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Split tolerance in chimeric GVH mice

Ch. Baron
Ph. Lang
V. Bierre
G. Rostoker
B. Weil

Ch. Baron · V. Bierre · G. Rostoker
B. Weil
Department of Nephrology,
Hôpital Henri Mondor, Creteil, France

Ph. Lang (✉)
INSERM U139, Hôpital Henri Mondor,
Creteil 94010, France

Abstract The i.v. inoculation of parental spleen cells into unirradiated adult F1 hybrid mice results in a graft-versus-host reaction (GVHR). In the strain combination B10D2 → (B10.BRx B10.D2) F1, this reaction is associated with thymic injury and transient but profound cellular immune deficiency. We further analysed the immune status of these mice 60 days after GVHR induction. Phenotypic studies of spleen cells showed that these mice were repopulated with parental lymphocytes resulting in a high degree of chimerism (85%). At this time, the mice looked healthy and recovered a normal cytotoxic T cell response (CTL) against allogeneic cells. GVH chimeric splenocytes were unresponsive against F1 hybrid cells in mixed lymphocyte culture (MLC), but exhibited anti-F1 CTL reactivity. We also analysed the anti-F1 reactivity of these mice in vivo. GVH chimeric splenocytes were unable to induce GVHR after injection into a new F1 hybrid and F1 GVH mice specifically rejected F1 bone marrow (BM) cells after lethal irradiation. Grafting a neonatal parental thymus prevented the rejection of F1 BM cells and restored CTL alloreactivity. It is concluded that the chimeric state induced by GVHR is associated with a split tolerance and that a radiosensitive thymic-dependent mechanism is involved in maintaining self-tolerance in these mice.

Key words GVHR · Tolerance
Thymus

Introduction

The injection of parental immunocompetent lymphoid cells into unirradiated adult F1 hybrid mice results in a graft-versus-host reaction (GVHR) which may involve the induction of a cellular and humoral immunodeficiency that is antigen non-specific [1]. The mice recovered normal CTL function 2 months after GVHR induction. The mechanism responsible for this functional recovery might involve repopulating donor T cells [2] as suggested by the high level of haematopoietic chimerism in these GVH mice [3]. This parental repopulation impedes the analysis of the maturation of the recipient T cells in a GVH environment. In view of the previously described thymic injury [4] and the inability to reconstitute these irradiated F1 GVH mice with F1 bone marrow (BM) cells [2], we investigated the role of the thymus in the restoration of self tolerance in these irradiated F1 GVH mice.

Materials and methods

Animals

B10D2, B10BR, (B10D2 × B10BR) F1 (BR × D2F1), C57BL/10 male mice 8–12 weeks of age were purchased from CNRS CSEAL laboratory (Orleans la source, France).

Tissue culture medium

Tissue culture medium (TCM) consisted of RPMI1640 (Gibco) supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids, 0.05 mM 2-mercaptoethanol (Gibco) and 20 mM HEPES, penicillin-streptomycin (Gibco) and 10% fetal calf serum (Seralab).

Preparation of spleen cells

Briefly, spleens were removed aseptically and processed into single-cell suspensions in Hank's balanced salt solution by mechanical disruption. Spleen cells were filtered, washed and resuspended in TCM.

Induction of GVHR

GVHR was induced by i.v. inoculation of 5×10^7 B10D2 spleen cells into unirradiated (BR × D2) F1 mice.

BM transplantation

Mice were lethally irradiated (9 Gy whole-body irradiation) 60 days after GVHR induction and inoculated i.v. with 3×10^7 normal (BR × D2) F1 or B10D2 parental cells.

Thymus grafting

Two neonatal thymus lobes were taken from 1-day-old DBA/2 mice (H2d) and transplanted under the kidney capsule of 60-day-old GVH mice. Thymus grafting was performed on the day following irradiation and reconstitution with BM cells.

Characterization of chimeras by flow cytometry

Recipients were characterized for chimerism using flow cytometry (Coulter, EPICS XL) to determine the percentage of splenocytes bearing (BR × D2) F1 MHC class I (H-2K^k). Briefly, spleens were prepared as previously described, red blood cells were ACK-lysed (ammonium chloride potassium carbonate lysing buffer) and the remaining cells were stained with an FITC-labelled anti-H-2K^k monoclonal antibody (clone 36-7-5, Pharmingen)

In vitro generation of CTL and assay for T-cell mediated lympholysis

Briefly, 5×10^6 spleen cells in TCM were stimulated with 10^6 irradiated allogeneic cells in 24-well tissue culture plates. After 5 days of culture, effector cells were assayed in a 4-h ^{51}Cr release assay against conA-stimulated splenic blasts as previously described [1].

Proliferation assay

Responder spleen cells (2×10^5) and irradiated spleen stimulator cells (2×10^5) were suspended in 200 µl TCM in a 96-well flat-bottomed microtissue culture plate (Falcon). The cultures were incubated at 37°C in humidified air containing 5% CO_2 for 4 days, then 1 µCi of ^{3}H-thymidine was added to each well for a period of 18 h. The samples were harvested onto a glass fibre filter and ^{3}H-thymidine uptake was measured by β scintillation counting (Beckman counter). Results are expressed as mean count disintegrations per minute for triplicate samples.

Assay for stem cell function

A ^{125}I iodo-2′-deoxyuridine (IUdr) uptake assay was carried out as previosly described [5], GVH marrow-injected recipients were injected i.p. with 5-fluorodeoxyuridine (FUdR) 5 days after marrow transplantation, and 1 h later were injected wit 0.5 µCi ^{125}IUdR i.p. The recipients were killed 5 h after IUdR injection and their spleen was removed and counted in a γ-spectrometer to assess the relative amount of IUdR incorporated in the donor-derived proliferating marrow cells. The percentage of isotope incorporation in the spleen of the marrow recipients was calculated using the formula:

$$\frac{\text{cpm recipient spleen}}{\text{cpm of injected amount}} \times 100$$

Results

Chimeric haematopoietic system of GVH mice

The splenic population was assayed 60 days after induction of GVHR for donor/host chimerism by Fluoro Cytometry analysis. Donor cells, that is cell failing to stain with antibody directed against H2K^k, comprised 80% of the total spleen.

Split tolerance of chimeric mice

GVH splenocytes were tested in vitro for anti-F1 reactivity. Figure 1 shows that in an MLR assay, 60-day F1 GVH splenocytes did not proliferate against normal F1 cells, but displayed a normal third-party alloreactivity (data not shown). In contrast, anti-F1 CTL were detected in these F1 GVH animals (Fig. 2).

This discrepancy led us to further analyse in vivo the reactivity of these F1 GVH animals. We used two approaches. First, we injected F1 GVH splenocytes into unirradiated F1 mice, and second we sought to determine whether F1 BM cells could grow in irradiated F1 GVH mice.

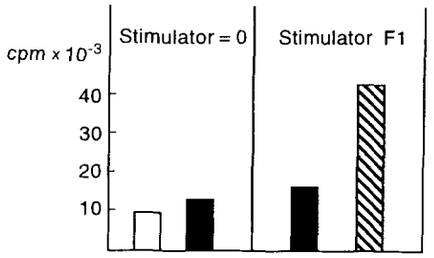

Fig. 1 F1GVH splenocytes do not proliferate against F1 target cells. Sixty days after GVH induction, 2×10^5 F1 GVH splenocytes (■) and 2×10^5 B10D2 splenocytes (▨) were cultured with 2×10^5 irradiated F1 cells (*right*). Spontaneous ^3H incorporation of F1 (□) and F1GVH (■) splenocytes alone is shown (*left*)

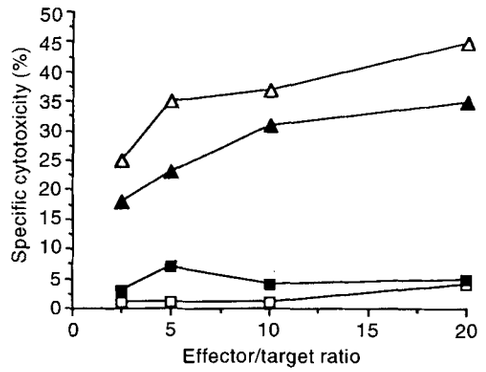

Fig. 2 Day 60 anti-F1 CTL reactivity in F1 GVH mice. In vitro generated CTL response to F1 splenocytes by F1GVH splenocytes (▲) and by normal F1 cells (■). CTL response of GVH splenocytes against B10 cells (△) and F1 splenocytes without stimulator cells (□)

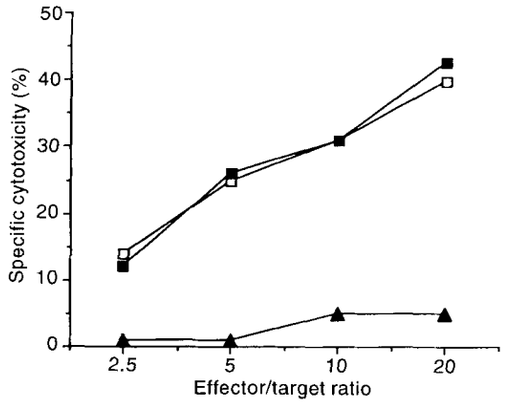

Fig. 3 F1 GVH splenocytes are unable to induce a GVH in normal F1 mice. Splenocytes (3×10^7) from 60-day F1 GVH mice were injected into (BR × D2) F1 mice (□) and CTL reactivity against B10 spleen cells was assessed 2 weeks later. Control mice were F1 mice injected with 3×10^7 B10D2 spleen cells (▲) and F1 mice injected with 3×10^7 F1 spleen cells (■) tested against B10 target spleen cells

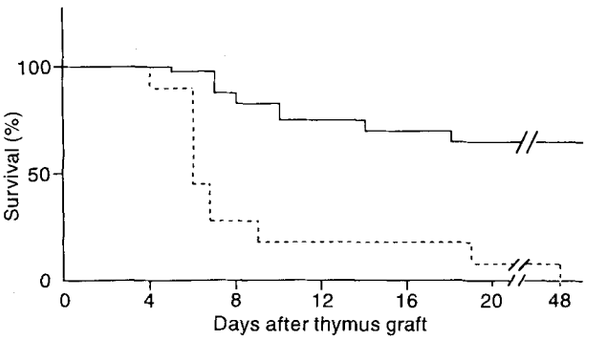

Fig. 4 Thymus graft prevents F1 bone marrow rejection. Sixty days after GVHR induction, (BR × D2) F1 mice were lethally irradiated and injected i.v. with 30×10^6 F1 bone marrow cells. One group of mice was grafted under the kidney capsule with parental thymic lobes (——) and the other group (– – –) was subjected to a sham operation

Responsiveness of F1GVH splenocytes in vivo to (BR × D1) F1 hybrid cells

In order to investigate the F1 reactivity of the chimeric cells, 30×10^6 splenocytes were injected i.v. into (BR × D2) F1 mice and the generation of allo-CTL was assessed 2 weeks later. The results shown in Fig. 3 indicate that F1 GVH splenocytes were unable to induce a GVHR in (BR × D2) F1 recipients.

Growth of F1 BM cells in F1 GVH recipients

In order to analyse further the mechanisms underlying the state of tolerance in these mixed chimeric animals, 60-day GVH mice were lethally irradiated (900 rad) and inoculated i.v. with either 3×10^7 F1 BM cells or 3×10^7 B10D2 BM cells. Figure 4 shows that mice grafted with F1 BM cells were not reconstituted. Most of the mice died within 2 weeks. In contast, grafting these mice with B10D2 BM cells resulted in permanent survival (data not shown). We evaluated the stem cells proliferative capacity in the repopulating spleens of the GVH mice using an in vivo (^{125}I iodo-2′-deoxyuridine) assay. Injection of 2×10^6 F1 BM cells in lethally irradiated F1 GVH mice gave 0.05% IUdr uptake, whereas injection of 2×10^6 parental B10D2 BM cells generated 0.5% IUdr incorporation.

Effect of thymus grafting on syngeneic BM rejection

The above findings suggested that irradiation abrogated a regulatory mechanism controlling the rejection of F1 BM

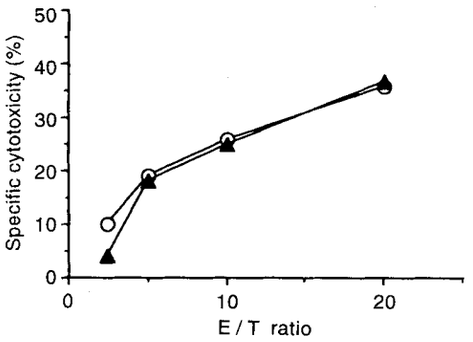

Fig. 5 Restoration of CTL alloreactivity of (BR × D2) F1 GVH mice after parental thymus grafting. Sixty days after GVHR induction, (BR × D2) F1 mice were lethally irradiated, reconstituted i.v. with 30×10^6 F1 bone marrow cells and parental thymic lobes were grafted under the kidney capsule. CTL activity was tested 2 months later against B10 cells (▲). CTL activity of control lethally irradiated (BR × D2) F1 mice injected with 30×10^6 F1 bone marrow cells (○) was also tested

cells. To investigate the thymus dependency of this radiosensitive mechanism (BR × D2) F1 mice were lethally irradiated and injected i.v. with 30×10^6 F1 BM cells 60 days after GVHR induction. One group of mice was grafted under the kidney capsule with parental thymic lobes and the other group was subjected to a sham operation. Results sown in Fig. 4 indicate that parental thymus grafting prevented the rejection of F1 BM cells and Fig. 5, demonstrates that these thymus-grafted animals were fully immunocompetent as assessed by their ability to generate CTL against allogeneic targets.

Discussion

Shortly after induction of GVHR, a profound immune deficiency develops, but after 6–8 weeks GVH mice recover their immune function as evidenced by the ability of the animals to generate allogeneic CTL responses [4]. The basis for the immune reconstitution might be the repopulation of GVH mice by the donor-derived T cells transferred in the GVH-inducing inoculum [2]. Indeed, phenotypic analysis of the splenic cell population on day 60 following GVHR showed. That most of the cells were of parental origin. This mixed chimerism, previously reported in other strain combinations [3], is associated with an apparent state of tolerance possibly related to a veto mechanism resulting in anergy or apoptosis [6]. GVH mice looked healthy. MLR and GVH reactivity against F1 cells could not be detected whereas they were fully immunocompetent against a third-party alloantigen. However, these animals displayed a CTL response against F1 cells, suggesting the existence tolerance can be associated with a significant level of anti-host CTL activity despite a low MLR response [7]. Both CD4+ and CD8+ cells are involved in the induction of GVH across a disparty in class I and class II antigens, but the present findings suggest that tolerance is only expressed in $CD4^+$ cells. This finding extends the observation of Fukuzawa et al. [8] showing in a milder GVH combination that the defective TNP self-response was selective for the CD4+ cell population. Because we have previously found no correlation between GVH and CTL activity in response to allogeneic cells, it is not unexpected that these GVH mice do not exhibit signs of GVH disease [9]. It remains unclear whether the observed cytotoxicity is the consequence of an allogeneic reaction or is the result of an autocytotoxic mechanism [10].

This split tolerance observed in unirradiated animals is also expressed in lethally irradiated F1 GVH mice. These mice reject F1 BM cells but not the parental cells used to induce the GVHR. The mechanisms underlying the rejection of allogeneic BM cells are complex and need further clarification. Various types of cells might be involved: NK cells [11], natural suppressor cells [12], radioresistant CTL [13] and $CD3^+/\alpha\beta^+$/NK 1.1^+ cells [11]. The specificity of the rejection, i.e. rejection of F1 cells but not parental B10 D2 cells, would suggest that the rejection is mediated by radioresistant CTL. However, F1 BM cells could also be rejected by an antibody-dependent cell-mediated type reaction [4] and recently the presence of allogeneic-specific NK cells has been reported [15]. Whatever the type of rejecting cells, the target antigen either is only present on the parental strain not used for inducing the GVHR, or is specific for hybrid cells and could, for example, result from the presentation of peptides from one parent within the class-I MHC of the other parental strain.

Autoreactive radioresistant CTL might also be involved in the rejection process. This model would then be related to the autoreactivity observed in syngeneic GVH rats following irradiation and cyclosporine treatment [16]. It is quite conceivable that the double insult GVH and irradiation could generate autoreactive cells as already described in humans developing syngeneic GVH [17]. In both models, major thymic abnormalities have been reported [4, 16], and it has been demonstrated in rats that thymic injury is causally related to syngeneic GVHD [16]. In our model, the role of the thymus in the imbalance between the production of autoreactive and autoregulatory lymphoid cells is further demonstrated by the

abolition of F1 BM rejection and full CTL restoration following thymus grafting of GVH-irradiated mice. Several mechanisms might be evoked for explaining this thymic-dependent radiosensitive mechanism and the dysregulation of the system in F1 BM reconstituted irradiated GVH mice. As previously reported, veto cells might induce or maintain tolerance and this phenomenon is T-cell dependent. Similarly, it is believed that natural suppressor (NS) cells play a role in tolerance induction possibly by regulating T cells or NK cells. NS cells require activated T-cell signals to express their maximum suppressive activity [18] and one could speculate that thymus grafting after irradiation would favour their activity. A direct role of the thymus must also be considered. The thymus may generate autoregulatory T cells capable of regulating the self-reactive cells in the periphery. Previous findings have indicated that the thymocytes responsive to the alloantigens of the host actively protect the marrow graft from rejection [13]. Whether this is accomplished by augmenting stem cell proliferation or through suppression of host effector cell function remains to be elucidated.

It is concluded that the chimeric state induced by GVHR is associated with split tolerance and that a radiosensitive thymic-dependent mechanism is involved in maintaining self-tolerance in these mice.

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