrow.

c. Fractionated AMSA treatment. Both normal and leukemic rats were treated with a daily dose of 5 mg AMSA per kg body wt i.v. for 1 up to 4 consecutive days. To determine normal and leukemic stem cell toxicity, CFU-S and LCFU-S assays were performed on femoral bone marrow 24 hr after the (last) injection of AMSA.

RESULTS

a. The LD_{50} *in normal- and leukemic rats*

In Fig. 1 the LD_{50} curves in normal rats (A) and leukemic rats (B) are plotted employing a computer program for probit analysis. In normal rats the LD_{50} dose is 26.4 mg/kg wih 95% confidence limits ranging from 23.2 to 29.6 mg/kg. In BNML rats this value is 28.3 mg/kg (95% confidence limits: 25.3–31.2 mg/kg). Thus, no significant difference was found for the LD_{50}-AMSA dose between normal and leukemic rats. With these dosages, death was due to irreversible aplasia. Deaths in the higher dose ranges were due to acute cardiac/respiratory failure and could not be prevented by isologous bone marrow transplantation.

b. Toxicity for normal hemopoietic stem cells and clonogenic leukemic cells

Figure 2 shows the surviving fraction of normal stem cells (CFU-S) and clonogenic leukemic cells (LCFU-S) as a function of the administered AMSA dose. It is clear that leukemic cells are more sensitive than normal stem cells. With a dose that is tolerable for all rats, i.e., 20 mg/kg, surviving fractions of 4.1×10^{-5} and 5.5×10^{-2} are obtained, respectively.

c. Fractionated AMSA treatment

In Fig. 3 the results of fractionated AMSA treatment in terms of normal stem cell toxicity and anti-leukemia activity are plotted. It appears that with fractionated treatment the difference in sensitivity between both cell populations (or: the therapeutic index) increases. This is especially clear when the 4 × 5 mg/kg regimen is compared with the 20 mg/kg single dose treatment (Fig. 2), i.e., a 4 log and a 3 log difference in cell kill, respectively. With the 2 × 5 mg/kg and 3 × 5 mg/kg treatment regimens a 2–3 log cell kill difference is observed between CFU-S and clonogenic leukemic cells (LCFU-S).

DISCUSSION

After AMSA had shown promising results in animal models [11], it subsequently showed sig-

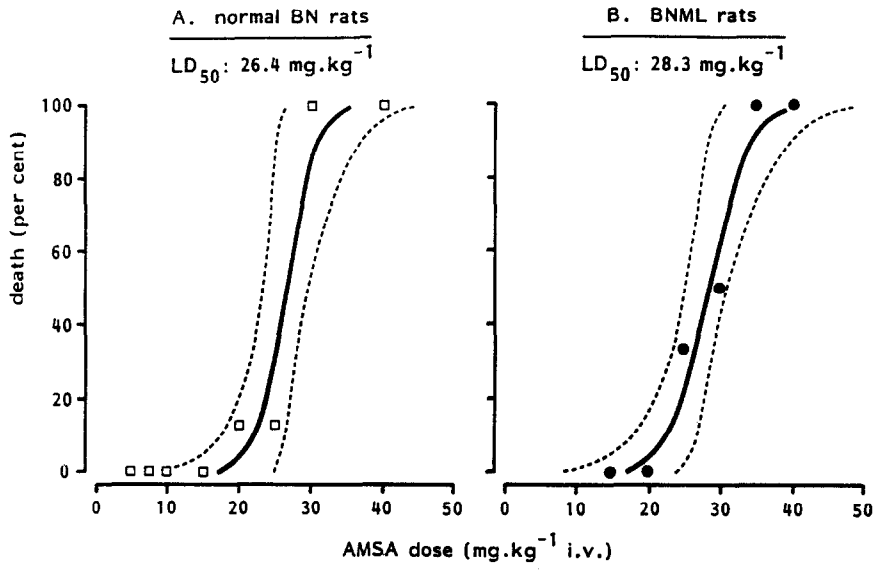

Fig. 1. The LD_{50} *values for AMSA in normal* (A) *and leukemic* (B) *rats. The area between the dotted lines represents* 95% *confidence limits. BNML: BN acute myelocytic leukemia.*

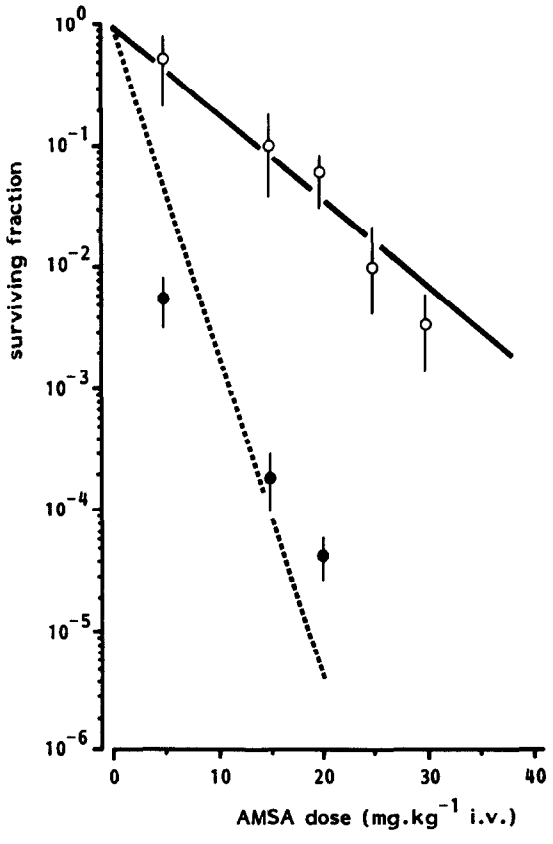

Fig. 2. AMSA: Sensitivity of normal pluripotent hemopoietic stem cells (CFU-S) and in vivo *clonogenic leukemic cells (LCFU-S). Vertical bars represent standard deviations.* o——o *CFU-S: colony forming units-spleen;* ●-------● *LCFU-S: leukemic colony forming units-spleen.*

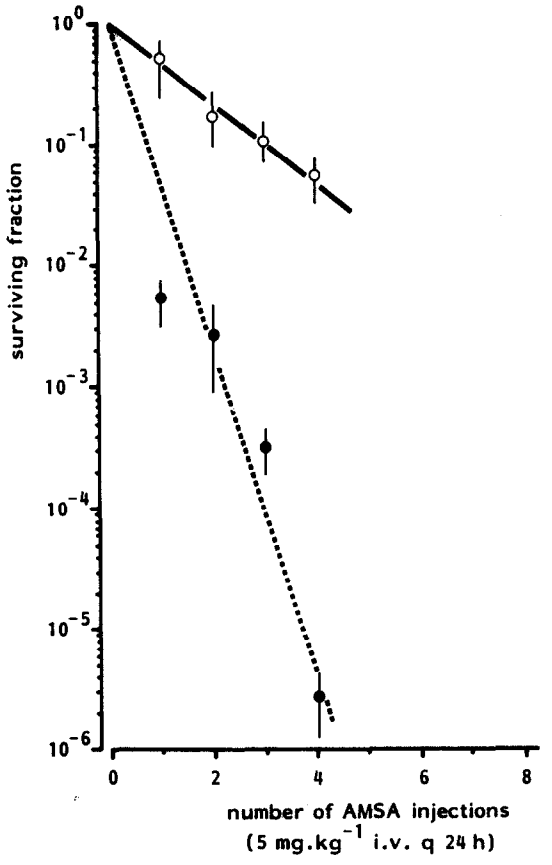

Fig. 3. Repeated injections of AMSA: Sensitivity of normal pluripotent hemopoietic stem cells (CFU-S) and in vivo *clonogenic leukemic cells (LCFU-S). Vertical bars represent standard deviations.* o——o *CFU-S: colony forming units-spleen;* ●-------● *LCFU-S: leukemic colony forming units-spleen.*

nificant activity in the acute leukemias and, to a lesser extent, also in patients with non-Hodgkin's lymphomas as studied in phase I and phase II studies [1–6]. From a number of phase II studies in patients with refractory/relapsed acute myelocytic leukemia the cumulative complete remission rate is 17% [5] and various treatment centers have now included AMSA in first line treatment regimens. Bone marrow toxicity is the major dose-limiting factor. As there are no clear-cut quan-

titative human data on the anti-leukemic efficacy of AMSA, the present study, employing a relevant rat model for human AML, was undertaken to evaluate the therapeutic index after single or multiple dose treatment. The BN acute myelocytic leukemia offers the advantage that both the pluripotent hemopoietic stem cell population and the *in vivo* clonogenic leukemic cells can be assayed quantitatively.

Firstly, the LD_{50} values were determined (Fig. 1). It appeared that infusion of isologous bone marrow cells could not prevent death in the higher dose ranges, obviously due to acute lethal cardiopulmonary damage. In this respect, the rat is much more sensitive than man. Based on the equivalent surface area dosage conversion factor [12] 25 mg/kg in the rat compares to 3.5 mg/kg in man. This human dose can be given repeatedly at consecutive days without lethal toxicity [5].

From single dose treatment experiments it became clear that clonogenic leukemic cells (LCFU-S) are much more sensitive to the drug as compared with hemopoietic stem cells (CFU-S; Fig. 2). With fractionated treatment (Fig. 3) the therapeutic index increases even further. Although the underlying mechanism of this difference in sensitivity is not clear, this is most probably not due to a difference in the proliferating fraction of normal rat stem cells and clonogenic BNML cells. As reported before, at day 13 after leukemia transfer 40–60% of the leukemic cells are in DNA synthesis phase [7, 8] as compared with 40% of the normal CFU-S [10]. Maximally, AMSA induced a 4 log difference in cell kill with the 4 × 5 mg/kg regimen. At this dose level, good for a 5–6 log leukemic cell kill, a sufficient number of CFU-S is left (surviving fraction: 5.0×10^{-2}) to restore normal hemopoiesis completely.

Based on previous studies in our group, AMSA is as effective as high-dose cytosine arabinoside [13] or high-dose cyclophosphamide [14]. This supports the inclusion of this new drug in the primary treatment of AML.

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