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CHANGES IN MURINE GRAFT-VERSUS-HOST DISEASE AFTER PARENTERAL INJECTION OF THE DONOR WITH INTESTINAL MICROBIAL FLORA

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SUMMARY

Graft-versus-Host Disease (GvHD) is a well-known complication of allogeneic bone marrow transplantation (allo-BMT). Mice engrafted with allogeneic bone marrow (BM) cells may suffer late onset GvHD (LO-GvHD), previously called secondary disease. LO-GvHD is known to be determined by the presence and the composition of intestinal microbial flora (I-MF) in the recipient as well as the donor. Anti-MF antibodies have been postulated to cross-react with tissue antigens and subsequently induce LO-GvHD. In this study allo-BMT experiments were carried out with specific pathogen free (SPF) C57Bl/6J (B6) (H-2b) donors and lethally irradiated SPF C3H/He (C3) (H-2k) recipients. In order to obtain different starting points at the donor site with regard to anti-MF antibody responses, B6 donors received single (day -10) or repeated (day -38 and -10) intra-peritoneal (i.p.) injections either with MF of their own (SELF-MF) or C3 recipients (RECIP-MF). Lethal LO-GvHD was found significantly ($p<0.01$) the highest (15/35=43%), in C3 recipients engrafted with BM from single SELF-MF injected B6 donors. On the other hand mortality was found the lowest in recipients engrafted with BM from donors twice injected with SELF-MF (1/25= 4%). Low mortality rates were also found in C3 recipients engrafted with BM from other B6 donors, i.e. saline injected controls (4/26=15%), and B6 donors injected once (6/32=19%) or twice (5/26=19%) with RECIP-MF. Isotype nor MF-specific serum antibodies in the different B6 donor groups showed any correlation with the occurrence of LO-GvHD in C3 recipients. It is discussed how LO-GvHD might have occurred in C3 recipients due to an interaction between RECIP-MF and 'activated' BM-cells from SELF-MF challenged donors.

INTRODUCTION

Graft versus Host Disease (GvHD) may occur as a serious complication after allogeneic bone marrow transplantation (allo-BMT) in lethally irradiated (9

Gy) mice. Transfer of allogeneic bone marrow (BM) cells together with spleen cells will cause death of all recipients within three weeks after transplantation (*Heidt* et al., 1981; *van Bekkum* et al., 1974a). However, only part of the recipients may die due to lethal GvHD after transfer of 10^7 allo-BM-cells without spleen cells (*Heidt* et al., 1981; *Veenendaal* et al., 1988) and clinical symptoms become manifest late, three weeks after transplantation. This type of GvHD, which has previously been named secondary disease, is therefore referred to as minor, delayed type, or late onset GvHD (LO-GvHD) (*Heidt* et al., 1981; *Pollard* et al., 1976; *Rappaport* et al., 1979; *van Bekkum* et al., 1974a).

LO-GvHD appears to be associated with the presence of intestinal microbial flora (I-MF) in the recipient, since it is strongly mitigated or even absent in germfree (GF), antibiotic decontaminated, and "clean" specific pathogen free (SPF)* mice (*Heidt*, 1989; *Jones* et al., 1971; *van Bekkum* et al., 1974a,b). LO-GvHD does not occur when GF or totally decontaminated recipients are (re)conventionalised 40 days after marrow transplantation (*van Bekkum* et al., 1974a). Thus there is a window of approximately 40 days during which the LO-GvHD 'inducing' capacity of the recipient's intestinal microbial flora decreases.

There is evidence that not only the recipient's I-MF but also the I-MF of the donor plays a role in MF-associated LO-GvHD (*Heidt* et al., 1989). Previous experiments in mice have shown that modulation of donor-MF by oral treatment with non-absorbable small spectrum antibiotics increased the occurrence of LO-GvHD (*Veenendaal* et al., 1988). Moreover, there is evidence that modulation of the I-MF by oral antibiotic treatment decreases myelopoiesis in the bone marrow. This effect may add to the changes of the cellular composition of the BM-graft (*Goris* et al., 1985; 1986a).

It is still unknown by which mechanism LO-GvHD occurs. Bacteria in the digestive tract have been postulated to share antigens common to the recipient host and thus induce cross-reacting antibodies (*van Bekkum* and *Knaan*, 1977). Since the induction of systemic humoral and cellular tolerance to intestinal antigens, generally referred to as oral tolerance, is found to be inhibited during GvHD (*Strobel* et al., 1985; *Strobel* and *Ferguson*, 1986), unopposed B-cell proliferation may contribute to high levels of anti-MF antibodies (*Hamilton* and *Parkman*, 1983; *van der Waaij* and *Heidt*, 1987). If so, immunostimulation by intestinal (bacterial) antigens is likely to occur in the first week(s) after marrow engraftment.

Whether or not engrafted BM-cells are able to react with MF-antigens present in the recipient host and thus induce and/or aggravate LO-GvHD may depend on the composition and the activated state of the engrafted cells. The BM as a major site of long living immunoglobulin (Ig) producing cells during secondary responses has been documented (*Benner* et al., 1981). An increase of IgA producing B-cells in the BM has been found during the secondary response against orally given antigens (*Alley* et al., 1986). So far there is a lack of information on the possible relationship between anti-RECIP-MF antibodies in the donor and LO-GvHD in allogeneic recipients.

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* Clean SPF means strict protection against (microbial) contamination from the outside world, which makes the flora exceptionally stable in composition. For generations the entire I-MF may be (immunologically) tolerated.

In the present study we investigated whether modulation of the immune response by allogeneic C57Bl/6J (B6) (H-2b) donors against their own I-MF (SELF-MF) or that of C3H/He (C3) (H-2k) recipients (RECIP-MF) affected LO-GvHD. A difference in the immunoreactivity against RECIP-MF and SELF-MF antigens could not be excluded on forehand with regard to primary or secondary responses upon parenteral challenge. For this reason B6 donors were injected intra peritoneally (i.p.) once or twice with either SELF-MF or RECIP-MF prior to allo-BMT. The I-MFs of both mouse strains used in this study were found to be similar regarding species of genera of facultative anaerobic bacteria such as *Enterobacteriaceae*. However, the composition of (highly concentrated) obligate anaerobic bacteria was found morphologically different at microscopic examination of Gram-stained faecal pellets.

MATERIALS AND METHODS

Mice

Five to seven weeks old male C57Bl/6J (B6) (H-2b) and C3H/He (C3) (H-2k) mice were purchased from the SPF unit of the Zentral Insitut für Versuchstierzucht (Hannover, FRG) and used at 10-12 weeks of age. Weights varied between 25 and 30 grams per mouse.

Housing

Animals were housed in a conventional animal room at the Central Animal Facility (University of Groningen, The Netherlands), fed with rodent pelleted food (RMH-B, Hope Farms B.V., Woerden, The Netherlands), and tap water *ad libitum*. No antibiotics were used.

Sampling of faeces

Fresh faeces from B6 donor mice and C3 recipient mice were sampled 2 weeks after arrival, pooled and stored at -70°C in small aliquots for immunisation and for detection of microbial flora (MF) specific antibodies.

Table 1: Outline i.p. injection of B6 donors with their own microflora (SELF-MF) or C3 recipient MF (RECIP-MF) before BMT

		Days before BMT		
Group	(donors)	38♥	10♥	
A	(2xsaline)	Saline♠	Saline	
B	(1xSELF-MF)	Saline	SELF-MF	
C	(2xSELF-MF)	SELF-MF	SELF-MF	→C3H/He♣
D	(1xRECIP-MF)	Saline	RECIP-MF	(10^7 BM-cells)
E	(2xRECIP-MF)	RECIP-MF	RECIP-MF	

♥) Injection dose: 0.5 ml of 1:50 suspension of (washed) faeces holding approximately $5x10^9$ m.o./ml.

♠) Saline = Pyrogen free saline.

♣) C3H/He recipients received 9 Gy total body irradiation (speed: 0.45 Gy/min.) 24 hrs before BMT.

Bacteriological culturing and typing

Faeces were cultured quantitatively for *Enterobacteriaceae*, *Staphylococci*, *Enterococci* and yeasts as described previously (*Veenendaal* et al., 1988). *Enterobacteriaceae* were biotyped according the API 20E classification system (Analytab Products Ind., France). The composition of highly concentrated obligate anaerobic species in the MF of B6 and C3 mice was examined microscopically after Gram-staining of washed faeces.

MF-immunisation

B6 donors were divided into 5 groups of 10-15 mice. Each mouse was injected intra peritoneally (i.p.) according the outline given in Table 1. Briefly, mice were injected i.p. either with 0.5 ml of washed faeces or with pyrogen free saline. Washing of faeces was carried out as follows: frozen aliquots of faeces were thawed, suspended in pyrogen free saline (1:9 w/v) and centrifuged at 12g for 5'. Supernatants were centrifuged 10,000 g for 20'. The pellets were re-suspended in pyrogen free saline (5x the volume of the supernatant). This 1:50 suspension of washed faeces contained approximately $5x10^9$ bacteria per ml. This processing procedure will have killed practically all anaerobic bacteria in the suspension.

Irradiation

C3 recipients received lethal total body irradiation 24 hr. before BMT as described previously (*Veenendaal* et al., 1988). The total dose given was 9 Gy; radiation speed 0.45 Gy/min.

Bone marrow transplantation

B6 donors were exsanguinated before harvesting BM-cells. Sera were stored at -20°C. Isolation, preparation, and pooling of BM-cells was carried out as described previously (*Veenendaal* et al., 1988). For each group (Table 1) of approximately 30 C3 recipients, 10-15 B6 donors were needed. Recipients were injected intravenously in the orbital plexus with 10^7 nucleated B6 BM-cells in 0.25 ml (conc. $4x10^7$ nucleated cells/ml). During injection, the recipients received general anaesthesia by N_2O, O_2 (2:1) and fluothane 3.0%. Radiation Controls received total body irradiation only. Chimaerism was assessed on day 35 and 100 by Hb-electrophoresis as described previously (*Veenendaal* et al., 1988).

Monitoring GvHD

Between day 0 and 100 after irradiation, recipients were daily screened for clinical symptoms, mortality and histopathological changes.

Clinical symptoms

Body weights were determined individually at weekly intervals. Mice showing progressive weight loss, hunched back, ruffled fur, diarrhoea, skin lesions, and persistent dullness after stimulation were defined as moribund and killed by cervical dislocation after exsanguination via the plexus orbitalis.

Mortality

Mortality was divided into three periods according to *Rappaport* et al. (1979); 1st phase in which mice die of graft failure (day 0-10), 2nd phase in which mice die when suffering from acute GvHD (day 11-18), and a 3rd phase in which mortality occurs delayed (day 19-100). Animals that died during the first phase were excluded from this study.

Histopathology

At autopsy mice were examined macroscopically for aplasia of the thymus. Histology was carried out on spleen,

thymus (if present), skin, liver, colon, small intestine, and lungs from moribund and surviving mice with and without symptoms. Organs were fixed in Zenker's solution, stained with H&E or Brachet (methylgreen-peponin) and histologically examined for GvHD features as described by *Hamilton* and *Parkman* (1983) and *Rappaport* et al. (1979).

Anti-MF immune response

In order to obtain information about possible differences in the immune response of B6 donors against SELF-MF or RECIP-MF the spleen weight index as well as immunoglobulins in serum were determined at the time of BM harvesting.

Spleen weight index (SWI)

The SWI of each donor mouse (formula C) was calculated by dividing the individual relative spleen weight (formula A) by the mean relative spleen weight of saline injected controls (formula B):

$$\text{(A):}\quad \text{Relative spleenweight} = \frac{\text{Spleenweight}}{\text{Bodyweight}}$$

$$\text{(B):}\quad \frac{\sum_{1}^{n} (\text{Relative spleenweight})}{n}$$

$$\text{(C):}\quad \text{SWI}\,(x) = \frac{\text{A}\,(x)}{\text{B (controls)}}$$

Serum immunoglobulins

Isotype serum immunoglobulin (Ig) M, IgG and IgA as well as microflora specific antibodies were detected by using an enzyme linked immuno sorbent assay (ELISA). Briefly, 96 wells flat bottom trays (Greiner, Nürtingen, FRG) were incubated with goat anti mouse (GAM) IgM, IgG or IgA isotype specific antisera (Sigma, St. Louis, USA) for 1.5 hr. at 37°C. The antisera were diluted (1:1000) in 0.1 M carbonate buffer (pH 9.6). Trays were coated with a suspension of washed faeces 1:80 in 50 mM citrate buffer (pH 4.6); incubation: 1 hr. 30' at 37°C, for detection of anti B6 (=SELF)-MF and anti C3 (=RECIP)-MF isotype specific antibodies. After coating and each subsequent step, the trays were emptied and washed 3x5' with washing buffer (0.01 M Tris, 0.15 M NaCl, 0.05% Tween-20). Incubation with test sera was carried out for 45' at 37°C. The test sera were two-fold serially diluted in incubation buffer (0.01 M Tris, 0.15 M NaCl, 0.05% Tween-20, 1% Bovine Serum Albumin) starting at: 1:1000 for total serum IgM and IgG levels, 1:100 for total serum IgA, and 1:10 for microflora specific antibodies. Trays were incubated for 30' at 37°C with Horseradish Peroxidase conjugated GAM-IgM, -IgG and -IgA isotype specific antibodies (Sigma, St. Louis, USA). Conjugates were diluted 1:1000 in incubation buffer. Substrate (0-phenylene diamine dihydro chloride 0.04% and ureum peroxide) was added to each well for the final step and incubated for 30'

Table 2: Analysis of pooled faeces from B6 and C3 mice

Microorganism	B6 (n=5)♥	C3 (n=5) ♥
E. coli (API: 5144552)	1.6×10^3	2.0×10^3
Prot. mirabilis (API: 0536000)	2.0×10^4	2.5×10^4
Enterococcus spp.	5.0×10^4	3.0×10^4
Staph. Aureus	1.0×10^4	1.5×10^4
Bacillus spp.♣	ND♦	ND
Obligate anaerobic bacteria♠	10^{11}	10^{11}

♥) Data represent concentrations in m.o./g. faeces.
♣) Qualitative aerobic culturing only.
♠) Microscopic analysis by eye revealed a morphological difference between highly concentrated obligate anaerobic fractions in B6 and C3 faeces.
♦) ND: not determined.

at room temperature. The substrate conversion was stopped by adding 4N H_2SO_4 and the extinction measured photometrically at OD492 on an ELISA reader (Titertek Multiskan). In each tray a pooled reference serum was measured to verify the ELISA procedure.

Statistical analysis.

Statistical evaluation of the significance of differences in survival rates between group was carried out by Kaplan-Meier analysis. Statistical evaluation of the occurrences of clinical manifestations of GvHD was carried out by the chi-square or Fischer-exact test with Bonferroni correction. Differences in the bacterial concentrations were evaluated by the Mann-Whitney rank sum test. Differences in total and anti-MF specific serum antibody levels, and relative spleen weight index were evaluated by Newman-Keuls analysis of variance with Bonferroni correction for multiple comparison. Significance levels were taken at $p<0.05$.

RESULTS

Faecal analysis

Culturing

Results of quantitative and qualitative culturing of B6 and C3 faeces are shown in Table 2. No differences were found in the concentrations or biotypes of *Enterobacteriaceae*; e.g. *Escherichia coli* (*E. coli*) API20E: 5144552 and *Proteus mirabilis* API20E: 0536000, and *Staphylococci* and *Enterococci*. Yeasts were not detected. *Bacillus* spp. were detected in either faeces, but not quantified nor identified.

Micromorphology

Micromorphological examination of Gram-stained samples of washed faeces showed a clear difference in the composition of highly concentrated, predominantly obligate anaerobic, bacteria in the faeces of B6 and C3 mice. Gram-positive and Gram-negative fusiform and long shaped bacteria were predominant in B6-MF, whereas Gram-negative small rods and coccoid bacteria predominated in C3-MF.

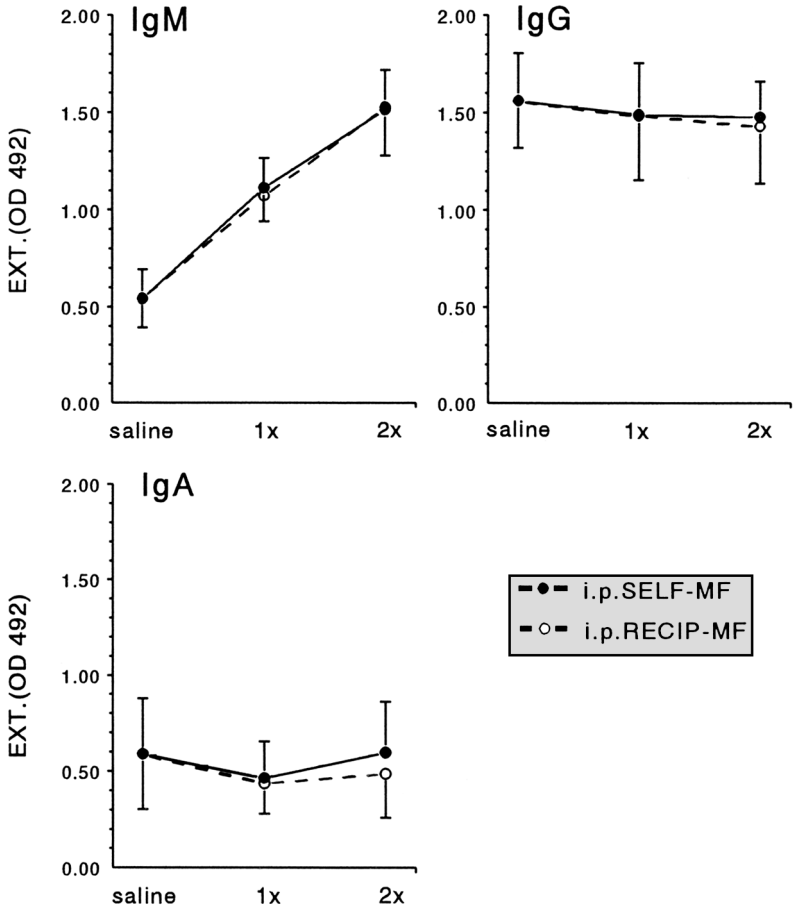

Figure 1: Isotype serum immunoglobulins in C57Bl/6J (B6) donors i.p. injected with saline (n=10), washed faecal flora of their own (SELF-MF) or washed faecal flora of C3H/He recipients (RECIP-MF). I.p. injection was performed 10 days before serum sampling for single injection; 1xSELF-MF (n=12), 1xRECIP-MF (n=16). Repeated injection was performed on day -38 and -10; 2xSELF-MF (n=12), 2xRECIP-MF (n=12). IgM antibodies increased significantly ($p<0.05$) after single injection and raised further ($p<0.05$) after repeated injection.

Clinical response to i.p. MF injection

Mice did not show clinical signs of disease upon i.p. injection of I-MF. This could be due to the fact that the majority of bacteria was dead due to contact with O_2 during the processing of the MF-suspension for injection.

Immune reactions

Isotype serum antibodies

Isotype IgG, IgM, and IgA levels in serum of donors were determined in 6 individual sera per group. Each serum was tested *in duplo*. The antibody levels are presented as mean extinction levels at OD492 for single serum dilutions (Figure 1). The following single serum dilutions were used: 1:2000 for IgM, 1:16,000 for IgG, and 1:400 for IgA. At these dilutions the highest differences were found between the various groups (data not shown). IgM antibodies in donors increased significantly ($p<0.05$) after single (group B,D) and repeated (group C,E) injection with

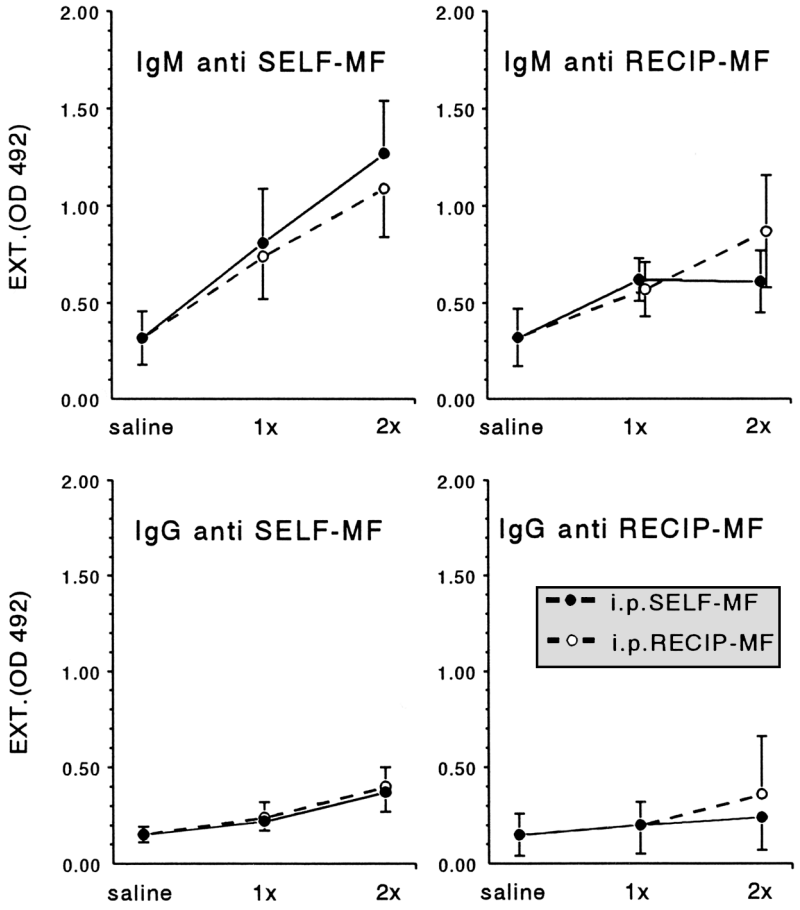

Figure 2: Anti-microflora antibodies in serum of C57Bl/6J (B6) donors i.p. injected with saline (n=10), washed faecal flora of their own (SELF-MF) or washed faecal flora of (C3H/He) recipients (RECIP-MF). I.p. injection was performed 10 days before serum sampling for single injection; 1xSELF-MF (n=12), 1xRECIP-MF (n=16) and 38 and 10 days before sampling in case of repeated injection; 2xSELF-MF (n=12), 2xRECIP-MF (n=12).

SELF-MF as well as with RECIP-MF compared to saline injected (group A) donors. No significant differences were observed for IgG or for IgA in each donor group.

Anti-MF serum antibodies

IgM, IgG, and IgA anti SELF-MF and anti RECIP-MF specific antibody levels are presented as mean extinction levels at OD492 for single serum dilutions (Figure 2). The serum dilutions were 1:20 for IgM and 1:10 for IgG. IgA anti-MF serum levels were not detectable at the 1:10 serum dilution i.e. stayed below the extinction rate of 0.2 at OD492. IgM anti-SELF-MF and IgM anti-RECIP-MF antibodies both increased equally significant ($p<0.05$) in all 1xSELF-MF (group B) and 1xRECIP-MF (group D) injected donors compared to saline injected controls (group A) (Figure 2). IgM anti-SELF-MF further increased significantly in donors twice injected with SELF-MF (group C) as well as with

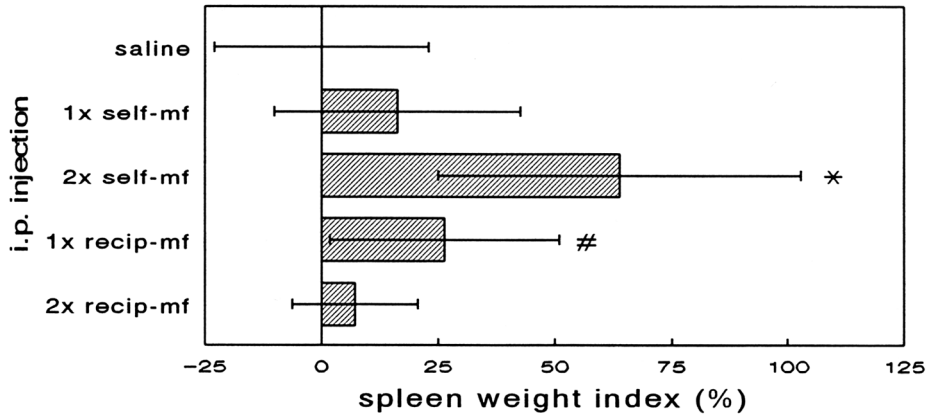

Figure 3: Spleen weight index in C57Bl/6J donors i.p. injected either with saline (n=7), 1xSELF-MF (n=13), 1xRECIP-MF (n=5), 2xSELF-MF (n=12), and 2xRECIP-MF (n=10). I.p. injection was performed on day -10 before BMT for single injection and day -38 and -10 for repeated injection. (*) = $p<0.01$ compared to all other groups; (#) = $p<0.05$ compared to saline injected animals.

RECIP-MF (group E). IgM anti-RECIP-MF antibodies increased further in 2xRECIP-MF injected donors (group E) but not in 2xSELF-MF donors (group C) (Figure 2). No significant differences were found in the IgG anti-SELF-MF levels in either donor group. However, IgG anti RECIP-MF antibodies significantly increased ($p<0.05$) in 2xRECIP-MF injected B6-donors compared to saline injected controls (Figure 2).

Spleen weight

Mean spleen weight index of each of the B6-donor groups is shown in Figure 3. The spleen weight index was found to be increased ($p<0.01$) in 2xSELF-MF injected donors compared to all other groups. The spleen weight index in 1xRECIP-MF injected donors was found significantly higher ($p<0.05$) compared to saline injected controls.

Chimaerism

Mice tested for chimaerism carried 100% the Hb pattern of B6 donors on day 35 after transplantation.

Clinical symptoms

Early mortality and symptoms

Irradiated control C3 mice, not engrafted with BM, all died by day 10; mean survival time 7.5±1.5; range 5-10 days. 'Engrafted' mice that died during this period were recorded as "graft failures" and were excluded from this study. By day 11 the graft will have taken and starts functioning. "Early mortality" was defined as mortality between day 11 and 18 after engraftment according to *Rappaport* et al. (1979); results shown in Table 3. Mortality during this period ranged from 8% (2/27) in group C (C3 recipients engrafted with BM from 2xSELF-MF injected donors) to 0% in group A (n=26) and B (n=35). There was no significant difference between the groups.

Weight loss was found in all mice that died before day 18. In surviving C3 recipients, however, early weight loss was found to be the strongest in 17/26 (65%) of the animals engrafted with BM from 2xRECIP-MF injected donors (group E). This was significantly the highest ($p=0.01$) compared to animals in group A, B and C (Table 3). Except

Table 3: 'Early' mortality and weight loss (>10%) in C3 recipients engrafted with bone marrow from MF injected B6 donors; 11-18 days after BMT

Group (donor)♥	'Early' mortality (%) 11-18 days after BMT	Weight loss in survivors 11-18 days after BMT
A (saline)	-/26 (-)	6/26 (23)
B (1xSELF-MF)	-/35 (-)	10/35 (29)
C (2xSELF-MF)	2/27 (8)	9/25 (36)
D (1xRECIP-MF)	1/33 (3)	14/32 (44)
E (2xRECIP-MF)	1/27 (4)	17/26 (65) ♣

♥) C57Bl/6J donors i.p. injected once (day -10) or twice (day -38 and -10) with washed faeces from their own (SELF-MF) or C3 recipients (RECIP-MF). Control donors were injected with saline.

♣) p=0.01 compared to group A, B, and C (Chi-square test with Bonferroni correction).

for one animal, which had been engrafted with BM from 2xSELF-MF injected B6-donors and died on day 16, no diarrhoea was seen before day 18 after transplantation in any of the C3 recipients.

'Late' mortality and symptoms at 18-100 days after BMT

Survival rates, weight loss and the occurrence of diarrhoea between day 18 and 100 after BMT are shown in Figure 4 and Tables 4 and 5 respectively. Mortality after day 18 was found the highest; 15/35 (43%) ($p<0.01$) in C3 recipients engrafted with BM from 1xSELF-MF injected donors (group B) (Figure 4). Non-surviving animals in this group also died earlier (mean 45.9 days, range 23-91) compared to mice suffering from lethal GvHD in other groups (overall mean: 65.0 days; range: 43- 100 days).

Table 4: Weight loss (>10%) 19-100 days after BMT in surviving and non-surviving C3 recipients engrafted with bone marrow from microflora injected B6 mice

		Weight loss (>10%) 19-100 days after BMT		
Group (donor)♥	Number of recipients (at day 19)	Non-survivors	Survivors	Total (%)
A (saline)	26	3	6	9 (35)
B (1xSELF-MF)	35	13♣	6	19 (54)♠
C (2xSELF-MF)	25	1	2	3 (12)
D (1xRECIP-MF)	32	3	4	7 (22)
E (2xRECIP-MF)	26	4♣	10	14 (52)♠

♥) C57Bl/6J donors i.p. injected once (day -10) or twice (day -38 and -10) with washed faeces from their own (SELF-MF) or C3 recipients (RECIP-MF). Control donors were injected with saline.

♠) p≤0.02 compared to group A, C, and D (Chi-square test with Bonferroni correction).

♣) p=0.03 between non-survivors and survivors in group B and E (Fisher-exact test).

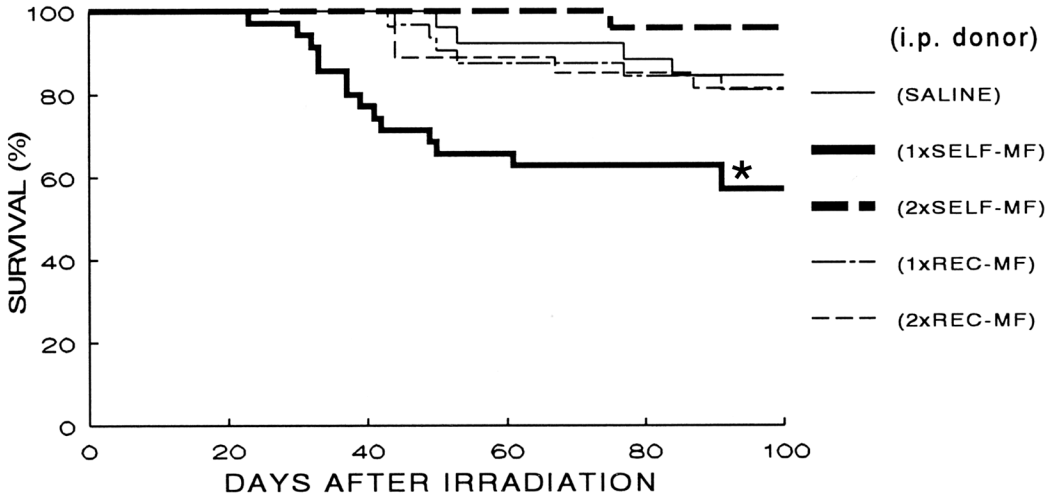

Figure 4: Survival rates of lethally irradiated (9 Gy) C3H/He (H-2k) recipients engrafted with 10^7 nucleated BM cells from C57Bl/6J (H-2b) donors 19-100 days after BMT. Donors had been pre-treated by i.p. injected with saline, washed faecal flora of their B6-strain (SELF-MF) or C3 recipients (RECIP-MF). I.p. injection was performed on day -10 for single injection and day -38 and -10 for repeated injection. The number of recipients at day 19 were: group A (2xsaline) n=26, group B (1xSELF-MF) n=35, group C (2xSELF-MF) n=25, group D (1xRECIP-MF) n=32, and group E (2xRECIP-MF) n=26. (*) = $p<0.01$ compared to all other groups (Kaplan-Meier analysis).

The overall occurrence of weight loss after day 18 was found to be the highest (p=0.003) in group B (1xSELF-MF donors) and in group E (p=0.02) (2xRECIP-MF donors) compared to recipients in the other groups (Table 4). With regard to survivors and non-survivors, however, weight loss appeared to be significantly the highest (p=0.03) in non-survivors in group B (1xSELF-MF injected donors) and significantly the lowest in non-survivors in group E (2xRECIP-MF injected donors).

Table 5: Diarrhoea 19-100 days after BMT in surviving and non-surviving C3 recipients engrafted with bone marrow from microflora injected B6 donors

Group (donor)♥	Number of recipients (at day 18)	Diarrhoea 19-100 days after BMT		
		Non-survivors	Survivors	Total (%)
A (saline)	26	3	2	5 (20)
B (1xSELF-MF)	35	5	4	9 (26)
C (2xSELF-MF)	25	1	-	1 (4)
D (1xRECIP-MF)	32	4	1	5 (16)
E (2xRECIP-MF)	26	4	2	6 (22)

♥) C57Bl/6J donors i.p. injected once (day -10) or twice (day -38 and -10) with washed faeces from their own (SELF-MF) or C3 recipients (RECIP-MF). Control donors were injected with saline.

In contrast to the early period after BMT (<day 18), diarrhoea was observed regularly in mice that died after day 18. The incidence of diarrhoea in the non-survivors varied between 33% and 100%. Like mortality, the overall occurrence of diarrhoea was found at the highest but not significant level in mice of group B (1xSELF-MF donors) (Table 5).

Arthritis

Around day 80 five out of all surviving mice that also showed skin changes as dyskeratosis of the ears and tail, started to display redness and swelling of the paws. These changes were self-limiting and healed spontaneously by day 100. There was no preference for arthritis to occur in any of the groups.

Histological examination

Lungs

Histological examination of lung tissue showed moderate to severe carnification in all mice. Only in moribund mice infiltrates with granulocytes were seen, whereas none of the tissues examined showed infiltrates with lymphocytes.

Thymus

All moribund mice that died after day 18 showed total aplasia of the thymus. Histologically, regeneration of the thymus was only found in mice that displayed clinical symptoms but survived until day 100. Animals surviving 100 days without any clinical symptom had a normal thymus.

Spleen

Histological examination of spleen sections of C3 recipients that died after day 18 revealed absence of follicle centres as well as the absence or a strongly decreased number of plasma cells. Evidence of haematopoiesis, however, was still found in these mice as granulocytes and megakaryocytes predominated in the splenic red pulpa.

Intestines

Changes in the intestines were only found in moribund mice that suffered from diarrhoea. A decrease was found in the number of goblet cells as well as a decrease of the length of the villi. No submucosal lymphocytic infiltrates were seen.

Kidneys

No histological changes were found in the kidneys of the animals examined.

DISCUSSION

This study provides additional evidence to our previous hypothesis (*Veenendaal* et al., 1988) that the incidence of intestinal microbial flora (I-MF) associated late onset GvHD (LO-GvHD) in mice (previously called secondary disease) is affected by both the I-MF of the recipient and the donor. LO-GvHD in C3 recipients was found to be affected when B6 donors had been i.p. injected with the autochthonous B6-strain I-MF (=SELF-MF) either once (10 days) or twice (38 and 10 days) before BM harvesting. On the one hand LO-GvHD mortality increased to 43% (control 15%) when using BM from once SELF-MF injected donors, whereas on the other hand LO-GvHD mortality decreased to 4% when twice SELF-MF injected donors were used. However, unlike our previous supposition, no correlation was found between LO-GvHD and the serological response by the donor against allochthonous

RECIP-MF or SELF-MF. Thus we conclude that anti-MF antibody levels alone are of no predictive value for I-MF associated LO-GvHD in mice. However, the presence or absence of anti-MF antibodies may still be a factor which indicates immune reactivity or immune suppression respectively.

An explanation for the donor I-MF to influence LO-GvHD is given by the assumption that the composition of the BM (graft) is determined by the composition of I-MF in the donor. Experiments in mice have shown that antibiotic modulation of I-MF not only affects haemopoiesis but also LO-GvHD when donor mice are treated as such (*Goris* et al., 1985, 1986a; *Heidt* et al., 1989; *Veenendaal* et al., 1988). A difference in the immunoregulation of autochthonous SELF-MF versus non-indigenous or allochthonous RECIP-MF may best be illustrated in B6 donors twice injected with either flora. IgM anti-RECIP-MF antibodies were found at a higher level in 2xRECIP-MF compared to 2x SELF-MF injected mice (Figure 2). On the other hand the mean spleen weight index was found significantly the highest in 2x SELF-MF injected donors, whereas it returned to a normal level in 2xRECIP-MF injected B6 mice (Figure 3). These data show that B6 mice respond immunologically different to allochthonous C3 recipient-MF (RECIP-MF) compared to their own autochthonous B6-MF (SELF-MF).

The bacterial subset in either SELF-MF or RECIP-MF to which the B6 immune system reacts differently, apparently should not be searched for within the low concentrated facultative anaerobes, like the *Enterobacteriaceae*, as this fraction was found to be similar in faeces of both mouse strains. This confirms previous reports on the minor role of *Enterobacteriaceae* in the pathogenesis of LO-GvHD (*Heidt* et al., 1989; *Veenendaal* et al., 1988). Instead, the highly concentrated fraction of the intestinal MF, predominantly containing obligate anaerobic bacteria, may be most important in this respect as this fraction was found to be morphologically different between the B6 and C3 mice used in this study. Within the fraction of highly concentrated obligate anaerobic bacteria, "non-immunogenic" and/or tolerogenic indigenous species may be of utmost importance in LO-GvHD, since any correlation was lacking between the levels of antibodies in serum mounted by B6 donors against immunogenic bacteria in either SELF-MF or RECIP-MF on the one hand and the induction of LO-GvHD in C3 recipients on the other.

In euthymic mice, there is evidence that the majority of the highly concentrated obligate anaerobic bacteria in the GI-tract are non-immunogenic. By using an indirect immuno-fluorescence technique we previously found that B6 as well as C3 mice, as were used in this study, both mount a detectable serum antibody response to only 10-15% of highly concentrated bacteria in their I-MF (unpublished results). B-cell tolerance has been described for autochthonous (host related) bacterial species as well (*Berg* and *Savage*, 1975; *Foo* and *Lee*, 1972), suggesting that these bacteria have become part of self and therefore are favoured to colonise the GI-tract of that particular host. The kind of defence against these indigenous species may be limited to granulocytes, macrophages and dendritic cells, i.e. the innate defence system.

The significant increase of the spleen weight index in 2xSELF-MF i.p. injected donors, may point at maximal stimulation of reticular endothelial cells, i.e. dendritic cells and macrophages, instead of lymphoid cells. This postulation is supported by the fact that only an increasing IgM response was found whereas an isotype switch to IgG or

IgA remained absent indicating the absence of T-cell mediated germinal centre reactions. However, unfortunately no histology was carried out on donor spleens in order to establish this postulation.

Stimulation of innate defence cells, i.e. granulocytes, macrophages, and dendritic cells together with the absence of a secondary response at the donor site still does not explain the differences in LO-GvHD in recipients engrafted with BM from single versus twice SELF-MF injected donors. Single i.p. injection with SELF-MF 10 days before BM harvesting in a way increased the LO-GvHD inducing capacity of the BM. However, this effect was fully reversed by repeated injection with SELF-MF. This indicates that the BM changes, which occurred after single i.p. injection with SELF-MF and aggravated GvHD, were only temporary. The first injection possibly created a new level of balance between I-MF and the immune "suppressor" system. Apparently, this level remained unchanged after i.p. challenge 28 days later.

LO-GvHD in this study was characterised by total aplasia of the thymus and depletion of germinal centres in the spleen. These features are similar to previous reports (*Hamilton* and *Parkman*, 1983; *Rappaport* et al., 1979; *Veenendaal* et al., 1988). Total lymphoid aplasia is responsible for T- and B-cell unresponsiveness during GvHD (*Hamilton* and *Parkman*, 1983; *Wall* et al., 1988). These findings correlated well with mortality, despite the persistent presence of granulocytes and macrophages in (the spleens of) mice suffering from lethal LO-GvHD. How LO-GvHD features are related to the temporary BM changes induced by SELF-MF remains subject of further study.

In conclusion, we postulate that the key for MF-associated LO-GvHD should be searched for within the non-immunogenic part of highly concentrated obligate anaerobic bacteria in the intestinal tract of the recipient. The degree at which engrafted BM cells in some way interact with this fraction and either cause or prevent LO-GvHD greatly depends on the level of 'activation' of BM cells mediated by the donor's own I-MF. The thymus becomes functionally damaged in the allo-transplanted recipient by allo-cells which have stimulated shortly before BMT. The "suppressor" system, which normally controls the response to SELF-MF, plays an important role in this process. If this is true, LO-GvHD in some part may be caused by I-MF antigens in the recipient which are shared by the donor, i.e. the degree of homology between donor and recipient-MF. Further study will be needed to elucidate this postulation.

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