Induction of Graft versus Leukemia Effect in Bone Marrow Transplantation: Dosage and Time Schedule Dependency of Interleukin 2 Therapy

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ABSTRACT

The present work is a continuation of our studies to improve the graft versus leukemia (GVL) effect in autologous bone marrow transplantation. We have recently shown that the GVL effect of bone marrow transplantation (BMT) with interleukin 2 (IL-2)-activated bone marrow (ABM) followed by IL-2 therapy immediately after BMT is superior to the GVL effect of BMT with fresh, syngeneic bone marrow, with or without IL-2 therapy, in mice with acute myeloid leukemia. The present studies show that institution of IL-2 treatment 1, 2, or 3 weeks after BMT with ABM resulted in shortening of survival and fall in cure rate as compared to IL-2 therapy instituted immediately after BMT with ABM. Increasing the dose of IL-2 did not improve results. However, reducing the frequency of IL-2 administration to once a day instead of twice a day affected the results adversely. Commencing IL-2 therapy 1, 2, or 3 weeks after BMT with fresh, syngeneic bone marrow did not improve the GVL effect as compared to IL-2 therapy started immediately after BMT with fresh, syngeneic bone marrow. Cryopreserved bone marrow was effectively activated with IL-2 and used successfully for BMT after thawing. The animals cured of leukemia by BMT with ABM and IL-2 therapy were not resistant to leukemia and died when reinfused with leukemic cells. Our findings suggest that for optimum GVL effect, activation of bone marrow is necessary and IL-2 therapy should be started immediately after BMT with ABM.

INTRODUCTION

Allogeneic bone marrow transplantation has long been recognized to offer a potential for cure for AML (1, 2). The concept of ABMT was prompted by the unavailability of suitable donors for a majority of patients and the morbidity and mortality associated with allogeneic bone marrow transplantation. However, the success of ABMT has been limited by frequent relapses (3). The high relapse rate following ABMT has been explained partly on the basis of the absence of GVHD and thus poor GVL effect (4). In animal models, strategies to improve the graft versus tumor effect of ABMT have included the induction of mild GVHD by administration of cyclosporin (5, 6). A similar attempt has been made in clinical ABMT, but the benefits of such therapy are yet to be defined (7). Ex vivo purging of harvested BM with 4-hydroperoxycyclophosphamide eradicates the leukemic cells from the graft (8, 9), but relapses continue to take place. This is probably because of reactivation of minimal residual disease escaping the preparative therapy for ABMT.

IL-2 has been used after ABMT for patients with AML and in a case of neuroblastoma, but its long term effect on the disease status remains unknown (10, 11). LAK cells have been suggested as adjuvant therapy in ABMT to improve the GVL effect (12). However, LAK cell therapy is not possible for a long time after ABMT due to prolonged lymphopenia. Thus, new modalities to improve the GVL effect in ABMT need to be explored.

We have previously shown that ABM is superior to spleen LAK cells against murine natural killer-resistant tumors in vitro and in vivo and that BMT with ABM followed by IL-2 therapy reduces the dissemination of established melanoma and sarcoma in mice better than BMT with FBM with or without IL-2 (13, 14). We have recently reported that BMT with ABM followed by IL-2 therapy is superior to BMT with FBM (with or without IL-2 therapy) in terms of survival and cure in mice with AML; the GVL effect after BMT with ABM did not improve in the absence of posttransplant IL-2 therapy (15).

The present investigation examines the influence of different dosage schedules and of the time of institution of IL-2 therapy in the posttransplant period on the survival of mice with AML. In addition, we have studied this experimental model under conditions which are relevant to the clinical setting, such as optimal sequence for cryopreservation and activation of bone marrow and response of cured animals to a rechallenge with leukemia.

MATERIALS AND METHODS

Leukemic Cells. C1498 (murine AML) cells were obtained from the American Type Culture Collection (Rockville, MD) and propagated in CM consisting of RPMI 1640, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 0.03% l-glutamine (Irvine Scientific, Santa Ana, CA), 5 x 10^-4 M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO), penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% heat-inactivated fetal calf serum (JR Scientific, Woodland, CA). The cells were cryopreserved at ~80°C. They were thawed immediately before in vitro experiments. Before in vivo use, the cells were allowed to grow in CM for 24 h at 37°C in a humid atmosphere with 5% CO2. They were harvested, washed 3 times with RPMI, and suspended appropriately in RPMI to infuse 2 x 10^5 cells/mouse in a volume of 0.5 ml via the lateral tail vein. (In our preliminary studies, 2 x 10^5 leukemic cells were found to be lethal for 100% of mice.) C1498 cells morphologically have features of typical myeloblasts in Wright-Giemsa stained smears, and are positive for chloroacetate esterase; they are negative for myeloperoxidase and Sudan black B.

Animals. The animals used in this study were female C57BL/6 mice between the ages of 8 and 12 weeks obtained from Charles River Laboratories (Wilmington, MA).

Interleukin 2. Recombinant IL-2 was a generous gift of the Cetus Corporation (Emeryville, CA). It had a specific activity of 3 x 10^8 units/mg (1.2 mg/vial), purity of 98%, and endotoxin level <0.01 μg/vial.

Bone Marrow: Collection, Activation, Cryopreservation. BM collection and IL-2 activation were carried out as described previously (13). Briefly, BM was collected by flushing femurs and tibias with RPMI and a single cell suspension was prepared by passing the BM through a single layer of 10-gauge nylon mesh. Erythrocytes were lysed by hypotonic shock with distilled water. For activation with IL-2, the BM
cells were suspended in CM (10^6 cells/ml) and placed in plastic flasks (Corning Glassware, Corning, NY). IL-2 (1000 units/ml) was added and the flasks were incubated at 37°C in a humid atmosphere with 5% CO2 for 24 h. The cells were harvested using Dispo cell scrapers (American Scientific Product, McGaw Park, IL), washed 3 times with RPMI, and resuspended in RPMI for i.v. infusion; these cells will be referred to as ABM. For in vitro experiments, the BM was activated for 1, 3, and 5 days, depending on the experiment. FBMs were collected in RPMI immediately before use.

For some experiments, the ABM cells were cryopreserved with 10% dimethyl sulfoxide in fetal calf serum at —80°C for 10–14 days before using them for BMT. For another group of experiments, the FBMs were cryopreserved for 10–14 days; these cells were thawed, activated with IL-2 for 24 h, and subsequently used for BMT. Cryopreserved BM was used for in vitro experiments as well. BM collected from mice cured of leukemia was also used for BMT (without in vitro IL-2 activation).

Spleen LAK Cells. Spleens were removed aseptically and crushed with the piston of a syringe in CM. The cell suspension was passed through 10-gauge nylon mesh. Erythrocytes were lysed with distilled water and the cells were finally suspended in CM (10^6/ml). IL-2 activation was carried out for 1, 3, and 5 days, as described for activation of BM.

FBM and spleen cells were also collected from mice after 45–100 days following BMT (with FBM, FBM plus IL-2, or ABM plus IL-2) and tested for their cytotoxic potential against C1498 cells in vitro.

Chromium Release Assay. A 4-h 51Cr release assay was carried out to compare the cytotoxic potential between ABM and LAK cells, among various groups of BM, and between FBM and spleen cells, against C1498 cells in vitro. Target cells (10^6) were labeled with 500 μCi of NaCrO4 (ICN Radio Chemicals, Irvine, CA) in 1 ml of CM for 1 h. They were washed 3 times with CM and added at a concentration of 5 x 10^5 cells/well in round-bottomed microtiter plates (Linbro Scientific Co., Hamden, CT). Effector cells were added at various effector:target ratios in a final volume of 200 μl/well. The plates were incubated for 4 h at 37°C in a humid atmosphere with 5% CO2. Maximum isotope release was ensured by addition of 0.1 N HCl, and spontaneous release was allowed by addition of CM, to the target cells. The culture supernatant was harvested with a Skatron Titertek System (Skatron A.S., Lierberg, Norway) and counted in a gamma counter (Packard Auto Gamma-500; United Technologies, Packard, Laguna Hills, CA).

The percentage of specific lysis was calculated as

\[
\frac{\text{Experimental cpm} - \text{spontaneous cpm}}{\text{Maximum cpm} - \text{spontaneous cpm}} \times 100 \text{/rectr}
\]

All determinations were made in triplicate and data were calculated as mean ± SE. Each experiment was done 3 times.

Design of the Survival Study. This study was composed of 10 experiments performed in sequence. There were 4–6 treatment arms in an experiment; every treatment arm was replicated more than once (see "Results"). There were always 4 mice under a treatment arm in every experiment. Every experiment included a control group that received no treatment.

Treatment. BMT was performed 3 days after infusion of leukemic cells. The conditioning regimen for BMT was composed of CTX, 1 mg/mouse i.p., and 500 rads of total body irradiation delivered at a rate of 150 rads/min from a Gammacel cesium irradiator (Atomic Energy of Canada, Ottawa, Ontario) 4 h after CTX. BM (5 x 10^6 cells in a volume of 0.5 ml/mouse) was infused via the lateral tail vein 8–10 h after CTX. IL-2 therapy was started immediately or 1, 2, or 3 weeks after infusion of BM as indicated under "Results"; it was continued for 7 days (unless specified otherwise).

Monitoring of Survival. The mice were examined daily until death or up to 100 days for their general appearance, signs of GVHD in the form of alopecia, erythroderma, or gross weight loss and for survival. Survival days were calculated using the day of infusion of leukemic cells as day zero. Mice surviving for ≥100 days were considered as cured. (In our preliminary studies carried out for optimization of different treatment schedules, no deaths were seen after 60 days, and autopsy of mice surviving for ≥100 days did not show evidence of leukemia.)

Rechallenge with Leukemia. Mice cured of leukemia following BMT (with FBM alone or FBM plus IL-2 therapy or ABM plus IL-2 therapy) were rechallenged with 2 x 10^5 leukemic cells; this was done between days 100 and 120 following the previous exposure to leukemia. Their survival was compared with that of naive mice receiving similar doses of leukemic cells.

Statistical Analysis. P values are based on two-sided log-rank test (16). Where indicated, a test for trend was performed (17). Survival curves and median survival were computed by the method of Kaplan and Meier (18). The cure rate was calculated as the probability of surviving 100 days, based on Kaplan-Meier survival curves.

RESULTS

Preliminary Studies. Preliminary experiments were carried out to compare the cytotoxic activity of ABM and spleen LAK cells against C1498 cells in vitro; ABM was always found to be superior to LAK cells (Fig. 1). Experiments were also performed to evaluate the effect of cryopreservation on the bone marrow. BM was cryopreserved for 10–14 days; it was thawed by standard techniques and incubated with IL-2 for 24 h. This BM showed higher cytotoxic activity in vitro against C1498 cells than FBM or unactivated, thawed BM, suggesting that BM that has been cryopreserved can be effectively activated with IL-2. BM cells activated with IL-2 were cryopreserved for 10–14 days and thawed, and their cytotoxic activity was tested against C1498 cells in vitro; these cells always showed cytotoxicity equal to or less than that of FBM. This suggests that the cytotoxic potential generated by IL-2 is lost during the process of cryopreservation and thawing. Data on the effect of cryopreservation on the cytotoxic potential of BM are shown in Fig. 2. FBMs and spleen cells harvested from mice 45–100 days after BMT with FBM alone, FBM plus IL-2 therapy, or ABM plus IL-2 therapy did not show any antileukemic activity in vitro (data not shown).

Results of Survival Studies. The data have been pooled from 10 experiments performed at different time points. Analysis
BM cryopreserved, thawed, unactivated
ABM
BM cryopreserved after activation
BM cryopreserved before activation

Fig. 2. Effect of cryopreservation on the IL-2-induced cytotoxic potential of bone marrow against C1498 cells in vitro. BM cells were collected from C57BL/6 mice and cryopreserved for 10–14 days. These cells were thawed, activated with IL-2 for 24 h, and used in cytotoxicity assay or were thawed at the time of assay and used without activation. BM cells activated for 24 h with IL-2 (ABM) were used without cryopreservation, or they were cryopreserved for 10–14 days and thawed at the time of cytotoxicity assay. FBM collected immediately prior to use was also tested in the assay. The cytotoxicity assay composed of a 4-h 51Cr release assay was carried out with various effector:target ratios in triplicate as described under “Materials and Methods.” Data are shown as percentage of specific lysis.

Fig. 3. Pattern of survival with sequential delay in the institution of IL-2 therapy after BMT with FBM. Mice were infused i.v. with 2 × 10^6 C1498 cells; BMT was performed 3 days later. Conditioning therapy composed of CTX (1 mg/mouse) and total body irradiation (500 rads). FBM (5 × 10^6 cells/mouse) was infused i.v. 8–10 h after CTX. IL-2, 10^6 units i.p. twice a day, was started immediately or 1, 2, or 3 weeks after BMT and was continued for 7 consecutive days. Control groups received no treatment. Mice were followed for survival for 100 days. P: a versus b, 0.91; a versus c, 0.99; a versus d, 0.86.

Effect of IL-2 Dosage Schedule on Survival. Table 1 shows the outcome with various forms of treatment. Untreated mice became sick toward the end of 3 weeks and died in the next 2–3 days; none of the untreated mice survived. BMT with ABM followed by IL-2 therapy twice a day for 7 days immediately after BMT significantly improved the results as compared to control (P < 0.001). When frequency of IL-2 administration was reduced to once a day instead of twice a day after BMT with ABM there was significant deterioration in the median survival and cure rates (P = 0.001). Increasing the dose of IL-2 or duration of IL-2 therapy did not improve the results (P for trend = 0.28).

IL-2 Therapy after BMT with FBM. Our previous study showed that institution of IL-2 therapy immediately after BMT with FBM did not improve the results over BMT with FBM alone (15). Experiments were performed to evaluate the influence of IL-2 therapy at different time points after BMT with FBM. The results are shown in Fig. 3. Institution of IL-2 therapy immediately and 1, 2, or 3 weeks after BMT with ABM resulted in median survival of 29, 30, 31, and 31 days, respectively. There was no difference in the cure rate (P for trend = 0.99).

IL-2 Therapy after BMT with ABM. To determine the optimum time for starting IL-2 therapy after BMT with ABM, (in order to achieve maximum GVL effect), experiments were carried out by starting IL-2 administration immediately and 1, 2, and 3 weeks after BMT with ABM. The outcome is shown in Fig. 4. Institution of IL-2 treatment 1 week after BMT with ABM resulted in a slight shortening of median survival (36 days versus 40 days) and fall in the cure rate (25 ± 9.7% versus 36.1 ± 8.0%) as compared to IL-2 administration immediately after BMT with ABM. However, institution of IL-2 2 and 3 weeks after BMT with ABM resulted in significant shortening of survival (33 days with each) and drop in the cure rates. Overall, increasing the delay in the institution of IL-2 therapy after BMT with ABM affected the survival adversely (P for trend <0.001).

When IL-2 therapy was started 3 weeks after BMT (with FBM or ABM), mice started dying even before completion of 1 week of IL-2 treatment.

Table 1 Results of survival and cure rates with various dosage schedules of IL-2 after BMT with ABM

<table>
<thead>
<tr>
<th>Group</th>
<th>Median survival (days)</th>
<th>Cure rate ± SE</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>1. Control</td>
<td>22 (18–25)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2. IL-2, 10,000 units twice a day</td>
<td>40 (28–45)</td>
<td>36.1 ± 8.0</td>
<td>1 vs. 2 &lt; 0.001</td>
</tr>
<tr>
<td>3. IL-2, 25,000 units twice a day</td>
<td>37 (22–41)</td>
<td>37.5 ± 17.1</td>
<td>2 vs. 3 0.7</td>
</tr>
<tr>
<td>4. IL-2, 50,000 units twice a day</td>
<td>33 (21–43)</td>
<td>25.0 ± 15.3</td>
<td>2 vs. 4 0.21</td>
</tr>
<tr>
<td>5. IL-2, 10,000 units twice a day, 5 days/week for 2 wk</td>
<td>37 (23–41)</td>
<td>25.0 ± 15.3</td>
<td>2 vs. 5 0.28</td>
</tr>
<tr>
<td>6. IL-2, 10,000 units once a day</td>
<td>32 (27–40)</td>
<td>12.5 ± 8.3</td>
<td>2 vs 6 0.001</td>
</tr>
</tbody>
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* Mice were infused with 2 × 10^6 C1498 cells i.v. Treatment was started 3 days later. Control groups received no treatment. Conditioning regimen for BMT composed of CTX, 1 mg/mouse i.p. followed 4 h later by 500 rads of total body irradiation. ABM cells (5 × 10^6 cells/mouse) were infused 8–10 h after CTX. IL-2 therapy in the indicated doses was started immediately after BMT and continued for 7 days, except where indicated otherwise.
* Data pooled from 10 experiments with 4 mice each.
* Data pooled from 2 experiments with 4 mice each.
* Data pooled from 4 experiments with 4 mice each.

Based on plots and the stratified log-rank tests revealed no significant interexperiment variability (P = 0.27). Experiments were carried out for optimization of BMT with FBM ± IL-2 therapy and with ABM ± IL-2 therapy in normal mice. Treatment regimens that showed less than 10% mortality have been used for studies in leukemic mice.
BMT with Cryopreserved BM. To test the applicability of this model to the clinical ABMT setting, experiments were performed by using BM that had been cryopreserved. The following two types of cryopreserved BM were used for BMT: (a) BM was activated with IL-2 for 24 h, cryopreserved for 10–14 days, and thawed and used at the time of BMT; (b) BM was cryopreserved for 10–14 days, it was thawed, activated with IL-2 for 24 h, and then used for BMT. The results of survival are shown in Fig. 5 and compared with BMT with FBM and with ABM; IL-2 therapy, 10^4 units twice a day where indicated, was started immediately after BMT. BMT with BM activated prior to cryopreservation yielded results similar to those obtained with BMT with FBM (P = 0.50); the median survival was 31 days (range of days to death, 27–58) and cure rate was 12.5 ± 8.3%. These observations were in agreement with our findings that the cytotoxic potential of cryopreserved ABM was not higher than that of FBM against C1498 cells in vitro.

BMT with BM activated after cryopreservation, followed by IL-2 therapy, yielded results superior to those obtained by BM with FBM (P = 0.003) and comparable to BMT with ABM followed by IL-2 therapy (P = 0.92). The median survival was 39 days (range of days to death, 33–46) and cure rate was 37.5 ± 12.1% in this group. These findings show that cryopreserved BM, when thawed, can be effectively activated with IL-2 and that this is the optimal sequence of cryopreservation and activation.

Rechallenge with Leukemia. Experiments were carried out to determine if the animals cured of leukemia developed antitumor immunity or if those receiving BMT with ABM could eradicate a subsequent challenge with the leukemic cells better than those receiving BMT with FBM. Cured mice were infused with 2 × 10^5 leukemic cells i.v. The results shown in Table 2 indicate that animals cured by BMT with FBM, BMT with FBM plus IL-2 therapy, and BMT with ABM plus IL-2 therapy were not resistant to this dose of leukemic cells. The survival of the previously cured mice was not different from that of the control group (P = 0.44).

BMT with BM from Mice Cured with ABM and IL-2. BM was harvested from mice previously cured of leukemia by BMT with ABM and IL-2 therapy. This BM was used for BMT (followed with and without IL-2 therapy immediately after BMT) in a group of mice with leukemia. Results were compared with BMT with FBM and with ABM and IL-2 therapy. Data shown in Table 3 indicate that BM from cured mice was equivalent to FBM as regards its influence on the survival of leukemic mice; it did not show higher antileukemic activity than the FBM, even when supplemented with IL-2 therapy after BMT.

Graft versus Host Disease. Signs of GVHD were not seen in any mice.

**DISCUSSION**

Our study shows that the antileukemic activity of ABM is superior to that of FBM in vitro. To maintain this antileukemic effect in vivo, systemic IL-2 therapy was necessary; in the absence of exogenous IL-2 therapy, the GVL effect of BMT with ABM was not different from that of BMT with FBM in mice with AML (15). BMT with ABM followed by IL-2 therapy also resulted in normal hematopoietic reconstitution. It did not result in GVHD.

Increasing the dose of IL-2 or prolonging the duration of administration of IL-2 did not increase the GVL effect of BMT.
with ABM. The exact reason for this is not known. IL-2 has a short half-life; administration by intermittent bolus doses does not maintain constant, detectable serum levels of IL-2 (19). It is possible that maximum IL-2 levels achieved by various doses used in this study were not much different from each other and thus the GVL effect was comparable in all these groups. A significant fall in the cure rate by reducing the frequency of IL-2 administration indicates that more frequent administration or a constant infusion of IL-2 may maintain higher serum levels of IL-2 and achieve better results.

Institution of IL-2 therapy immediately after BMT with FBM did not increase the antileukemic effect over BMT with FBM alone (15). It could be argued that in the presence of bone marrow aplasia following conditioning therapy for BMT, there were not enough cells to be activated by IL-2 and hence no improvement in GVL effect. In patients undergoing BMT, endogenously generated killer cells have been reported to be circulating 4–6 weeks after BMT (20). Also, the lymphocytes circulating soon after ABMT have been found to be capable of developing LAK activity in vitro (21). However, even sequential delay in the institution of IL-2 therapy after BMT with FBM did not improve the GVL effect in our study. It appears that parenteral IL-2 therapy does not achieve adequate serum IL-2 levels to generate killer cells with optimum antileukemic activity in vitro. With maximum tolerated doses of IL-2 in humans, serum levels of 5–10 units of IL-2/ml are detected (19); these are far below the levels required for generation of LAK or ABM cells in vitro (13). Although a direct comparison between in vivo and in vitro settings is not possible, our data indicate that BM should be activated with IL-2 in vitro before BMT to increase the GVL effect. The serum levels attained by IL-2 therapy are probably adequate to maintain the antileukemic potential of ABM rather than generate antileukemic effect from FBM.

IL-2 therapy instituted immediately after BMT with ABM apparently maintains higher antileukemic effect leading to eradication of minimal residual disease after high dose chemo/radiotherapy. Once the residual leukemic cells start proliferating, further continuation of IL-2 therapy does not seem to make a significant impact. IL-2 alone did not show any antileukemic effect in the presence of florid disease (15). Thus, delaying the institution of IL-2 therapy after BMT with ABM may result in reactivation of residual disease which may not be possible to control. This was evident from a downward trend in survival and cure rates by postponement of IL-2 after BMT with ABM. A significant observation of the present study relevant to the clinical setting is that cryopreserved BM can be effectively activated with IL-2 after thawing, and used successfully for BMT. However, if the BM is activated with IL-2 and cryopreserved, it does not show any cytotoxic activity at the time of thawing. We have made similar observations on the human ABM (22). The loss of cytotoxic activity is probably related to the procedures of cryopreservation and thawing. Thus, if the ABM must be used in clinical ABMT to increase the GVL effect, the activation should be carried out 1 day prior to BMT and not at the time of BM harvest.

The mechanism of increased GVL effect following BMT with ABM plus IL-2 therapy is not known. IL-2 therapy leads to proliferation of T-cells and adoptively transferred LAK cells in vivo (19, 23–25). A similar phenomenon possibly follows BMT with ABM and IL-2 therapy. It is uncertain how long the adoptively transferred ABM cells maintain an antileukemic effect. However, if they serve a function of immune surveillance to prevent a leukemic relapse, they possibly do so in the early posttransplant period. BM and spleen cells harvested from mice living ≥45 days after BMT did not show antileukemic activity in vitro. Similarly, these mice did not show any evidence of protection from a rechallenge with leukemia. Analysis of immune reconstitution following BMT with ABM is presently under investigation in our laboratory.

IL-2 therapy in patients has been reported to induce secretion of γ-interferon and tumor necrosis factor; both these cytokines have antileukemic activity (26) and enhance the effect of IL-2 in the generation of ABM (27). A recent study has shown that IL-2 therapy after BMT for hematological malignancies was tolerated well and did not compromise engraftment (10). These observations and the findings of the present study suggest that IL-2 therapy should be instituted immediately after BMT with ABM in order to maintain maximum GVL effect and to eradicate residual leukemia. Clinical studies should be carried out to determine the optimum dose and duration of IL-2 therapy after BMT.

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REFERENCES

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