Mitigation of Secondary Disease of Allogeneic Mouse Radiation Chimeras by Modification of the Intestinal Microflora

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SUMMARY—Conventional CBA mice subjected to lethal whole-body irradiation and allogeneic bone-marrow transplantation developed delayed secondary disease, which caused 95% mortality within 100 days. Symptoms of secondary disease as well as mortality were virtually absent in similarly treated mice kept in the germfree state or given a colonization-resistant (CR) flora. Conventionalization of these mice as early as 40 days after transplantation did not induce a significant degree of secondary disease except in 1 group of CR mice derived from conventional mice by antibiotic treatment. The acute form of secondary disease occurring after transplantation of allogeneic spleen cells was much less influenced by the gnotobiotic conditions, which confirmed the concept that the mortality was caused primarily by severe graft-versus-host reactions. The implications of these findings for the treatment of patients receiving bone-marrow grafts are discussed.—J Natl Cancer Inst 52: 401-404, 1974.

THE DELAYED MORTALITY of allogeneic mouse bone-marrow chimeras is significantly reduced by continuous treatment with antibiotics (1). Jones et al. (2) reported that germfree (GF) allogeneic chimeras survived for at least 120 days, but conventionalization of these survivors 4–10 months after transplantation caused their deaths within 4 weeks. Recently Heit et al. (3) observed a significant reduction of mortality from delayed secondary disease (SD) in mouse radiation chimeras made bacteria-free by antibiotic treatment. These authors also mentioned that conventionalization of 10 of these mice 4 months after transplantation did not have adverse effects.

Interest in the clinical implications of these findings recently increased; on the one hand, the major limitation in bone marrow grafting is delayed rather than acute SD (4, 5), and on the other hand, effective methods now can completely remove the microflora of animals and man (6) or replace it by less pathogenic bacteria (7). Accordingly, we compared the development of delayed SD in conventional and GF mice and in mice colonized by an intestinal microflora that accomplished “colonization resistance” (CR). A CR flora protects against colonization of the intestinal tract by limited numbers of other (outside) microorganisms to which the animals are exposed (8). In this respect, mice carrying a CR flora react differently from GF mice. The CR flora consists of an incompletely identified number of anaerobic bacteria.

In view of the findings of Jones et al. (2), we also investigated the time that the microflora had to be controlled to prevent mortality from SD. Most of our experiments were performed in lethally irradiated mice grafted with allogeneic bone marrow; these mice develop a classic delayed SD. Because mortality from this complication was most effectively prevented both in GF and CR animals, additional experiments were done with a model representing acute SD, produced by grafting allogeneic spleen cells and bone marrow into lethally irradiated mice.

MATERIALS AND METHODS

Recipients of the bone marrow were CBA/Rij 3 male and female mice 10–14 weeks old, exposed to whole-body irradiation with 900 rad 2+4 hours before the intravenous administration of 107 bone marrow cells or a mixture of 107 bone marrow cells and spleen cells from C57BL/Rij donor mice. The physical parameters of the X-irradiation were: 300 kV; 10 mA; half-value layer, 3.0 mm Cu; dose rate, 60 rad/minute; focus-target distance, 50 cm. The mice were irradiated in Petapex boxes under conditions of maximal backscattering, and the dose was calculated as midline tissue dose according to the recommendations of the EULEP Dosimetry Standardization Committee (9). The whole-body dose of 900 rad is supralethal for conventional CBA mice (LD99 = 750 rad). For GF CBA mice, 900 rad is also supralethal. (The LD99 has not been determined accurately because of scarcity of GF CBA mice, but the available dose-mortality data indicate that the LD99 is between 800 and 850 rad.)

The gnotobiotic animals were obtained by a variety of methods (table 1). The GF mice were from our germfree breeding colony, which was originally started by cesarean section and foster-nursing the babies with GF random bred Swiss mice (ND2).

In the “decontaminated” group of mice that received bone marrow and spleen cells, the entire intestinal microflora was removed by feeding conventional mice nonadsorbable antibiotics in the drinking water.

CR mice had 2 origins. Some groups were derived from GF mice by contamination with CR flora and were designated CR-GF+d. Other groups were derived from conventional animals (CR-conv d) by treatment with antibiotics according to the regimen in table 1.

GF mice were conventionalized in 2 steps. First, the animals were orally contaminated with CR flora. After continuation of strict isolation for another 4 days while the implanted microflora colonized the gastrointestinal tract, they were removed from the isolator and housed in a conventional mouse room. CR-flora mice were conventionalized by transfer directly from the isolator to a conventional experimental animal room.

1 Received May 14, 1973; accepted September 28, 1973.
2 Radiobiological Institute TNO, 151, Lange Kleiweg, Rijswijk (Z-H), The Netherlands.
3 This strain is not H-2†, but an unknown H-2 type.
The bone marrow and spleen cells were obtained from conventional donor mice by removal of femurs and spleens aseptically and preparation of cell suspensions in a laminar flow unit. These suspensions were put into sterile closed bottles, which were introduced into the isolators after surface sterilization by routine techniques used in gnotobiotic research. The mice were housed 5 together in transparent plastic cages in laminar cross-flow isolators (11) or closed metal isolators (12). The groups were inspected daily for recording and removal of dead animals. The bedding and feces of all cages were cultured for bacteria weekly and before conventionalization to confirm the gnotobiotic status of the animals.

Blood samples of all groups were taken at the end of the experiment to determine the electrophoretic pattern of the hemoglobin (13). In all cases this was the donor's (C57BL) type, and thus confirmed the presence of chimerism in the erythropoietic system. In our experience with the same strain combination and similar conditions of irradiation and transplantation, complete erythropoietic chimerism was always found in long-term survivors; when tested, such animals also had donor-type leukocytes exclusively.

Dead animals were autopsied, and tissue specimens were fixed in buffered formalin for histologic processing. In some experiments animals were killed for histologic examination of fresh tissues.

RESULTS

Text-figure 1 presents the mortality pattern for several experiments on conventional mice grafted with allogeneic bone marrow. No difference was found between the male and female groups. Symptoms of SD such as wasting and diarrhea appeared between 25 and 30 days after transplantation and increased in severity. By the 90th day >95% of the animals died from classic delayed allogeneic SD.

In contrast, little or no mortality occurred in all gnotobiotic groups given allogeneic bone marrow (table 2). Moreover, conventionalization did not elicit a significant degree of SD in 5 of 7 groups. Nearly all mice developed graying of the fur (as seen in animals given isogeneic bone marrow); exceptions were the CR-GFd group conventionalized at 60 days and the CR-conv d group, in which several mice had diarrhea and gradually developed severe wasting. Some were killed for histologic examination. CR-GFd allogeneic chimeras could be conventionalized as early as 40 days after transplantation without adverse effects.

Histologic lesions in animals from the gnotobiotic groups receiving bone marrow only were always minimal, even in those inspected after conventionalization. Slight but characteristic lesions of graft-versus-host (GvH) activity were regularly observed in the skin, but only occasionally in the gut and liver.

The mortality pattern of acute SD after the grafting of bone marrow and after 2 doses of spleen cells is depicted in text-figures 2 and 3, respectively. Comparison of the mortality curves for conventional mice
Table 2.—Survival data of gnotobiotic allogeneic radiation chimeras before and after conventionalization

<table>
<thead>
<tr>
<th>Status (number of mice)</th>
<th>Percent survivors between days 20 and 90</th>
<th>Day of conventionalization (number of mice)</th>
<th>Effect of conventionalization</th>
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<tbody>
<tr>
<td>Conventional (56)</td>
<td>10</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>GF (69)</td>
<td>100</td>
<td>86 (30)</td>
<td>80 100</td>
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<tr>
<td>CR from GF (58)</td>
<td>93</td>
<td>79 (28)</td>
<td>80 91</td>
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<td></td>
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<td>40 (15)</td>
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<td></td>
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<td>100 (15)</td>
<td>100 100 180 100</td>
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<tr>
<td>CR from conventional (31)</td>
<td>90</td>
<td>66 (15)</td>
<td>100 57</td>
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Text-Figure 2.—Influence of gnotobiotic state on mortality from acute SD. CBA recipients were exposed to 900 rad of whole-body irradiation and received $10^7$ bone-marrow cells and $10^7$ spleen cells from C57BL donors. Curve 1 represents 25 conventional mice; curve 2, 23 GF mice; and curve 3, 26 completely decontaminated conventional mice.

Text-Figure 3.—Influence of gnotobiotic state on mortality from acute SD. CBA recipients were exposed to 900 rad of whole-body irradiation and received $10^7$ bone-marrow cells and $5 \times 10^6$ spleen cells from C57BL donors. Curve 1 represents 23 conventional mice, and curve 2, 24 GF mice.

showed that the groups receiving $5 \times 10^6$ spleen cells died later than those grafted with $10^7$ spleen cells, as expected. The acute SD was less severe in the former group, which could be more susceptible to prevention. GF as well as decontaminated mice subjected to the same treatment developed classic symptoms of acute SD, but the mortality was always less than in the corresponding conventional groups. Even in the groups receiving $5 \times 10^6$ spleen cells, control of microflora did not prevent 100% mortality. The histologic changes in gnotobiotic mice dying with acute SD were characteristic for acute GvH uncomplicated by infections (12).

DISCUSSION

Our findings concerning delayed SD confirm and extend those of Jones et al. (2) and Heit et al. (3) insofar as the animals were kept under gnotobiotic conditions. The decontaminated mice in the latter study had a much higher mortality (about 50% at 10 wk) than in any of our groups or in the experiments of Jones et al.; however, they cannot be strictly compared, since our chronic GvH experiments on delayed SD did not include totally decontaminated animals.

Our observations that in most groups conventionalization of the gnotobiotic animals did not significantly induce SD or mortality are in striking contrast to those of Jones et al., who conventionalized their chimeras between 150 and 180 days post transplantation. This method resulted uniformly in death. We suggest that these animals succumbed from infection due to sudden conventionalization, which was prevented in our experiments by the introduction of a preliminary step with CR flora in the conventionalization process. Without this precaution, normal GF animals were observed to die soon after removal from the isolators. Although the clinical condition and the mortality pattern during the postconventionalization period were not significantly different in the CR-conv d groups from those in the other gnotobiotic groups, the slightly higher number of deaths as well as delayed...
depigmentation of the fur in some animals merits attention. If it is assumed that these symptoms reflect some degree of SD, further detailed investigations of this gnotobiotic condition are required, since this method is the most realistic for clinical application.

In fact, a difference in the composition of the intestinal flora between CR-GFd and CR-conv d mice cannot be excluded because we could not isolate and identify the anaerobic microorganisms in the CR flora. CR-conv d mice conceivably retain 1 or more strains other than those in the CR flora used for contamination of GF mice, and such microorganisms—which may vary from individual to individual—may influence the manifestations of SD symptoms. The mortality in the CR-GFd group conventionalized at 60 days remains unexplained, since it was handled in the same way as the other conventionalized groups that showed no adverse reactions.

It seems particularly important that mice conventionalized as early as day 40 after transplantation did not show significant deterioration of their condition and certainly not the pattern of mortality developing at that exact time in the conventional groups (text-fig. 1). If the classic symptoms and the mortality of delayed SD in the conventional condition were due to infectious complications of GvH-induced tissue defects, normal delayed secondary disease would be expected to develop in the GF mice following conventionalization during that early period after transplantation. Our results suggest that the severity of the GvH reaction itself may be determined by the presence or absence of microflora, e.g., via a non-specific stimulation of lymphoid cells by bacterial antigens.

Our results have interesting implications for patients undergoing allogeneic bone-marrow transplantation. In babies with combined immune deficiency disease (CID) and treated with bone marrow from non-HL-A identical donors, we reported the prevention of acute GvH disease by grafting small numbers of purified stem cell concentrates (14). These patients developed delayed GvH disease and died between 40 and 120 days after grafting. At autopsy the lesions were suggestive of severe infections complicating characteristic GvH lesions which, per se, were not considered life-threatening. It seems logical that the lives of such patients could be prolonged if the source of infections is removed. The present results in mice justify further attempts to treat severe CID with a combination of purified stem cells, bacteriologic decontamination, and isolation, if HL-A identical siblings are not available as donors. Similarly patients receiving full-size bone-marrow grafts from HL-A identical siblings should be decontaminated and isolated as protection against GvH disease, which develops in 50% of such cases and kills 20% (15).

A change in microflora was not effective in protecting against acute SD. This agrees with the concept that mortality from this syndrome is due primarily to tissue damage from severe GvH rather than to secondary infections (12).

REFERENCES

(9) Committee for Dosimetry Standardization, European Late Effects Project Group: Protocol for EULEP X-ray Dosimetry, August 1972