Comparison of Cyclophosphamide, Cytarabine, and Etoposide as Immunosuppressive Agents Before Allogeneic Bone Marrow Transplantation

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Etoposide and cytarabine have been shown to exert high antileukemic activity and are currently under study as preparatory agents before allogeneic bone marrow transplantation. However, data concerning their engraftment-promoting potency are scarce. Therefore, we tested these agents in LEW rats receiving a myeloablative dose of busulfan followed by transfer of F1 (CAP × LEW) marrow, which is unable to induce a graft-vs-host reaction (GVHR). Since busulfan by itself has only minor immunosuppressive potency, graft rejection ensues unless etoposide, cytarabine, or cyclophosphamide provide additional immunosuppression to facilitate durable engraftment. Before allogeneic bone marrow transplantation in humans, 120 mg/kg of cyclophosphamide, 60 mg/kg of etoposide, or 900 mg/kg of cytarabine are the standard doses given in conjunction with total body irradiation. Seventy-five percent of these doses administered in addition to busulfan resulted in rejection rates of 75% for cytarabine and 58% for etoposide, respectively, whereas no rejections were observed with cyclophosphamide. These data indicate that etoposide and cytarabine are inferior to cyclophosphamide in their rejection-preventing potential. Using either of these agents as substitutes for cyclophosphamide before allogeneic bone marrow transplantation may increase the risk of graft rejection in HLA-mismatched bone marrow transplantation and, in case of HLA identity, if T-depleted marrow is administered.

Since 1977 the standard preparatory regimen preceding allogeneic bone marrow transplantation for patients with hematologic malignancies has consisted of high-dose cyclophosphamide and total body irradiation (TBI), the latter administered at doses between 7.5 and 12 Gy, depending on dose rate and fractionation schedule. The results obtained with various modifications of this combination were encouraging, with a considerable proportion of the patients being cured. Nevertheless, the unacceptably high rate of relapse, especially in patients who receive transplants for advanced leukemias, clearly indicated that a search for alternative preparatory regimens was necessary.

Because cyclophosphamide is no agent of first choice in the conventional treatment of both acute myelogenous and acute lymphocytic leukemia, attempts have been made to replace this drug with other cytostatic agents in the transplant situation as well. Combinations of fractionated TBI and high-dose etoposide or high-dose cytarabine have been shown to exhibit substantial antileukemic activity that may be superior to that of the standard regimen. In the context of allogeneic bone marrow transplantation, however, the preparatory regimen must also provide considerable immunosuppressive activity to allow durable engraftment of the donor marrow.

To address the question of the immunosuppressive potency of drugs with known high antileukemic efficacy, we looked for a preclinical model that might allow a comparison of the engraftment-promoting potency of various cytostatic agents administered before allogeneic bone marrow transplantation. The prerequisite for such an approach is a myeloablative regimen that, however, has limited immunosuppressive potency and does not allow engraftment of allogeneic marrow. Tutschka and Santos have reported that busulfan-treated mice and rats do represent an experimental model exactly fulfilling these criteria. However, due to the toxicity of some agents of potential interest, this model had to be refined to enable us to distinguish between deaths due to toxicity and deaths due to rejection of the grafted marrow. We have succeeded in doing this by carefully monitoring the hematocrit and granulocyte counts of the grafted animals.

Materials and Methods

Experimental design. LEW rats received a lethal dose of busulfan that by itself was not sufficiently immunosuppressive to allow engraftment of allogeneic bone marrow. Increasing doses of TBI, cyclophosphamide, cytarabine, and etoposide were added and tested for their capacity to promote durable engraftment of 4 × 10⁶ allogeneic marrow cells. To exclude graft-vs-host reaction (GVHR)-induced morbidity and mortality, F1 (CAP × LEW) marrow was used to reconstitute the parental strain LEW rats.

Since preliminary experiments revealed that the preparatory regimens tested would be highly toxic, with a substantial proportion of the rats dying even after syngeneic bone marrow transplantation, it proved necessary to determine whether death was due to graft rejection or due to toxicity of the preparatory regimen in each individual rat. Consequently, hematocrit determinations and leukocyte and differential blood counts were done on each animal on days 4, 7, 10, 13, 16, 19, 23, 32, 42, 52, 62, 72, and 82 posttransplant. The day of marrow grafting was termed day 0.

All rats surviving the 100-day observation period received an allogeneic CAP skin graft to indicate persistence or to ascertain rejection of the transplanted marrow since rejection might otherwise have been masked by autologous recovery.

Experimental animals. Female LEW rats aged 10 to 16 weeks were used as recipients, and the F1 crosses of LEW and CAP rats (CAP × LEW) of either sex aged 10 to 25 weeks served as bone marrow donors. Busulfan. Tablets of busulfan containing 0.5 and 2 mg were crushed in a mortar, suspended in 3 mL of tap water, and applied via a gastric tube 24 hours before intravenous (1V) injection of the bone marrow. If not otherwise stated, a supralethal dose of 35 mg/kg was administered before allogeneic bone marrow transplantation.
administered. Lower doses were used only to establish the lethal dose of the drug.

**TBI.** TBI was administered immediately before marrow grafting. Five rats each were placed in plastic boxes (25 x 25 x 5 cm) and irradiated with a 60Co source (source-cage distance, 90 cm; field size, 30 x 30 cm; dose rate, approximately 1 Gy/min).

**Cyclophosphamide.** Cyclophosphamide was dissolved in distilled water, diluted in normal saline and injected intraperitoneally on day -2 if the dose did not exceed 60 mg/kg. Higher doses were divided equally and administered on days -3 and -2.

**Etoposide.** With the exception of special experiments indicated in Results, etoposide was administered intraperitoneally on day -2 after dissolution of the substance in normal saline.

**Cytarabine.** Cytarabine, 75 mg/kg, equivalent to the clinically used dosage of 3 g/m², was administered twice daily intraperitoneally for a total of 8, 12, or 16 doses starting on days -5, -7, or -9, respectively. The last dose was administered on day -2.

**Bone marrow preparation.** Rats were killed by cervical dislocation, and the marrow was rinsed from the tibia and femurs with 0.5 mL of normal rat serum. Cells were washed once and injected into the lateral tail vein of the recipients.

**Skin grafting.** Full-thickness skin grafts, 10 to 15 mm in diameter, were transferred from the anterior chest wall of the donor to that of the recipient. Some animals received a second graft of third-party origin. The transplanted skin was observed for signs of rejection until day 100 after skin grafting. In case of rejection, skin grafting was repeated to exclude infection or mechanical destruction as the cause of skin graft failure.

**Blood counts.** Blood was drawn by puncture of the lateral tail vein. A drop of blood was collected in a microhematocrit tube and a leukocyte pipette. The hematocrit and leukocyte and granulocyte counts were determined by routine techniques.

**Definitions.** Primary rejection (engraftment failure) is a failure to attain a granulocyte count >500/μL and a hematocrit >30%. Secondary rejection is death in secondary aplasia (granulocytes <500/μL and hematocrit <30%) after initial engraftment as defined by the aforementioned criteria. Deaths due to other reasons include all deaths occurring in animals with blood counts not fulfilling the rejection criteria and were counted as due to "other" reasons. Usually these deaths were due to toxicity of the preparatory regimen. Deaths occurring before day 7 posttransplant were considered toxic irrespective of hematologic parameters.

**Statistics.** Rejection rates were compared by using the χ² test.

**RESULTS**

**Establishment of the experimental model.** First, we determined the lethal dose of oral busulfan in female LEW rats. Death rates were 68% after 20 mg/kg and 80% after 25 mg/kg. No animal out of 13 and none of 19 survived a dose of 30 or 35 mg/kg, respectively. On the basis of these data, we chose 35 mg/kg of busulfan for all further experiments.

Death in marrow aplasia could easily be prevented by 2 x 10⁸ syngeneic bone marrow cells administered IV 24 hours after the administration of 35 mg/kg of busulfan (n = 13). In contrast to the rapid regeneration of hematopoiesis after syngeneic bone marrow transplantation, allogeneic transplant of CAP x LEW bone marrow were not successful if preparation consisted of oral busulfan only (n = 19). Granulocyte counts and hematocrit values were indistinguishable from those of controls receiving no bone marrow at all. Obviously, allogeneic marrow was rejected by rats treated with busulfan alone, and death caused by hematopoietic failure ensued. Thus, this experimental model appeared suitable for studying the engraftment-facilitating potency of any agent of interest. In this study, cyclophosphamide, cytarabine, etoposide, and TBI were tested for their immunosuppressive engraftment-promoting activities when added to busulfan.

**TBI.** TBI doses of 1.5 to 9 Gy were administered in addition to busulfan on day 0 immediately before injection of 4 x 10⁸ allogeneic F1 (CAP x LEW) bone marrow cells. Figure 1 shows the survival curves with respect to the additional TBI dose administered. In conjunction with Fig 2 it demonstrates that survival alone is not a valid end point for studying the beneficial role of TBI in the engraftment of allogeneic bone marrow since deaths due to TBI dose-related toxicity counteract the preventive effects of higher TBI doses on graft rejection. The careful assessment of engraftment or rejection by determining hematocrit, leukocyte count, and differential blood counts, according to the schedule outlined in Materials and Methods, allowed us to differentiate between these causes of death.

As shown in Fig 2, the total rejection rate fell from 100% after 1.5 Gy to 64% after 3 Gy. No rejection was observed after doses of 4.5 Gy or greater. After 1.5 Gy rejection was always of the engraftment failure type (primary rejection), while after 3 Gy primary and secondary rejections were seen. However, secondary rejections after the administration of TBI occurred very early within the first ten days. One late rejection masked by autologous recovery was observed, as indicated by rejection of a CAP skin graft (Table 1). All other rats surviving the full observation period retained CAP skin grafted at day 100 indefinitely, thus indicating the persistence of allogeneic CAP x LEW hematopoiesis (Table 1).

**Cyclophosphamide.** Cyclophosphamide was added to 35 mg/kg of busulfan administered in doses of 10 to 60 mg/kg on day -2 or in divided doses of 90 to 360 mg/kg on days -3 and -2. After 60 mg/kg, the total rejection rate was 26%,
with the primary and secondary rejection rates being 13% each. No rejection at all was observed after doses of at least 90 mg/kg (Fig 3). Secondary rejections occurred as early as those seen after TBI; late rejections were not observed. CAP skin grafted at day 100 was retained in any rat surviving the observation period (Table 1).

Cytarabine. Cytarabine, 75 mg/kg, equivalent to the clinically used dose of 3 g/m², was administered twice daily for a total of 8, 12, or 16 doses in addition to the lethal dose of busulfan. The last dose of cytarabine was always administered on day -2. As shown in Fig 4, the primary rejection rate ranged from 58% to 67% regardless of the total dose of cytarabine used. For the two lower doses, the total rejection rate was 75%; for the higher dose the secondary rejection rate could not be determined due to early mortality resulting from excessive nonhematologic toxicity. Cytarabine administered in this setting did not interfere with durable engraftment of syngeneic bone marrow as tested in 13 animals. The survival rate for these animals 100 days after syngeneic bone marrow transplantation was three of four after 8 x 75 mg/kg, five of five after 12 x 75 mg/kg, and two of four after 16 x 75 mg/kg.

Etoposide. On the basis of pharmacokinetic considerations, etoposide was administered on day -3 in the clinical setting. In syngeneic transplant experiments we tested whether etoposide would interfere with graft survival if the time between injection of the drug and the bone marrow was shortened. Busulfan was administered as described, and 60 mg/kg of etoposide were administered alternatively on day -3, -2, or -1 and followed by syngeneic bone marrow transfer. Nonengraftment was observed in one of seven rats receiving etoposide on day -1, while all rats showed durable engraftment after the application of etoposide on day -2 (n = 11) or -3 (n = 3).

In the allogeneic setting, after the addition of increasing doses of etoposide to busulfan, the following primary rejection rates were observed: 90% after 30 mg/kg, 26% after 45
mg/kg, and 10% after 60 mg/kg. The secondary rejection rate was 32% after 45 mg/kg, based on the total number of animals receiving this etoposide dose. Due to the overwhelming toxicity, follow-up periods were too short to determine the secondary rejection rate for 60 mg/kg of etoposide (Fig 5).

All animals not dying of rejection experienced prolonged secondary pancytopenia with slow recovery, presumably due to autologous reconstitution. Three animals surviving the 100-day observation period rejected allogeneic CAP skin grafts, strongly indicating prior rejection of the transplanted marrow.

Comparison of the immunosuppressive potency of cyclophosphamide, cytarabine, and etoposide. The preparative regimens for allogeneic bone marrow transplantation in humans usually include 120 mg/kg of cyclophosphamide,1 12 × 75 mg/kg of cytarabine,9,10,12 or 60 mg/kg of etoposide6,13 in conjunction with TBI. Fifty percent and 75% of the clinically used dose of etoposide are significantly inferior in their engraftment-promoting potency to equivalent doses of cyclophosphamide (P < .01), as were 75% and 100% of cytarabine when compared with the respective doses of cyclophosphamide (P < .01).

DISCUSSION

Our data confirm that the busulfan-treated rat is a useful model for determining the engraftment-promoting potency of immunosuppressive agents before allogeneic bone marrow transplantation, as was first demonstrated by Floersheim and Ruszkiewicz19 and later especially by Tutschka and Santos.14–16 These investigators relied mainly on the survival of the animals to indicate successful and durable engraftment. In addition, persistence of the marrow graft was documented by cytotoxic alloantisera. Except for cyclophosphamide, this original experimental design does not allow an estimation of the immunosuppressive potency of cytostatic agents at higher dose levels since concurrent systemic toxicity leads to a high rate of deaths even after syngeneic transplantation. The decisive point in testing the engraftment-promoting potency of high-dose cytostatic agents in combination with lethal doses of busulfan, however, is to distinguish death caused by rejection of the grafted marrow from death due to toxicity of the preparatory regimen. This can be achieved by monitoring the hematocrit, leukocyte count, and differential blood count according to a rigid time table, as demonstrated by the 36 controls (see Results), all of which showed rapid and durable engraftment of syngeneic marrow. In addition, the reproducibility of the method is indicated by the clear cutoff points with regard to engraftment and rejection of allogeneic marrow after increasing doses of both TBI and cyclophosphamide (Figs 2 and 3).

Our experiments revealed that cytarabine and etoposide, if added to lethal doses of busulfan, were clearly inferior to cyclophosphamide in their potency to promote engraftment of allogeneic bone marrow. If approximately 50% of the clinically used dose of cytarabine, etoposide, or cyclophosphamide was added to 35 mg/kg busulfan, which by itself is lethal but not sufficiently immunosuppressive to allow engraftment of allogeneic marrow, primary rejection rates were 58% for cytarabine, 90% for etoposide, and 13% for cyclophosphamide. Increasing the dose of etoposide and cyclophosphamide reduced the rejection rate substantially; no dose-response effect was observed for cytarabine.

The presumably enhanced antileukemic efficacy of cytarabine and etoposide as compared with cyclophosphamide before allogeneic bone marrow transplantation thus has to be weighed against the inferior immunosuppressive potency of the respective agents.25 For the future it would appear reasonable to abandon the current policy of using an identical preparatory regimen in all patients scheduled for bone marrow transplantation. Instead, disease status,16 the risk of development of severe GVHR, as well as the risk of graft rejection26–36 would have to be considered, and the preparatory regimen administered to any individual patient should reflect the final evaluation of the aforementioned criteria.

A number of limitations regarding the presented data need to be considered. First, all experiments were performed on one rat strain combination. Since animals belonging to a given inbred strain are genetically identical, our data are equivalent to the outcome of transplantation in a single patient. Other combinations of donors and recipients might have produced different results. Second, what is true in rats may not necessarily be true in other species or in humans. Third, cyclophosphamide, etoposide, and cytarabine were investigated in conjunction with busulfan. We cannot exclude the possibility that etoposide or cytarabine might be equivalent to cyclophosphamide in its immunosuppressive potency if tested in combination with TBI.

At present, other experimental data dealing with this problem are not available. The observation, however, of four graft failures in four patients receiving either mismatched or T-depleted marrow after conditioning with etoposide and TBI13 strongly indicates that our findings do have implications for the clinical situation. Indeed, it is our concern that preparatory regimens in which cyclophosphamide is replaced by etoposide or cytarabine may lead to an increased risk of rejection, especially in the case of HLA-mismatched and/or T-depleted bone marrow transplantation.
REFERENCES

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