Transient Graft-Versus-Host Reaction in the Treatment of Leukemia in Mice

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SUMMARY—Inbred RF mice, each bearing a transplanted myeloid leukemia, received 530 rads of X rays and then injections of C57BL spleen cells (10–60 × 10⁶) and bone marrow cells (40–60 × 10⁶). Most of these animals died with clinical and histological signs of secondary disease. Leukemia incidence, as judged by morphological criteria, varied with the spleen cell dose and ranged between 50% and 0. The severity of the secondary disease could be diminished, without recurrence of leukemia, by the immunosuppressive agents cyclophosphamide and heterologous antilymphocyte serum. Administration of recipient-type hematopoietic cells and blood after the course of cyclophosphamide abolished the chimeric state and prevented secondary disease, without recurrence of the leukemia. It is concluded that, in the experimental system used, permanent chimerism and the chronic graft-versus-host reaction are not necessarily prerequisite to the suppression of a transplanted leukemia.—J Nat Cancer Inst 41: 421-437, 1968.

COMPLETE elimination of leukemic cells in animal or human leukemia cannot be obtained by whole-body irradiation unless very high doses, 3000 R or more, are used (1). Animals exposed to such high doses succumb within a few days or even hours because of intestinal and central nervous system damage (2). With lower X-radiation doses the leukemic cells are not completely eliminated, but death from the bone marrow syndrome may be prevented by establishment of a foreign hematopoietic tissue graft. This treatment may also result in an immunological reaction of the transplanted allogeneic cells against the host and host tumors (3). A number of workers have tried to use this immunological reaction to eradicate tumor cells that have survived irradiation (3–9). Although this treatment frequently resulted in disappearance

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4 I am indebted to Drs. M. J. de Vries, D. W. van Bekkum, and H. Balner for valuable discussions and help in preparing this paper, and to Dr. D. van der Waaij for advice in bacteriological problems and barrier-nursing. I wish to thank Mrs. E. Buikj-Tempelaars for her skillful and reliable technical assistance, also Mrs. W. L. G. Levert-van Eyk and Mr. H. Dersjant for typing the chimeras. The International Atomic Energy Agency's authorities, represented by Mr. F. J. Remick, approved this work for publication.
of the neoplastic disease in mice, it also caused high mortality from secondary disease. Also, human leukemias have been treated by whole-body irradiation followed by transplantation of bone marrow obtained from normal individuals, with similar complications as observed in mice (3, 8). From his clinical trials, Mathé termed the graft-versus-host reaction, or secondary disease, "the stumbling block in the treatment of leukemia by whole-body irradiation and transfusion of allogeneic hematopoietic cells" (3).

In this paper a modification of the experimental treatment was devised (with Dr. V. Stanković from the Institute R. Bošković) toward the induction of a vigorous but transient graft-versus-host reaction, i.e., of temporary instead of permanent chimerism. It was hoped that the reaction would be severe enough to eliminate the leukemic cells, while its limited duration would allow the recipients to recover from secondary disease.

In the experiments to be reported, mice bearing a transplanted myeloid leukemia were subjected to sublethal irradiation and injection of allogeneic spleen cells. A similar treatment was shown by Woodruff et al. (6, 7) to be effective against an ascites tumor and mammary carcinoma in mice. After an interval it was attempted to end the graft-versus-host reaction by the treatment with cyclophosphamide and antilymphocyte serum, as well as by administration of recipient-type hematopoietic cells and blood. Immunosuppressive drugs (10-11), antilymphocyte serum (15), and recipient-type hematopoietic cells (16, 17), have been described by many investigators as effective in the treatment of the graft-versus-host reaction in non-leukemic animals. The present paper will show that, in some cases, both eradication of leukemia and survival of the mice were achieved, whereas the chimeric state was artificially abolished.

MATERIALS AND METHODS

Experimental design.—Mice were inoculated intravenously with a strain-specific, transplantable myeloid leukemia. After 4 days, when small foci of leukemic cells were already found in histological sections of the liver, the animals were irradiated and then received injections of allogeneic spleen cells and, usually, of bone marrow cells. The day of irradiation was considered as day 0. During the subsequent 4 weeks the treatment aimed at termination of the graft-versus-host reaction was instituted. The chimeric state was usually assessed on day 60. Mice surviving 100 days were considered free from disease, as both secondary disease (18) and leukemia (see below) are not likely to become manifest after that time.

Whole-body irradiation.—A General Electric Maxitron X-ray unit was used—250 kvp, 30 ma, HVL 2.1 mm Cu, field diameter 23 cm, with maximal backscatter. The animals (16 per group) were confined in a shallow, circular plastic box at 55 cm distance from the X-ray source, and received 530 rads at a rate of 50 rads per minute. The dose was measured in the midline of a phantom mouse.

Cell suspensions.—These were prepared and counted according to the routine procedure used in this laboratory (19). Cell counts are expressed as the number of eosin-negative cells unless otherwise specified. Irradiated animals were given injections 4-6 hours after X-ray exposure.

Animals.—Inbred male and female RF mice, 12-14 weeks old, bearing a strain-specific transplantable myeloid leukemia, were used. Mice in an individual experiment were of the same sex, but the sexes of cell recipients and donors were not matched.

Allogeneic hematopoietic cells were provided by inbred C57BL/Rij mice, which differ from the RF strain in both H-2 and H-3 histocompatibility loci, as well as in R and Z blood group specificities (20).

During the experimental procedure the mice were supplied with antibiotics in the drinking water as well as with autoclaved food pellets. They were maintained in groups of 2-3 in autoclaved cages provided with autoclaved sawdust, and were barrier-nursed as described elsewhere (21). All injections were carried out at a desk covered with sterilized linen; sterile surgical gloves and sterile instruments were used. Animals were autopsied and their heart blood was cultured. Organs for histology were taken from all autopsied mice and processed as described (21).

Myeloid leukemia.—Originating in an irradiated RF male mouse, this leukemia has been carried in

1 Obtained in 1963 from Dr. C. C. Denny, Oak Ridge National Laboratories, Oak Ridge, Tenn., and bred by strict brother × sister mating.
both male and female animals of that strain for more than 70 transplant generations. It seldom affects lymph nodes and thymus and closely resembles that recently described by Siegler and Rich (22). Titration experiments (23) showed that 2-20 spleen cells from leukemic animals were sufficient to induce leukemia in 50% of isogeneic recipients. In animals surviving 100 days post inoculation, the incidence of myeloid leukemia was not higher than in uninoculated mice of this strain. No evidence was obtained for antigenicity of the tumor, since mice treated with irradiated leukemic cells did not resist challenge with viable leukemic cells (23).

Cyclophosphamide.—After cyclophosphamide (Endoxan, Asta-Werke, Brackwede, Germany) was dissolved in distilled water or sterile physiological saline, it was immediately injected intraperitoneally. The drug was administered on days 4-15 or 16 after irradiation and allogeneic cell injection, according to the following plan: day 4, 2.0 mg per mouse; days 5 and 6, 1.0 mg per mouse per day; later on, 0.5 mg per mouse per day. Since the animals weighed approximately 20 g at the time of this treatment, the total dose administered was 425-450 mg/kg.

Antilymphocyte serum (ALS).—Obtained from rabbits immunized with mouse lymph node cells (15), ALS was used, without further purification, by subcutaneous injection according to the following schedule: days 16-25 post irradiation, 0.5 ml per mouse per day, and thereafter 0.7 ml per mouse once weekly for 4 weeks. The batch of ALS agglutinated mouse thymocytes in vitro (24) up to and including the titer of 1:32. Although comparatively large amounts were used, no toxic effects were seen.

Dose schedule of normal rabbit serum (NRS) was similar to that of ALS. The NRS did not agglutinate mouse thymocytes.

Immunized donors of cells or blood.—RF mice received 4 injections of C57BL spleen, thymus, and lymph node cells at weekly intervals. Approximately 100 X 10^6 nucleated cells per mouse per injection were administered intraperitoneally as well as subcutaneously. The animals were exsanguinated by heart puncture under ether anesthesia 10-14 days after the last injection. The blood was drawn into a syringe containing 0.1-0.2 ml of 3.8% sodium citrate per ml of blood obtained from a single animal. Hematopoietic cells of bled animals were processed in the usual manner.

Chimerism.—Erythrocytes were typed according to the Gorer-Mikulska technique (25) or by hemoglobin electrophoresis (26). Blood for testing was taken from the orbital venous sinus 2 months or more after irradiation. Sometimes peritoneal cells were also typed according to a technique described by Balner (27). The method of Billingham (28) was used for skin grafting.

Diagnostic criteria.—Presence or absence of leukemia in treated animals was assessed morphologically, i.e., by autopsy and histology. Since treatment with irradiation and allogeneic hematopoietic cells commonly results in a considerable extramedullary myelopoiesis in the spleen and liver, it was sometimes difficult to decide whether animals dying without massive leukemia still possessed leukemic cell foci. The decision was based on the following arbitrary criteria: a) Leukemia was probable if autopsy showed a soft spleen weighing 150 mg and more, pale nodules in the liver, or both. b) Probability that myeloid cell foci in the liver and spleen were leukemic was low if they consisted of erythroblasts, mature myeloid cells, and neutrophiles with segmented nuclei.

The histopathology of secondary disease in sublethally irradiated, nonleukemic RF mice treated with C57BL spleen and bone marrow cells has been described (27).

Thus one of four different postmortem diagnoses could be made: 1) clear evidence of leukemia, 2) probable or possible leukemia, 3) secondary disease, and 4) death attributable to other causes, e.g., toxicity of cyclophosphamide or radiation syndrome. The leukemia incidence indicated in the results includes both unquestionable and suspected cases of leukemia.

Terminology.—Acute graft-versus-host reaction, chronic graft-versus-host reaction, and graft-versus-host reaction with signs of graft rejection as described for nonleukemic mice (27) have all been termed secondary disease. Animals bearing a transplanted myeloid leukemia are sometimes referred to as leukemic or leukemia-bearing animals. Spleen cells obtained from massively enlarged spleens of leukemic mice are denoted as leukemic cells. In this study, reversal is defined as the return
of the chimera hematopoiesis to the animal's own genotype, whether this is accomplished by regeneration of the autogeneic hematopoietic cells or by repopulation by the cells of an isogeneic donor.

RESULTS

Text-figure 1 shows the results of experiments 1–7. Each group consists of mice pooled from several experiments.

Experiment 1, Untreated Leukemia

Intravenous inoculation of \(1 \times 10^6\) leukemic cells into 55 RF male and female mice resulted in 100% mortality with evident leukemia after 10–19 days. In the irradiated mice of the different treatment groups, the day of irradiation was considered as 0. For comparison, mortality of the controls was tabulated in a similar fashion, the time axis starting at day -4 as the day of inoculation.

Experiment 2, Irradiation

Irradiation of 48 leukemia-bearing RF mice with 530 rads postponed death from leukemia for approximately 10 days. Five mice (10%) probably died from irradiation-induced bone marrow depletion rather than from leukemia, but, in three of these animals, histologically the liver showed multiple foci of leukemic cells.

Comment.—The dose of radiation (530 rads) was sublethal for nonleukemic RF mice (21). Although the mortality histograms of experiments 1 and 2 probably indicate that the interval between inoculation and death from leukemia varied slightly, no spontaneous recovery was observed. As expected, leukemia could not be eradicated by 530 rads.

Experiment 3, Allogeneic Bone Marrow

Of 32 leukemic RF mice that received 40–60 \(10^6\) allogeneic (C57BL) bone marrow cells after 530 rads, 16 mice (50%) died with leukemia (14 evident, 2 suspected cases). Eight mice (25%) died with secondary disease or graft rejection, and eight mice (25%) survived 100 days. Erythrocyte typing in 9 animals surviving 65 days showed 6 cases of chimerism and 3 of partial reversion. One of these 3 spontaneously reverting animals died subsequently with secondary disease (21), while in the other 2 the reversion process was completed, as judged by peritoneal cell typing on day 100, which also showed 4 other partial reversions among 6 animals found to be chimeras on day 65. None of these animals developed leukemia and all survived in good health until they were killed after more than 150 days.

Experiment 4, Allogeneic Spleen Cells

In a pooled group of 87 leukemia-bearing RF mice treated with 530 rads and 10–40 \(10^6\) allogeneic (C57BL) spleen cells, 85 animals (98%) died. Seventy-two (85%) of the deaths occurred before day 21. Except for 5 clear-cut cases of leukemia and 2 suspected cases (8%), these animals died with changes typical of secondary disease and, usually, with bone marrow depletion. Mice dying with leukemia were treated with the lowest dose (10 \(10^6\)) of allogeneic spleen cells. The hemoglobin pattern on electrophoresis showed that the two 100-day survivors were chimeras.

Experiments 5–7, Allogeneic Bone Marrow and Spleen Cells

Forty-eight leukemic RF mice received 40–60 \(10^6\) C57BL bone marrow cells on days 0, 3, or 5 after irradiation with 530 rads and injection of 10 \(10^6\) C57BL spleen cells (expt. 5). Only 10 animals (21%) died before day 21. Half these mice, i.e., all those dying on day 9, received bone marrow on day 5. Most deaths occurred during the 2d and 3d month after treatment, with postmortem evidence of a chronic secondary disease. The leukemia incidence (3 unquestionable and 3 suspected cases, 13%) was not significantly different from that in the group receiving no bone marrow in addition to spleen cells (expt. 4). According to erythrocyte typing, 7 mice dying around day 80 and 2 mice surviving more than 100 days were chimeras.

In an attempt to diminish the incidence of leukemia, the number of allogeneic spleen cells injected after irradiation was increased to 40 \(10^6\)
TEXT-Figure 1.—The fate of RF mice inoculated with $1 \times 10^6$ myeloid leukemia cells, irradiated 4 days later with 530 rads and given injections, 4–6 hours after irradiation, of C57BL spleen cells (S), bone marrow cells (BM), or both (S + BM). The day of irradiation is considered as day 0. Mice surviving 100 days and more were free from disease for several months, until they were killed. (See text for details.)
cells. On day zero, 40–60 $\times 10^6$ donor-type bone marrow cells were injected together with the spleen cells (expt. 6). No leukemia was recorded in 41 treated animals. Mortality was low within 3 weeks post treatment (12%), but all mice died with secondary disease after this period.8

After the injection of $60 \times 10^6$ allogeneic spleen cells together with 40–60 $\times 10^6$ allogeneic bone marrow cells (expt. 7), most (85%) of 20 irradiated leukemic mice died with secondary disease within 3 weeks. No death with leukemia was recorded.

Comment.—Results presented here and elsewhere (21) indicate that bone marrow depletion may be an important factor in death of sublethally irradiated mice treated with allogeneic spleen cells. The injection of allogeneic bone marrow in addition to spleen cells appears to reduce such mortality. Only after the high doses of spleen cells ($60 \times 10^6$) did the mortality pattern of mice treated with allogeneic spleen and bone marrow cells resemble that of mice receiving spleen cells only. In contrast to the animals treated with spleen cells only, the animals in experiment 7 died with a fully repopulated bone marrow. It can be assumed, therefore, that the mortality in this experiment was caused by the severe graft-versus-host reaction induced by the large number of allogeneic lymphoid cells.

As to the antileukemic effect, bone marrow is inferior to the spleen cells. The bone marrow graft was probably frequently rejected (21). However, in late reversion, leukemia did not recur. This has also been observed by Wallis et al. (9).

Under the conditions of these experiments, only the injection of more than 40 $\times 10^6$ spleen cells together with bone marrow completely prevented the recurrence of leukemia. The following test was carried out to determine whether all the malignant cells were eradicated by this treatment: Ten leukemia-bearing RF mice were irradiated with 530 rads. Of these mice, 5 were treated with $40 \times 10^6$ C57BL spleen cells and 40 $\times 10^6$ C57BL bone marrow cells, and 5 with similar amounts of RF spleen and bone marrow cells. After 14 days all animals were splenectomized and their spleens cut into small pieces in 1.0 ml of Simms' solution. Each spleen was individually processed. The spleen fragments were injected subcutaneously through a large-gauge needle into normal RF mice. All 5 recipients of spleens from animals treated with isogeneic cells developed tumors at the site of injection (fig. 1) and died with leukemia 28–36 days after inoculation. The splenectomized spleen donors also died with leukemia. Recipients of spleens from animals treated with allogeneic cells remained healthy for 100 days,7 whereas the splenectomized donors died with secondary disease.

Thus, under the conditions of this experiment, a 14-day residence of allogeneic cells in irradiated mice with leukemia diminished the number of leukemic cells in the spleen below that required to transfer leukemia subcutaneously to normal mice. Barnes and Loutit (4) noticed the same fact. These data indicated that the end of the chimeric state at about day 14 might result in termination of secondary disease without recurrence of leukemia. To test this assumption the following experiments were performed: About day 14 after irradiation and injection of allogeneic spleen and bone marrow cells, RF mice with leukemia received a second dose of X ray (300–350 rads), or were treated with a single injection of cyclophosphamide (250–300 mg/kg). In both cases, isogeneic hematopoietic cells and blood were then administered. These experiments yielded negative results, in that 86% of 42 treated animals died with anemia and bone marrow depletion 5–14 days after the treatment, and reversal occurred only occasionally. Davis and Cole (17) carried out similar experiments on nonleukemic mice and concluded that the treatment aimed at reversion should be applied in the early phase of the graft-versus-host reaction, i.e., within the first week. However, this proved impractical in leukemic mice, since leukemia frequently recurred (data to be published).

Subsequent experiments showed that prolonged daily administration of cyclophosphamide in small

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8 Mice bearing leukemia appeared to tolerate higher doses of allogeneic spleen cells than nonleukemic mice (21). This difference might be explained by the assumption that the graft-versus-host reaction was partially “buffered” in leukemic animals due to either a) the greater volume of target cells, i.e., the rapidly expanding population of leukemic cells in addition to the cells of normal tissues, or b) the fact that space in the animal’s hematopoietic tissue wherein the allogeneic spleen cells could proliferate was diminished by proliferation of the leukemic cells.

7 After the inoculation, 1 animal developed an abscess at the site of injection and was killed on day 14 post inoculation.
doses during the first 2 weeks after irradiation and injection of allogeneic hematopoietic cells successfully conditioned the animals for reversal in the 3rd week. In addition, treatment with cyclophosphamide virtually abolished the acute mortality seen in mice receiving high doses of allogeneic spleen cells, as others have observed under comparable experimental conditions (12, 14). Results of the experiments aimed at termination of the secondary disease are illustrated in text-figure 2.

**Experiment 8, Cyclophosphamide**

The drug was administered to 21 leukemic mice on days 4–15 or 16 after irradiation and injection of $60 \times 10^6$ allogeneic spleen cells and 40–60 $\times 10^6$ bone marrow cells (see “Materials and Methods”). Mortality of these mice, pooled from 3 experiments, was postponed approximately 5 weeks beyond that of controls not receiving the drug. Postmortem examination showed a mild secondary disease, but leukemia was completely

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Text-figure 2.—The effect of cyclophosphamide (CYCLO), antilymphocyte serum (ALS), and recipient-type hematopoietic cells and blood (C + B) on the fate of leukemia-bearing RF mice receiving 530 rads and injections of $60 \times 10^6$ C57BL spleen cells (S) and 40–60 $\times 10^6$ C57BL bone marrow cells (BM). NRS = normal rabbit serum. The control group, not treated with immunosuppressants or isogenic cells and blood, consists of the mice of experiment 7 in text-figure 1. The mice in experiment 11 received isogenic (i.e., RF) spleen and bone marrow cells after irradiation. Time axis is the same as that in text-figure 1. See text for details.
absent. In two mice, one of which died during and the other shortly after the cyclophosphamide administration, severe anemia and bone marrow depletion were found. Death apparently due to drug toxicity was also seen in other groups receiving cyclophosphamide (text-fig. 2, early deaths in expts. 9, 10, and 11).

Erythrocyte typing showed the existence of chimerism in 6 animals surviving approximately 2 months.

Experiment 9, Cyclophosphamide and Anti-lymphocyte Serum (ALS)

In this experiment, 10 mice bearing leukemia received 530 rads and were treated with $50 \times 10^6$ allogeneic spleen cells and $40 \times 10^6$ bone marrow cells. After the cyclophosphamide course on days 4–15 post irradiation, ALS was administered for 6 weeks (see “Materials and Methods”). Mortality in this group was delayed as compared with animals receiving cyclophosphamide only. Deaths usually occurred between days 60 and 80, and 1 mouse survived 100 days. When tested on day 60 by erythrocyte typing, the animals were chimeras. Postmortem examination showed no leukemia, and only 1 case of mild secondary disease. The mortality in this group was probably due to inanition as a result of tooth fracture (see below).

Normal rabbit serum (NRS) failed to suppress secondary disease in 8 cyclophosphamide-treated mice (expt. 9a).

Comment.—Experiments 8 and 9 show that in leukemia-bearing mice treated with allogeneic hematopoietic cells the graft-versus-host reaction can be controlled by the administration of cyclophosphamide and ALS. Nevertheless, leukemia does not recur. A graft-versus-host reaction suppressed by cyclophosphamide and tolerated by the recipient might be expected to have a weaker antileukemic effect. This might, however, be counterbalanced by the added antileukemic effect of the drug itself (29). This explanation cannot be applied to the effect of ALS on the later occurring phase of the graft-versus-host reaction, because ALS has no known antitumor action. Under the conditions of this study, therefore, a chronic graft-versus-host reaction may not be needed for the permanent suppression of leukemia.

Experiment 10, Cyclophosphamide and Recipient-Type Hematopoietic Cells and Blood

This treatment was aimed at termination of the chimeric state in irradiated animals treated with allogeneic spleen and bone marrow cells. A total of 36 leukemic mice in 3 separate experiments (10a, 10b, and 10c) received 530 rads and injections of $60 \times 10^6$ C57BL spleen cells and $40-60 \times 10^6$ C57BL bone marrow cells. Cyclophosphamide was administered as specified in “Materials and Methods.” Table 1 gives details of the dose schedule for the treatment with recipient-type hematopoietic cells and blood. The mortality data are pooled in text-figure 2.

In addition to 2 deaths around day 10 due to cyclophosphamide toxicity, early mortality resulted between days 26 and 31, i.e., during or shortly after the treatment with recipient-type cells. In this period, most treated animals became extremely pale and 6 died. Autopsy showed gastrointestinal hemorrhage in 3, and large amounts of liquid

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Mice treated</th>
<th>Donor</th>
<th>Day of treatment with cells and blood*</th>
<th>Average cell dose per injection (×10⁶) and range†</th>
<th>Blood (ml/injection)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10a</td>
<td>10</td>
<td>Immunized‡</td>
<td>16, 17, 21, 24</td>
<td>15 (10-25) 60 (40-75) 40 (20-55) No</td>
<td>0.6 §</td>
</tr>
<tr>
<td>10b</td>
<td>7</td>
<td>Nonimmunized</td>
<td>17, 18, 21, 25</td>
<td>40 (25-50) 90 (75-110) 60 (20-100) No</td>
<td>0.6 (0.5-0.7)</td>
</tr>
<tr>
<td>10c</td>
<td>19</td>
<td>Immunized‡</td>
<td>16, 25, 28</td>
<td>35 (20-50) 90 (25-160) 60 (10-70) 25 (5-50)</td>
<td>0.8 (0.7-1.2)</td>
</tr>
</tbody>
</table>

*Day of irradiation is zero.
‡Nucleated cell counts. B.M. = bone marrow. L.N. = lymph node. Bone marrow and blood were administered intravenously. Spleen, lymph node, and thymus cells were injected intraperitoneally.
†Against C67BL antigens.
§Injected only on day 24.
blood in the peritoneal cavity of the other 3. The blood in the peritoneal cavity could be ascribed to a ruptured spleen. In all 6, the bone marrow was almost acellular and showed deposits of a fibrinoid substance. In 4 mice small thromboemboli containing young myeloid cells and erythroblasts were found in the brain. These deaths, attributable to acute graft rejection (21), occurred only in experiments 10a and 10c, where recipient-type hematopoietic cells were obtained from animals immunized against the donor (C57BL).

After this mortality, immediate transfusion of as much as 1.2 ml of isogeneic blood per mouse was carried out in the other mice. A few days later only 1 more animal died with leukemia.

A total of 27 mice (75%) survived approximately 2 months or more, and 13 mice (36%) survived more than 100 days. (These animals are still alive and in good health, 5 months post irradiation.) Chimerism was tested by erythrocyte and peritoneal cell typing on day 60 post irradiation and occasionally retested shortly before death. In all cases only RF-type cells were found, i.e., all 27 animals appeared to be reversals.

No explanation could be found for the mortality of 14 apparently reverted mice between days 60 and 90 in experiments 10a and 10b, since there were only 2 cases of leukemia, 1 suspected and 1 unquestionable. Twelve other animals died after 3–5 days of illness characterized by progressive weight loss and immobility (fig. 2). Postmortem examination only showed atrophy of the lymph nodes and spleen, as well as atrophy of the liver, kidney, and muscles. The skin frequently displayed hyperplastic hair follicles (fig. 3). Animals treated with cyclophosphamide and ALS (expt. 9) also died in the same period and with similar symptoms (including the occurrence of hyperplastic follicles in the skin) as the animals discussed here.

Only in experiment 10c did the probable cause of death become apparent. Autopsy of 3 mice that died on day 65 revealed that the teeth of these animals were loosened or broken close to the roots. Examination of the other 10 living mice from this experiment showed similar changes (fig. 4). These mice were thereafter supplied with food pellets softened with water (dough). The 10 animals rapidly regained weight and all survived more than 100 days (table 2). At about day 90 the teeth reappeared. In 4 cases supernumerary teeth grew (fig. 5). These ectopic teeth became 1–2 cm long and had to be clipped off to prevent ineffective mastication.

<table>
<thead>
<tr>
<th>Pellets (expts. 10a and 10b)</th>
<th>Alive on day 65</th>
<th>Surviving 100 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pellets ground with water (expt. 10c)</td>
<td>10/19</td>
<td>10/19</td>
</tr>
</tbody>
</table>

*Numbers indicate the number of living mice/the total number of mice per group on day 0.

Comment.—Under the conditions of these experiments a permanent chimeric state is apparently not prerequisite to the definitive suppression of the transplanted myeloid leukemia.

Experiment 11, Antileukemic Effect of Cyclophosphamide

When cyclophosphamide was used to suppress secondary disease, leukemia might have disappeared because of an antitumor effect of this drug (29), and not because of the graft-versus-host reaction.

The following experiment was carried out to test this possibility. Fourteen leukemic RF mice were irradiated with 530 rads. Thereafter, 8 mice received $40 \times 10^6$ isogeneic (RF) spleen cells and $40 \times 10^6$ RF bone marrow cells. Six mice were not treated with cells. On days 4–16 all animals received cyclophosphamide injections according to the same dose schedule used in experiments 8, 9, and 10. In the first group, treatment with isogeneic hematopoietic cells was repeated on days 17, 18, and 21. Blood was also given. Doses of cells and blood were the same as in experiment 10b (table 1). Because the animals not receiving cell injections became very anemic and 2 of them died with bone marrow depletion, it was necessary to transfuse 0.5 ml of isogeneic blood per recipient on days 11 and 14.

The incidence of leukemia was 100% in both groups (disregarding the animals dying with drug
toxicity during cyclophosphamide administration). The data were therefore pooled (text-fig. 2). The 8 animals treated with isogeneic cells tended to survive longer than the 6 animals not receiving the cells, a phenomenon described by de Vries and Vos (5).

Comment.—Cyclophosphamide in a dose used to control the acute graft-versus-host reaction does not account for the eradication of leukemic cells in the previously described experiments in which irradiated animals were treated with allogeneic hematopoietic cells and cyclophosphamide.

In this experiment, the recipients were males and the donors were females. Therefore, in the other experiments a possible difference in sex-linked histocompatibility antigens is not likely to prevent the regrowth of leukemia.

Barrier-nursing, antibiotic supply, and maintenance in autoclaved cages were apparently effective in preventing bacterial infections from interfering with experimental procedures. Only 15% of animals autopsied in this study died with a positive blood culture.

DISCUSSION

Data published by Barnes and Loutit (4), de Vries and Vos (5), Woodruff et al. (6, 7), Mathe et al. (8, 30, 31), and Wallis et al. (9) on the treatment of mouse tumors by total-body irradiation and injection of allogeneic or xenogeneic hematopoietic cells show the following: a) About 50% of treated animals survive 3 weeks post irradiation, i.e., the period of the acute radiation syndrome and the graft-versus-host reaction. b) Only 1–6% of animals survive 100 days, i.e., the period of chronic secondary disease. c) The frequency of tumor recurrence ranges between 10 and 60%, depending on the dose of irradiation, the interval between tumor inoculation and irradiation, and the dose of injected hematopoietic cells. d) Spleen and lymph node cells are more effective in reducing the tumor recurrence, but bone marrow affords a longer survival.

Comparable results were obtained in the present study in experiments 3–7, where the leukemic mice were treated only with irradiation and allogeneic spleen and bone marrow cells. Of 228 mice, 117 (51%) survived 3 weeks post irradiation and 12 (5%) survived 100 days. Leukemia recurred in 29 animals (13%), but all these were treated with bone marrow only or with a small number (10 × 10⁶) of spleen cells (expts. 3–5). Animals surviving 2 months or more were predominantly chimeras, and spontaneous reversion without recurrence of leukemia was only occasional (expt. 3).

The following two modifications of the treatment were explored in experiments 8–10:

1) The use of immunosuppressive agents by which the acute secondary disease could be controlled, resulting in permanent chimerism.—Similar experiments were carried out by Mathe et al. (8, 32). Their data show a 10% long-term (200 days) survival of chimeras receiving cyclophosphamide or amethopterin, whereas the leukemia recurrence (80%) was not appreciably different from that in their controls not receiving the drugs. The present study (expts. 8 and 9) clearly shows that treatment with cyclophosphamide and ALS can significantly prolong the survival of chimeras without any recurrence of leukemia. Of 39 treated animals, 27 (70%) survived 5–8 weeks longer than the appropriate controls. These data show that recipients tolerate a suppressed graft-versus-host reaction while it still exerts an antileukemic effect. The antileukemic effect cannot be attributed to the drug only (expt. 11).

2) Termination of the chimeric state.—Preliminary data (to be published) showed that artificial reversion could be produced by the administration of antidonor serum and recipient-type hematopoietic cells approximately 1 week after irradiation and injection of a small amount (10 × 10⁶) of allogeneic spleen cells, but leukemia recurred in most cases. Since the graft-versus-host reaction probably starts about day 5 post treatment (17, 33), conceivably measures directed at early elimination of allogeneic cells are likely to diminish or even abolish the antileukemic effect.

Reversal without recurrence of leukemia could be obtained in experiment 10 of this study where isogeneic hematopoietic cells were administered during the 3d week after irradiation and injection of allogeneic cells, after a course of cyclophosphamide. Of 36 treated mice, 9 died during the treatment, and 27 (75%) became reversals, as judged by erythrocyte and peritoneal cell typing on
day 60 post irradiation. Only 3 of these animals died with leukemia, and 13 (36%) survived in good health more than 150 days post irradiation. Therefore, eradication of leukemia may possibly be achieved without establishment of permanent chimerism, as already noted by Wallis et al. (9). The following objections might be raised against this conclusion.

1) Reversion of the erythrocyte and peritoneal cell population may not imply reversion of the other cell lines (34). Split chimerism, however, is not likely, since all 13 animals surviving 100 days and more rejected C57BL skin grafts within 9–10 days.

2) The mortality of 12 apparently reverted mice (33%) without evidence of leukemia between days 60 and 100 post irradiation might be evidence against true reversal in these animals. This late mortality was shown, however, to be caused in all probability by inanition due to tooth damage (fig. 4 and table 2). The causative factor may have been the cyclophosphamide, a drug known to cause epilation (29). Teeth, like hairs are epithelial derivatives and in mice grow throughout life. Cyclophosphamide toxicity to the hair follicles, as evidenced by late hyperplastic changes of these structures (fig. 3) in mice receiving the drug, might also affect the tooth epithelium and thereby explain the loosening and fracture of the teeth. Tooth damage was apparently also followed by an abnormal repair (fig. 5). When these mice received a suitable diet, they survived in good health and without recurrence of leukemia.

Data by Alexander et al. (35) indicate that in mice with a strain-specific tumor having the host’s transplantation antigens but also tumor-specific antigens, treatment with allogeneic immunocompetent cells can eradicate the tumor by a “graft-versus-tumor-antigen” reaction, and not necessarily by a graft-versus-host reaction, i.e., by a “graft-versus-H-2-antigen” reaction. The first reaction could also be elicited by isogeneic immunocompetent cells immunized against the tumor-specific antigen. Thus the antileukemic effect was potentiated, and host damage accompanying eradication of leukemia was compatible with life. Isogeneic lymphoid cells injected to terminate chimerism, or regenerating autogeneic cells (37, 38) may have eliminated any remaining leukemic cells by reacting against the tumor-specific antigen (39).

Leukemia used in this study, however, does not seem to possess such a specific antigen (23). So far, eradication of this leukemia could be explained only in terms of a graft-versus-host reaction. Thus only a quantitative difference between the leukemic cell population and the mass of the animal’s organs sensitive to graft-versus-host reaction (intestines, hematopoiesis, skin, liver) appears as a critical factor.

REFERENCES


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Figure 1.—RF mice inoculated subcutaneously with minced spleens obtained from leukemia-bearing RF mice 14 days after irradiation and injection of isogeneic (a) or allogeneic (b) spleen and bone marrow cells. All animals from figure 1a have a tumor in the right flank.
Figures 2-5: Aspect, skin histology, and tooth changes of leukemia-bearing RF mice treated with cyclophosphamide and isogeneic hematopoietic cells after irradiation and injection of allogeneic cells (expt. 10).

**Figure 2.**—Mouse from experiment 10a on day 75, a few hours before death. Note apathetic appearance and emaciation.

**Figure 3.**—Skin of a mouse from experiment 10a, which died on day 63 post irradiation. Hyperplastic hair follicles in the deep dermis. Hematoxylin-phloxine-saffron. ×120
FIGURE 4.—Living mouse from experiment 10c, 66 days post irradiation. Lower left incisor was broken and gums were swollen. The other 3 teeth were loose.

FIGURE 5.—Living mouse from experiment 10c, 97 days post irradiation. A supernumerary tooth growing alongside the lower right incisor.