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# THE INFLUENCE OF THE MICROFLORA ON GRAFT-VERSUS-HOST DISEASE IN EXPERIMENTAL AND CLINICAL BONE MARROW TRANSPLANTATION

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## INTRODUCTION

Allogeneic bone marrow transplantation (BMT) is currently being used as treatment for many fatal diseases of the haemopoietic system, among them severe aplastic anaemia (*Löwenberg* and *Gale*, 1985) and leukaemia (*Dicke* et al., 1985). Furthermore, patients suffering from fatal hereditary diseases that are associated with a dysfunction of the lymphoid system, like severe combined immunodeficiency (*Dooren* and *Vossen*, 1985) and patients with inherited severe metabolic disorders (*Barranger*, 1984) are being treated with bone marrow grafts. In total about 2500 patients are transplanted annually world-wide in 160 BMT centres.

One of the major complications of allogeneic BMT is graft-versus-host disease (GvHD), which is caused by donor type lymphocytes which react against the recipient's tissues. According to an evaluation of data from 2036 recipients of HLA identical sibling bone marrow transplants reported to the International Bone Marrow Transplant Registry, moderate to severe GvHD occurred in about 45% of these patients. In 48% of them, GvHD was related to their death (*Gale* et al., 1987). The severity of GvHD is influenced by several factors, which include the degree of immunogenic disparity (*Uphoff* and *Law*, 1958), the number of cells grafted (*van Bekkum*, 1964), the number of T-lymphocytes present in the graft (*van Bekkum*, 1964, 1972), the donor's sex (*Gale* et al., 1987) and the age of the recipient (*Gale* et al., 1987).

## EXPERIMENTAL BONE MARROW TRANSPLANTATION

Another important factor influencing GvHD is the recipient's gastrointestinal microflora. This was originally observed in gnotobiotic mice. In contrast to the bone marrow of primates, rodent bone marrow contains a low proportion of immunocompetent T-lymphocytes. As a consequence, its GvHD-inducing potential is comparatively low (*van Bekkum* and *de Vries*, 1967). Infusion of $10^7$ H-2 incompatible bone marrow cells into lethally irradiated (9.0 Gy X-rays) conventional mice results in a late onset type GvHD which does not give rise to symptoms until about three weeks after BMT. This disease kills the

majority of the recipients during the next two months but those that survive for more than three months seem to have recovered (*van Bekkum* and *de Vries*, 1967; *van Bekkum* et al., 1974). This type of GvHD is called delayed GvHD to distinguish it from the acute GvHD which can be induced in mice by supplementing the allogeneic bone marrow graft with donor derived spleen or lymph node cells. Mortality attributable to delayed GvHD can be completely prevented if the recipients are germfree mice (*Jones* et al., 1971; *van Bekkum* et al., 1974; *Truitt*, 1978; *Veenendaal* et al., 1988), or when they are conventional animals which have been subjected to complete (*Heit* et al., 1973; *Truitt*, 1978) or selective gastrointestinal decontamination (*van Bekkum* et al., 1974) by means of orally administered non-absorbable antibiotics prior to transplantation. In these experiments, selective decontamination resulted in animals which only harboured a strict anaerobic microflora. Mitigation of GvHD is also observed when the recipients of allogeneic H-2 mismatched bone marrow are conventional mice which have been associated with a strictly anaerobic microflora after a period of complete decontamination, or if they are germfree animals which have been associated with this flora (*van Bekkum* et al., 1974). This anaerobic microflora was originally obtained from selectively decontaminated conventional mice and was named CRF (colonisation resistance factor) flora after its capacity to provide animals with resistance against newly colonising microorganisms (*van der Waaij*, 1971). This CRF flora is largely composed of spore forming Gram-positive rods (mainly different *Clostridium* spp.; *Wensinck* and *Ruseler-van Embden*, 1971). The most striking difference between the conventional mice on the one hand, and the selectively decontaminated and the CRF mice on the other hand was the absence of aerobic Gram-negative rods (i.e. *Enterobacteriaceae*) in the second and third group.

If the bone marrow graft of conventional mice is supplemented with $10^7$ spleen cells so as to provide a graft composition which is, like primate marrow, rich in immunocompetent T-lymphocytes, the recipients suffer from an early onset GvHD which takes a fatal course within three weeks after transplantation. The absence of a gastrointestinal microflora in the recipients (i.e. germfree or completely decontaminated animals) delays this mortality by two weeks, but does not prevent it (*van Bekkum* et al., 1974).

*Jones* et al. (1971) showed that conventionalisation of germfree or completely decontaminated recipients of allogeneic H-2 mismatched bone marrow at 150 and 180 days after BMT caused their death within 4 weeks. It is conceivable that this mortality was the result of infection caused by uncontrolled colonisation of the gastrointestinal tract, since *Heit* and colleagues (1973) showed that reconventionalisation of the completely decontaminated chimeras, starting 175 days after BMT, did not produce mortality. We investigated the time period after BMT during which the recipients had to be maintained germfree or decontaminated in order not to loose the mitigating effect of the gnotobiotic state on GvHD. It became clear that reconventionalisation at day 40 or later after BMT did not influence the beneficial effect of the decontaminated or germfree state; also >90% of these chimeras survived after reconventionalisation without showing any signs of GvHD (*van Bekkum* et al., 1974). We also studied the effect of earlier reconventionalisation after BMT (i.e. days 8, 20 and 26 after BMT). Reconventionalisation at days 8 and 20 resulted in a mortality pattern after BMT, which was

identical to that of conventional recipients of allogeneic BMT. Reconventionalisation at day 26 after BMT gave a protective effect in about 50% of the recipients, the remaining animals survived for >200 days after BMT (*van Bekkum*, 1977). The results of the described experiments show that the severity of GvHD is determined by the presence or the absence of (some constituents) of the recipient's microflora. These findings suggested that not only histo-incompatibility is determining the occurrence and severity of GvHD, but that microflora-related factors also are of major importance. The hypothesis was that donor type lymphocytes could be stimulated or activated by antigens of bacteria from the gastrointestinal tract which cross-react with antigens present on the recipient's epithelial tissues, being the main targets in GvHD. A key role in this phenomenon was attributed to the *Enterobacteriaceae*, which were absent in the germfree, selectively or completely decontaminated, and CRF recipients of allogeneic mismatched bone marrow in which GvHD was mitigated, while this group of bacteria was always present in the conventional recipients of which 90% died from severe GvHD.

The hypothesis that GvHD is the result of T-cell stimulation, or of activation by cross-reactive antigens present on enteric bacteria and the recipients epithelium was confirmed by experiments performed by *van Bekkum* and *Knaan* (1977). In (CBA x C57BL) F1 hybrid mice they implanted CBA and F1 foetal gut fragments. After the implants were established (about 15 to 30 days after implantation), the mice were irradiated (9.0 Gy X-rays) and transplanted with 2x $10^6$ CBA bone marrow plus 2x $10^6$ CBA spleen cells, so that they developed acute GvHD. This was done in conventional as well in completely decontaminated carriers of foetal gut implants. As a control, conventional and completely decontaminated F1 carriers of foetal gut implants were transplanted with similar amounts of F1 bone marrow and spleen cells. Scoring of GvHD in the different groups was done by counting the numbers of degenerated and intact crypts in sections of the implanted F1 or CBA guts. After transplantation of CBA bone marrow and spleen cells in conventional F1 mice, the damage in the F1 foetal gut implant was twice as great as in F1 foetal gut implants of the decontaminated recipients. In conventional recipients of CBA bone marrow and spleen cells, the CBA foetal gut implants which were not in direct contact with any microflora, showed a significant score of GvHD lesions, while in decontaminated chimeras the CBA implant showed no histological lesions. None of the implants of the control animals, which had been treated with isogeneic cells, showed histopathological lesions characteristic for GvHD. It was concluded that the presence of a microflora at a distant site is capable in magnifying GvHD lesions in the germfree F1 gut implant, and is even capable of inducing donor type immune cells to cause GvHD lesions in the germfree CBA implant which is syngeneic to these cells.

The above-summarised observations all clearly indicate an important role of the microflora in the development and severity of GvHD after allogeneic bone marrow transplantation.

To investigate the general applicability of the observations in mice, we studied the effect of gastrointestinal decontamination on GvHD after allogeneic BMT, using beagle dogs as a preclinical model (*Vriesendorp* et al., 1981). The dogs were studied under three different gnotobiotic conditions: conventional, selective gastrointestinal decontamination, and complete gastrointestinal decontamination. Both selective and com-

**Table 1**: Incidence of lethal GvHD in different gnotobiotic groups of monkeys (different donor/recipient combinations)

| Donor/recipient combination | | | Gnotobiotic state | | |
|---|---|---|---|---|---|
| RhLA A/B | RhLA D/DR | Family relationship | Complete GID | Selective GID | Clean conventional |
| = | ≠ | none | 0/4[a] | - | 5/9[b] |
| ≠ | = | sibling | 0/2[c] | 0/1[d] | 4/5[e] |
| ≠ | ≠ | none | 6/8[f] | 1/1[g] | 5/5[h] |

Chi-square test: (a+c+d) vs. (b+e): $p<0.05$
(f+g) vs. h : $p>0.05$ (n.s.)

plete gastrointestinal decontamination were discontinued in surviving animals on day 40 after BMT, since experiments in mice had shown that after this period no extra beneficial effect on GvHD can be expected from the gnotobiotic state of the recipient (*van Bekkum*, 1977). Donors and recipients were typed for the major histocompatibility complex (MHC).

After conditioning with total body irradiation, the animals were given bone marrow cells to which lymph node cells were added to mimic the human situation. Selective gastrointestinal decontamination was found to mitigate acute GvHD in this study with dogs; a small effect on the incidence of GvHD and on the mortality resulting from this disease was observed in the groups in which $10^8$ lymph node cells per kg body weight were added to the bone marrow graft. It is to be assumed that complete decontamination gives the same degree of protection if not more. We did not observe any effect of complete GID on the incidence of GvHD and subsequent mortality after grafting of allogeneic bone marrow to which 2x $10^8$ lymph node cells per kg body weight were added. Therefore, the impression is gained that the acute GvHD caused by $10^8$ donor lymph node cells per kilogram body weight from a MHC identical donor is the maximum severity of GvHD that can be prevented by GID. This is in agreement with experiments in mice, which showed that the effect of the gnotobiotic state on GvHD is limited by the number of T-lymphocytes present in the graft (*Heidt* et al., 1981).

As a final pre-clinical model, we studied the influence of gastrointestinal decontamination on GvHD after (partially) mismatched allogeneic BMT in rhesus monkeys (*Macaca mulatta*). Twenty-five monkeys were either subjected to complete or to selective decontamination, irradiated with a single dose of 8.5 Gy X-rays (n=10) or 2 fractions of 7.0 Gy separated by 3 days (n=15), and transplanted with stem-cell enriched, lymphocyte depleted bone marrow. The donors were either unrelated mismatched (n=12), unrelated A/B matched (n=6), or related D/DR matched (n=7). The cell dose was 5x $10^7$/kg body weight.

GvHD could be studied in 16 of the 25 transplanted animals; of the remaining animals, 4 did not establish a take of the donor bone marrow while 5 monkeys were not evaluable due to early death caused by other complications being mostly severe electrolyte imbalance. The incidence of lethal GvHD in the different donor/recipient combinations is given in Table 1. Decontamination of the gastrointestinal tract resulted in the prevention of lethal GvHD in re-

**Table 2**: Characteristics of 94 evaluable patients

| | Bone marrow failure | | | Haematological malignancy | |
|---|---|---|---|---|---|
| | Severe aplastic anaemia | Fanconi's anaemia | Myelodisplastic syndrome | Leukaemia | Non-Hodgkin lymphoma |
| Selective GID (n=18): | 9 | 2 | 1 | 6 | 0 |
| Complete GID (n=76): | 14 | 4 | 7 | 47 | 4 |

cipients of partially matched T-lymphocyte depleted allogeneic bone marrow grafts. A possible difference between the two types (complete and selective) gastrointestinal decontamination could not be evaluated due to the small individual groups in this study. Gastrointestinal decontamination showed not to be effective in preventing lethal GvHD after transplantation with partially T-lymphocyte depleted completely mismatched unrelated bone marrow grafts. This failure was ascribed to the comparatively large number of T-lymphocytes which remained present in these grafts after using discontinuous albumin density gradient centrifugation for lymphocyte depletion (*Dicke* and *van Bekkum*, 1971).

## CLINICAL BONE MARROW TRANSPLANTATION

In man, the effect of GID on GvHD has been controversial for a long time. Several studies reported a reduction of the incidence of acute GvHD after allogeneic BMT (*Mahmoud* et al., 1984; *Schmeiser* et al., 1984; *Storb* et al., 1983). However, such an effect was not observed in other studies (*Leblond* et al., 1987; *Skinhøj* et al., 1987; *Storb* and *Thomas*, 1985). Recently, we reported on a retrospective evaluation of the efficacy of GID in a protective environment for the prevention of GvHD in 65 children and adolescents, grafted consecutively for either severe bone marrow failure (n=29) or leukaemia (n=36) (*Vossen* et al., 1990). It was concluded that, in contrast to selective gastrointestinal decontamination (Group I, n=21), complete gastrointestinal decontamination (Group II, n=44) in a strict protective environment is a very effective method for preventing acute GvHD in children and adolescents; it resulted in a cumulative frequency of ≥2 grade II acute GvHD of 17.5%, a low transplantation-related mortality of 26% and a good quality of survival in 69% of the graft recipients.

More recently the influence of complete and selective gastrointestinal decontamination in a strict protective environment on acute GvHD after allogeneic BMT was re-evaluated in a larger group of patients, which were transplanted in the Leiden Paediatric BMT Centre over a period of about 20 years. Since the above-mentioned report, 37 more completely decontaminated children have been grafted (Group III). Major differences between the former study groups and group III were the use of methotrexate plus cyclosporin-A for GvHD prophylaxis in 25 graft recipi-

**Table 3**: Antimicrobial drugs for complete and selective gastrointestinal decontamination (daily dose)

| Group I | Group II | Group III |
|---|---|---|
| Nalidixic acid (90 mg[a,b]) | Neomycin (200 mg) | Gentamicin (800 mg) |
| Co-trimoxazole (12/60 mg[a,b]) | Polymyxin B (2000 mg) | Cephaloridin (2000 mg) |
| Neomycin (15 mg[a]) | Cephaloridin (2000 mg) | Amphotericin B (2000 mg) |
| Polymixin B (20 mg[a]) | Amphotericin B (2000 mg) | |
| Amphotericin B (2000 mg) | | |

[a]: per kg body weight.
[b]: used only in a limited number of patients.

ents of group III, and the substitution of systemic (i.v.) antimicrobial prophylaxis for peroral GID early after BMT in recipients of group III with gastrointestinal complaints and bad compliance for oral antimicrobial drugs. Patients in all three groups received full bone marrow grafts from HLA genotypically identical siblings, following the usual pre-treatment regimens. The characteristics of the 94 evaluable patients are given in Table 2.

All patients were nursed in a strict protective environment, i.e., in laminar down flow isolators (*van der Waaij* et al., 1973), using aseptic nursing techniques and sterilisation of food, beverages and all other items brought into the isolator (*Vossen* and *van der Waaij*, 1972).

Antimicrobial drugs and dosages administered orally for selective gastrointestinal decontamination in group I, complete gastrointestinal decontamination of patients in group II, and complete gastrointestinal decontamination in group III are given in Table 3. Young children below the age of 2 years received half of the indicated dosages. The drugs were administered in four divided doses per day, except for co-trimoxazole, which was given twice daily. When during the early period after BMT children were unable to swallow the drugs for complete gastrointestinal decontamination due to nausea and vomiting, the suppression of the gut microflora was continued by i.v. administration of co-trimoxazole and cefamandole in usual therapeutical dosages; this was only done in children of group III. In individual cases of the same group, i.v. 5-flucytosine was added to the oral administration of amphotericin B, when elimination of yeasts from the gut had not been successful. This was done to suppress further growing of these microorganisms.

Decontamination started ≥ one week before the date of BMT and was given for at least a total of 40 days after BMT, based on experimental data (*van Bekkum*, 1977). Both complete and selective GID were considered successful when in the period from 7 days before until 40 days after BMT the target-microorganisms could not be isolated from more than two consecutive faecal samples. GvHD was diagnosed by clinical

**Table 4**: Composition of the SPF and conventional (HF) microflora

| SPF-Flora | Houston-Flora |
|---|---|
| Anaerobic microflora | Anaerobic microflora |
| CRF-flora (not defined) | Not defined |
| Aerobic microflora | Aerobic microflora |
| Streptococcus faecalis (7173711[a]) | Streptococcus faecium (7355510[a])<br>Streptococcus faecium (7317550[a]) |
| Staphylococcus aureus (6726153[b])<br>Staphylococcus epidermidis (6706133[b]) | Staphylococcus xylosus (6736552[b])<br>Staphyloccus haemolyticus (6632171[b]) |
| Escherichia coli (5144572[c]) | Escherichia coli (5144572[c])<br>Escherichia coli (5144532[c])<br>Proteus mirabilis (0536000[c])<br>Proteus mirabilis (0534000[c]) |
| | Pasteurella pneumotropica (1220000[d])[1] |

[a]: Biotype (API 20 Strep; API System, Montalieu-Vercieu, France).
[b]: Biotype (API Staph; API System, Montalieu-Vercieu, France).
[c]: Biotype (API 20 E; API System, Montalieu-Vercieu, France).
[d]: Biotype (API 20 NE; API System, Montalieu-Vercieu, France).
[1]: Isolated from nasal washings only.

symptoms. The severity of acute GvHD was graded according to *Thomas* et al., (1975).

According to the criteria used, GID was successful in 14 out of 18 evaluable children with selective GID in group I (78%); in 11 out of 40 evaluable children with complete GID in group II (27.5%) and in 19 of 36 evaluable children with complete GID in group III (53%).

The occurrence of ≥ 2 grade II acute GvHD was 6/18 (33%) in group I, 7/40 (17%) in group II and 1/36 (3%) in group III. More relevant was the finding that 0/30 successfully completely decontaminated children developed ≥ 2 grade II acute GvHD versus 6/14 (43%) successfully selectively decontaminated children. The latter is not different from the incidence (45%) in 2036 recipients of HLA identical sibling bone marrow transplants analysed by the International Bone Marrow Transplant Registry (*Gale* et al., 1987). From our observations it can be concluded that complete gastrointestinal decontamination is superior to selective gastrointestinal decontamination in preventing ≥ 2 grade II acute GvHD.

## THE MECHANISM

To study the mechanism, which underlies the influence of the gastrointestinal microflora on GvHD, H-2 different donor and recipient mice with a SPF and a conventional microflora were employed (*Heidt*, 1989). For this purpose a conventional murine microflora was imported from the M.D. Anderson Cancer Institute, Houston, TX, USA, called "Houston flora" (HF), since in our institute only SPF animals are being bred. The composition of the SPF flora

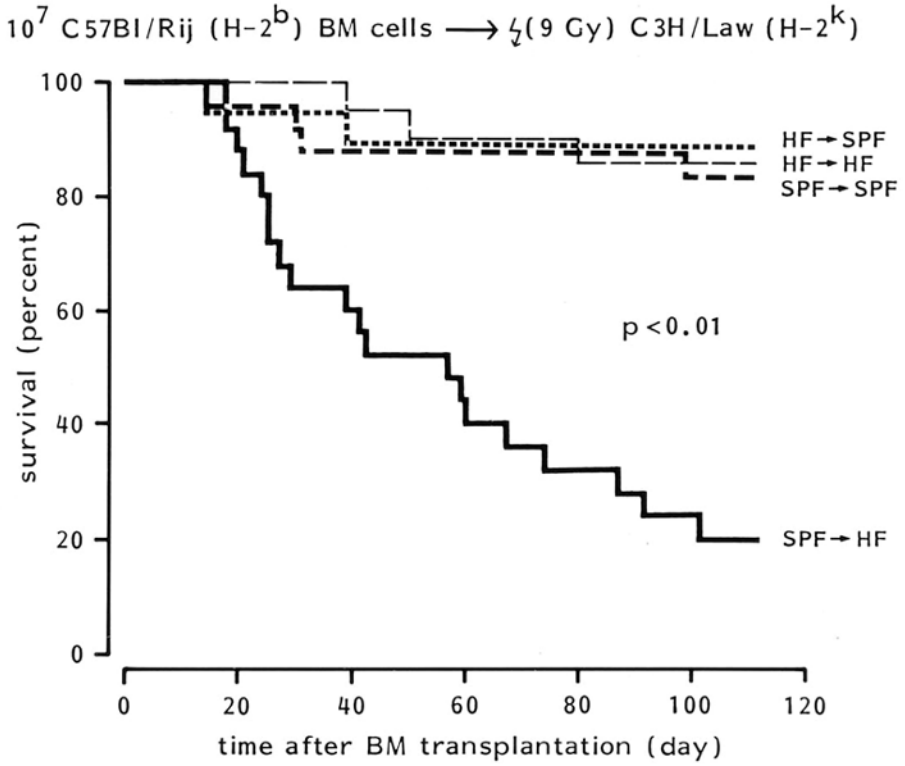


**Figure 1**: Mortality in the different microbiolically defined donor-recipient combinations.

and this HF is given in Table 4.

Germfree C3H/Law breeding pairs were associated with the conventional flora (HF). HF-bearing C57BL/Rij mice were obtained by foster nursing caesarean derived C57BL/Rij newborns by HF C3H/Law mothers. The HF C57BL/Rij animals obtained in this way were used as breeding animals to produce the experimental animals. Before entering the experiment, all mice were kept in Trexler type plastic film isolators to prevent undue association with any other microorganisms. Beside the above mentioned experiments using donor and recipient mice with the SPF flora or the HF, a second series of experiments was carried out to study the effect of complete and selective GID of HF recipients on GvHD. During both series of experiments, all recipients were housed under conditions of strict reverse isolation in a laminar cross flow isolator to prevent contamination of the animals with any new microorganisms (*van der Waaij* and *Andreas*, 1971). The animals received autoclaved (10 min., 134°C) AM-II food pellets (Hope Farms B.V., Woerden, The Netherlands) and acidified (pH 2.8) sterile drinking water.

The recipients were lethally (9 Gy) irradiated as a conditioning for BMT. The next day, they were injected i.v. with $10^7$ bone marrow cells from C57BL/Rij donor mice. Irradiation of the mice and transplantation of the bone marrow cells were also performed under conditions of strict reverse isolation.

According to the microbiological status of the donors and the recipients, there were four different experimental groups in the first series of experiments. They were: HF recipients of

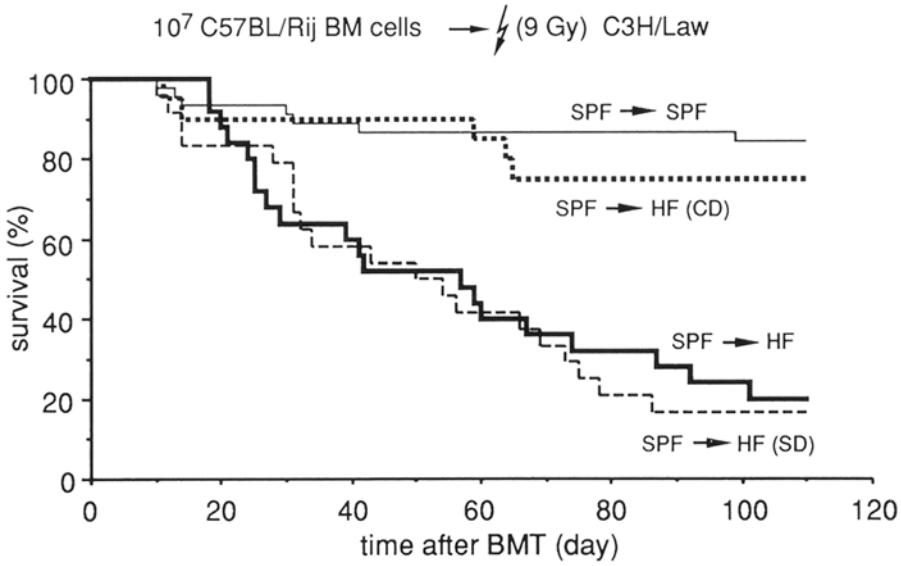


**Figure 2**: Influence of selective and complete GID on mortality of HF-bearing C3H/Law recipients of SPF-bearing C57BL/Rij bone marrow grafts.

SPF donor bone marrow (SPF to HF), HF recipients of HF donor bone marrow (HF to HF), SPF recipients of SPF donor bone marrow (SPF to SPF), and SPF recipients of HF donor bone marrow (HF to SPF).

No significant mortality from GvHD occurred in HF to HF recipients, SPF to SPF recipients and HF to SPF recipients, but the mortality of SPF to HF recipients was 80% (Figure 1). In the second series of experiments, prevention of GvHD (with a mortality-pattern identical to that in the HF to HF recipients, SP to SPF recipients and HF to SPF recipients) was observed in the completely decontaminated HF C3H/Law recipients of SPF C57BL/Rij bone marrow, while no effect of selective GID of the HF recipients was seen (Figure 2).

In the first series of experiments, lethal GvHD occurred in only one of the four transplanted groups of mice. This was the group of "conventional" (HF) recipients of SPF bone marrow (SPF to HF), in which severe diarrhoea was also observed and which showed the most distinct histopathological lesions. When the donors and recipients of the bone marrow were both HF carrying animals (HF to HF), more than 90% of the recipients survived after BMT. Also in the two other groups in which the recipients were SPF (i.e., SPF to SPF and HF to SPF), more than 90% of the recipients survived. This is comparable with observations made when recipients of allogeneic H-2 mismatched bone marrow are germfree or have been decontaminated (*van Bekkum* et al., 1974). The results show that mortality due to GvHD after allogeneic H-2 mismatched BMT is significantly prevented when there is identity between the microflora of the donor and recipient (HF to HF and SPF to SPF) or when the recipients do not harbour any aerobic microorganisms other than those present in the donors (HF to SPF).

In previous publications it was hypothesised that certain bacteria belonging to the microflora of the recipient may play a role in the induction of de-

layed type GvHD after allogeneic BMT because they carry antigens which are cross-reactive with epithelial antigens. After transplantation of allogeneic bone marrow from a donor which was matched for the major histocompatibility complex (MHC), a mitigation of GvHD was observed in selectively decontaminated dogs (*Vriesendorp* et al., 1981), which confirmed the findings in mice (*van Bekkum* et al., 1974) suggesting that *Enterobacteriaceae* might play a role in the induction and severity of GvHD. The observation that selective GID of HF recipients did not mitigate GvHD, in contrast to complete GID of HF recipients does not support this hypothesis since *Enterobacteriaceae* are being eliminated by selective GID. Therefore, it has to be assumed that other microorganisms than *Enterobacteriaceae* are involved in the induction of GvHD. It is likely that bacteria belonging to the complex anaerobic microflora are responsible for the induction of GvHD. *Foo* and *Lee* (1974) found that antigenic cross-reaction exists between mouse intestine and a *Bacteroides* spp., one of the anaerobic members of the autochthonous microflora of the rodent gastrointestinal tract. The above mentioned conclusion is supported by the earlier mentioned observations that selectively decontaminated human recipients of allogeneic bone marrow developed significantly more ≥ 2 grade II acute GvHD than completely decontaminated patients. The most striking difference between these two groups of patients was the presence of an anaerobic microflora in the first group of recipients (*Vossen* et al., 1990).

## HYPOTHESIS

The high mortality due to GvHD in HF recipients of SPF bone marrow in the first series of experiments can be explained by a double mechanism in evoking GvHD after allogeneic BMT. The first mechanism is the reaction of donor type T-lymphocytes against histocompatibility antigens. This graft-versus-host reaction gives rise to minimal intestinal lesions and subsequent limited mortality, as was the case in the completely decontaminated recipients. If however, the flora of the recipient of the allogeneic bone marrow contains anaerobic bacterial species which do not also belong to the indigenous flora of the donor, and which carry antigens that are cross-reactive with tissue antigens of the host, the donor T-lymphocytes are activated by these cross-reactive bacterial antigens and become reactive against host tissues. This results in a clinically and histologically more severe graft-versus-host reaction, leading to the death of the majority of the recipients.

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