Transpl Int (2004) 17: 341–350
DOI 10.1007/s00147-004-0724-5

ORIGINAL ARTICLE

Lymphadenectomy prior to rat hind limb allotransplantation prevents graft-versus-host disease in chimeric hosts

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Received: 9 April 2003
Revised: 14 November 2003
Accepted: 9 March 2004
Published online: 29 July 2004

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Abstract In previous rat studies, the use of mixed allogeneic chimerism (MAC) to induce host tolerance to hind limb allografts has resulted in severe graft-versus-host disease (GVHD). The purpose of this study was to determine if immunocompetent cells in bone marrow (BM) and/or lymph nodes (LNs) of transplanted limbs were responsible for inducing GVHD in mixed chimeric hosts. [ACI → Wistar Furth] chimeric rats received ACI hind limbs that were non-irradiated, irradiated (1050 cGy) or lymphadenectomized. Rejection, GVHD and donor chimerism was assessed. Chimeric hosts rejected none of their limbs. However, hosts of non-irradiated hind limbs succumbed to GVHD 22.4 ± 0.8 days after transplantation. In contrast, chimeras that received irradiated or lymphadenectomized ACI hind limbs showed no clinical or histological signs of GVHD at 5 months. We conclude that mixed chimeric hosts are susceptible to GVHD due to the immunocompetent cell load provided by the LNs, not the BM, of hind limb allografts.

Keywords Bone marrow · Chimerism · Composite tissue allotransplantation · Graft-versus-host disease · Lymph node · Transplantation

Abbreviations *BM*: Bone marrow · *CPM*: Counts per minute · *CTA*: Composite tissue allograft · *FACS*: Fluorescence activated cell sorter · *FITC*: Fluorescent iso-thiocyanate · *GVH*: Graft-versus-host · *GVHD*: Graft-versus-host disease · HVG: Host-versus-graft · *LN*: Lymph node · *MAC*: Mixed allogeneic chimerism · *MHC*: Major histocompatibility complex · *MLR*: Mixed lymphocyte reaction · *MoAb*: Monoclonal antibody · *PBL*: Peripheral blood lymphocytes · *SEM*: Standard error of mean · *TBI*: Total body irradiation · *TCD*: T-cell depletion/T-cell depleted · *TCR*: T-cell receptor ·

Introduction

Composite tissue allograft (CTA) procedures would provide optimal treatment for patients suffering from large tissue defects caused by trauma, tumor resection or congenital defects by providing identical tissue parts for reconstruction. In CTA procedures, multiple tissue types such as skin, subcutaneous tissue, nerve, blood vessels,

lymphatics, bone and muscle are transplanted in the form of extremities, larynx or facial tissues. The major disadvantage of these procedures is the need for immunosuppressive drugs to prevent rejection. These toxic drugs are associated with an increased occurrence of neoplasms, opportunistic infections, end-organ toxicity and do not prevent chronic graft rejection effectively [1]. Although these risks are considered to be justified in life-saving organ transplant procedures, their use in life-enhancing reconstructive CTA procedures remains controversial [2].

A promising approach to successfully eliminate the need for toxic immunosuppressive drugs is through induction of donor specific tolerance, which possibly could make composite allotransplantation the preferred treatment for many reconstructive procedures. One of the best-studied and most effective methods of inducing tolerance is through mixed allogeneic chimerism (MAC) achieved by bone marrow (BM) transplantation [3]. In spite of the promising potential of MAC, there still exist complications associated with its use that must be overcome to achieve widespread clinical application. One such complication is that chimeric hosts develop graft-versus-host disease (GVHD) after transplantation of hind limb allografts. Experimentally, non-chimeric and chimeric transplant models have been used to study GVHD. The F_1 hybrid model is the classic non-chimeric transplant model used, in which hosts develop GVHD after transplantation of lymphocyte-rich allografts.[4; 7]. Similarly, chimeric hosts have also been shown to be susceptible to GVHD after transplantation with these types of allografts [8; 11].

Tolerance induction through MAC cannot be applied clinically in CTA procedures unless this complication associated with its use is overcome. Risk factors associated with GVHD are the amount of transplanted lymphocytes, histocompatibility, host and donor age, and host environmental factors such as bacterial and viral contamination. Protocols aimed at preventing GVHD in experimental animals and in the clinical setting have focused mainly on reducing the number of donor lymphocytes within the graft. In BM transplantation, GVHD has been effectively prevented by ex-vivo T-cell depletion (TCD) and UV-irradiation [12, 13]. In small bowel transplantation, GVHD has been prevented by irradiation of grafts [5, 14], lymphadenectomy [4] or donor pretreatment with antilymphocyte serum or anti-T-cell monoclonal antibodies [15]. Foster et al. [8] speculated that intact BM in CTAs was responsible for GVHD in chimeric hosts, whereas Hewitt et al. [16] speculated that BM or other tissues, such as lymph nodes (LNs), were responsible for GVHD in F_1 hybrid hosts.

The purpose of this study was to determine in mixed chimeric hosts whether the BM and/or LNs within transplanted limbs carry a large enough lymphocyte load to cause GVHD. We tested the feasibility of surgically excising the LNs to eliminate the threat of GVHD in chimeric hosts receiving CTAs. This approach could be a better alternative to graft irradiation for prevential GVHD.

Materials and methods

A total of four groups were used. Three groups of [ACI → WF] chimeric rats received ACI hind limbs that were irradiated (1050 cGy), non-irradiated, or had all LNs removed. All animals were inspected daily for signs of rejection and GHVD. Group 4 (naïve ACI rats) was used to enumerate lymphocytes present in BM and LNs of a hind limb.

Animals

Male (5–7 week) ACI (RT1 A^b) and Wistar Furth (WF, RT1 A^u) rats weighing between 200 and 350 g were used. Animals were housed in separate cages at 24°C, with light 12 h a day and in air-flow regulated rooms. They were fed standard rat chow and given water ad libitum. All handling of animals was done in accordance with the guidelines of the Animal Care and Use Committee of the Louisville School of Medicine and with the *Guide for the Care and Use of Laboratory Animals* (Department of Health and Human Services, Publication No. [NIH] 86-23).

Groups

In groups 1, 2, 3, [ACI → WF] chimeras were prepared by irradiating host WF rats with 950 cGy of total body irradiation (TBI) and reconstitution with ACI BM (depleted of $\alpha\beta$ and $\gamma\delta$ TCR^+ T cells). Donor limb transplantation was done at least 28 days after BM reconstitution. In group 1 (controls $n=10$), host [ACI → WF] chimeras were transplanted with non-irradiated limbs from donor ACI rats that were syngeneic to the BM donor. In group 2 (controls, $n=8$), host [ACI → WF] chimeras were transplanted with irradiated (1050 cGy) limbs from donor ACI rats that were syngeneic to the BM donor. In group 3 ($n=6$), host [ACI → WF] chimeras received non-irradiated ACI limbs from which the popliteal and inguinal LNs were surgically removed prior to transplantation. In group 4 ($n=3$), six ACI limbs were harvested to calculate and characterize the cells present in the BM and LNs of one hind limb.

T-cell depletion (TCD) of BM in vitro

TCD was carried out as described elsewhere [9]. Briefly, aliquots of 200×10^6 unseparated ACI BM cells were

incubated with purified anti-$\alpha\beta$-TCR MoAb (R73; mouse IgG_1; BD PharMingen) and anti-$\gamma\delta$-TCR monoclonal antibodies (MoAb) (V65; mouse IgG_1; BD PharMingen) for 30 min at 4°C. Cells were incubated for 60 min at 4°C with immunomagnetic beads (Dynabeads M450, Dynal ASA, Oslo, Norway) at a bead/T-cell ratio of 20:1 and placed in a magnetic cell separator for 2 min to negatively select T cells. Cells were washed, counted and resuspended in Medium 199 (Life Technologies, Rockville, Md., USA) plus gentamycin at a concentration of 100×10^6 BM cells per ml.

Verification of bead depletion using flow cytometry

To confirm the adequacy of TCD, aliquots of BM cells were set aside for analysis prior to TCD, after incubation with primary MoAbs, and after TCD. Cells were incubated with either anti-$\alpha\beta$-TCR FITC (R73; mouse IgG_1; BD PharMingen), anti-$\gamma\delta$-TCR FITC (V65; mouse IgG_1; BD PharMingen) or rat-adsorbed goat anti-mouse IgG FITC (BD PharMingen) for 30 min and analyzed on a fluorescence activated cell sorter (FACS Calibur, Becton Dickinson, Belford, Mass., USA).

Preparation of mixed allogeneic chimeras [ACI → WF]

Mixed allogeneic chimeras were prepared according to previously established methods [16]. Briefly, WF hosts were conditioned with unfractionated 950 cGy of TBI. Using sterile technique, irradiated hosts were reconstituted within 4 to 6 h of TBI, with 100×10^6 ACI rat BM cells (diluted in 1 ml modified Eagle's medium) via penile vein infusion.

Characterization of chimerism after BM reconstitution

Engraftment of allogeneic BM was confirmed 4 weeks after BM reconstitution using flow cytometry to determine the percentage of peripheral blood lymphocytes (PBL) bearing ACI or WF major histocompatibility complex (MHC) Class I antigens. Whole blood aliquots of 100 µl were stained with anti-$RT1A^{abl}$ FITC (B5 LOU/cN IgM, BD PharMingen) and purified anti-$RT1A^u$ (NR3/31, rat IgG_{2a}, Serotec) MoAb for 30 min. Cells were washed and fixed in 1% paraformaldehyde. The threshold for detection of donor cells was 0.5%. Flow typing was repeated at 60 and 90 days after BM reconstitution to confirm stable chimerism before limb transplantation.

Irradiation of donor limbs

ACI donors were treated with 1050 cGy of TBI. Hind limbs were procured from these animals after the TBI and served as donor limbs.

Hind limb transplantation

Donor (ACI) animals and host [ACI → WF] chimeras were anesthetized with pentobarbital 60 mg/kg.

Donor operation

The skin was incised proximally to the mid-thigh area, the femoral artery, vein and nerve were dissected, and the individual muscle groups divided proximally. The limb was flushed for 10 min with heparinized Ringer's Lactate. The femur was divided at the mid-shaft.

Host operation

The hind limb was removed in a similar fashion as described above and the donor femur was fixed using a 2 mm Kirschner wire. Femoral vessels and nerves were anastomosed using microsurgical technique (10–0 Nylon). The muscles and tendons were approximated using 5–0 Nylon, and the skin was closed using absorbable suture (5–0 Monocryl). Automutilation was prevented by daily spraying a Chew Guard solution (Summit Hill Laboratories, Navesink, N.J., USA) on the transplanted, insensate limb for 60 days.

Surgical removal of lymph nodes

Studies were carried out to localize all LNs within the rat hind limb. Lymphatics were selectively stained by injecting 0.5–1.0 ml isosulfan blue 1% dye (Ben Venue Labs, Bedford, Ohio, USA) into the footpad, using a 30 Gauge needle. Shortly thereafter, the limb was dissected and the LNs identified. With the information from these studies, we could confidently remove all LNs from the hind limb prior to transplantation using microsurgical techniques.

Characterization of chimerism after limb transplantation

Chimeras were characterized by flow cytometry of PBL after limb transplantation at 15, 30, 60, 120 and 150 days to determine levels of donor macrochimerism.

Clinical and histopathologic assessment for rejection and GVHD

Animals were monitored daily for signs of acute rejection of the limb and for signs or symptoms of acute or chronic GVHD. Important clinical signs of rejection included edema, erythema, escharification and necrosis [17], and signs of GVHD included dermatoerythema, weight loss,

diarrhea, or general unkempt appearance [9]. Histopathologic grading for cutaneous rejection and GVHD were based on previously described criteria [9]. Skin and muscle biopsies from the limb CTA were taken at 14 days, 28 days and every month thereafter, and ear wedge skin biopsies were taken once every month. All animals were weighed daily and assessed visually for signs of rejection and GVHD. Target tissues for GVHD including tongue, ear, liver and small intestine were harvested at the end of the study, fixed in 10% buffered formalin and processed routinely for hematoxylin and eosin (H&E) staining. As previously reported, allogeneic CTA hosts manifested the most severe characteristics of GVHD in skin and tongue specimens [7]. Accordingly, these tissues were evaluated for GVHD in our experiments.

In vitro assessment of tolerance

Mixed lymphocyte reaction (MLR) assays were done at the end of the study and/or when the animals where killed. Spleens were sterile harvested, crushed, and the isolated lymphocytes were ACK-lysed, washed and resuspended in cMLR medium. Cultures were incubated at 37°C in 5% CO_2 pulsed on the fourth day with 1μCi [^{3}H] thymidine (PerkinElmer, Boston, Mass., USA), harvested on the 5th day with an automated harvester (PHD Cell Harvester, Technology, Cambridge, Mass., USA) and counted in a beta scintillation counter (Beckman, Palo Alto, Calif., USA). Results were expressed as counts per minute (CPM) + SEM.

In vivo assessment of tolerance

Three to six months after BM reconstitution, [ACI $\rightarrow$ WF] chimeras were transplanted with non-irradiated ACI limbs to assess for donor specificity of tolerance. Skin grafting was performed on non-transplanted chimeras and on chimeras of group 3 ($n=2$) to test donor specificity of tolerance. Full-thickness skin grafts (1 cm diameter) were harvested from the dorsum of ACI and (third-party) Fisher donors and placed on each recipient animal, separated by a 3-mm skin bridge. Tapes were carefully removed and grafts were scored daily for rejection.

Enumeration of cells within BM and LNs

BM was flushed from femoral and tibial bones taken from ACI rats with DMEM (Life Technologies, Rockville, Md., USA) and the inguinal and popliteal LNs were dissected and crushed between frosted slides in DMEM. Cells were counted and the total amount of BM and LN cells per limb was calculated. Samples of BM and LN cells were taken to enumerate the percentage of $\alpha\beta$ TCR$^+$ T cells and B cells. Aliquots of 1×10^6 were stained with anti-$\alpha\beta$-TCR PerCP MoAb (R73, mouse IgG_1; BD PharMingen) and anti-CD45RA PE MoAb (OX-33, mouse IgG_1; BD PharMingen) for 30 min and analyzed using FACS.

Killing criteria

Animals were killed at 150 days, which was the end point of the study. Animals with obvious signs of rejection or GVHD and/or failure to thrive were killed when these signs appeared.

Statistical analysis

Continuous variables were expressed as means ± SEM, and experimental data was evaluated for significant differences using analysis of variance (ANOVA) and the post-hoc Tukey's test. Differences were considered to be significant if $P<0.05$.

Results

FACS analysis was performed before transplantation to detect the percentage of PBL bearing ACI or WF MHC Class I antigens. All irradiated WF hosts reconstituted with TCD donor ACI rat BM cells demonstrated high levels of chimerism (85±3%). Chimerism levels remained stable in the animals that received limbs from which all LNs were excised, (group 3) which was similar to the non-GVHD control animals that received irradiated limbs (group 2). In contrast, GVHD-control animals (group 1) showed more than 15% increase in chimerism.

Clinical detection of GVHD after limb transplantation in chimeric hosts

As in previous studies [9, 10], weight loss was the most reliable predictor for the onset and progress of acute GHVD. In control group 1, chimeras transplanted with non-irradiated hind limbs succumbed to GVHD at 22.4±0.8 days post-transplantation. These animals lost over 25% of their initial weight during the first 3 weeks and had to be killed. In control group 2, none of the animals receiving irradiated (1050 cGy) hind limbs developed clinical or histopathologic signs of acute or chronic GVHD. These animals also experienced some weight loss (approximately 10%) during the first 2 weeks following limb transplantation, but they gradually regained weight and recovered, thriving for over 150 days post-transplantation. In group 3, chimeras transplanted with lymphadenectomized limbs, showed a similar weight

loss and gain pattern as group 2. None of these animals developed signs of acute GVHD, and all survived over 150 days post-transplantation. However, in 2/6 animals, mild clinical signs of a possible graft-versus-host (GVH) response were observed. These signs were less apparent, hair growth, a slight scruffy appearance, and mild weight loss (approximately 15%) compared to group 2, but without diarrhea, dermatoerythema, or hyperkeratosis. Clinical appearance of rats in group 3 is compared to that of group 1 (GVHD controls) in Fig. 1.

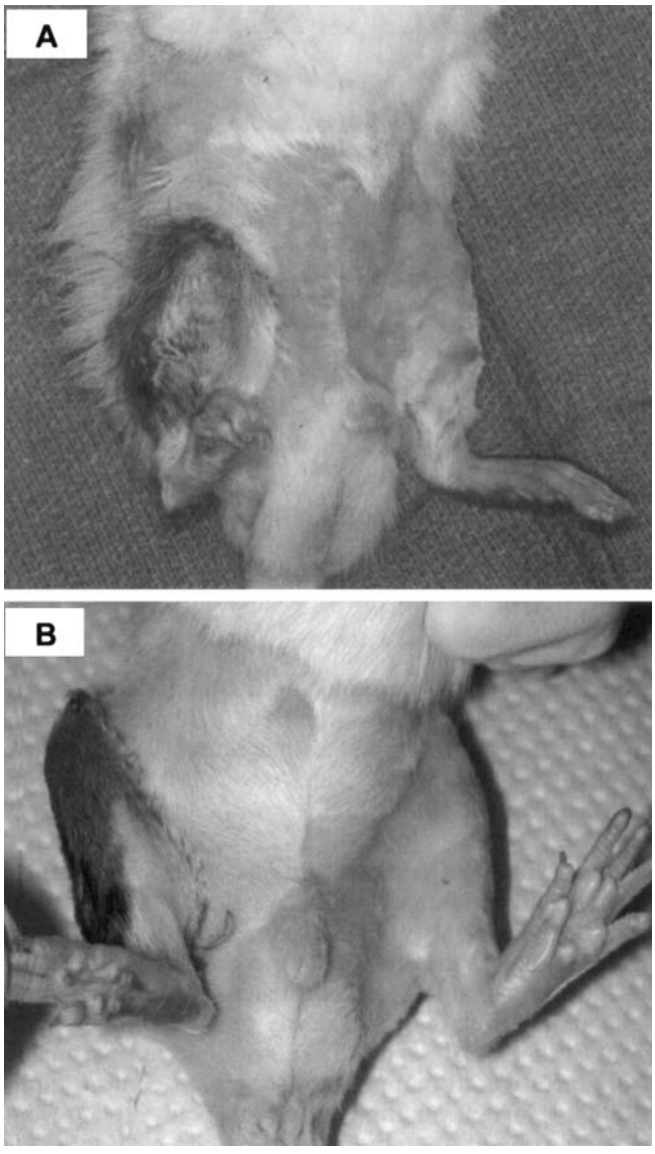

Fig. 1 Clinical assessment of GVHD. **A** Lethal GVHD in chimeras receiving non-irradiated limbs (group 1). Note the severe dermatoerythema on the abdomen and non-transplanted paw and its scruffy appearance at 21 days post-transplantation. **B** No signs of GVHD and prolonged survival in chimeras receiving lymphadenctomized limbs (group 3)

Histological detection of GVHD after limb transplantation in chimeric hosts

Histopathological examination of skin and tongue from animals that received limbs without lymph nodes did not reveal signs of GVHD. In Fig. 2, representative histopathological findings are compared to those of the controls (group 1 and 2) and animals from group 3.

Assessment of donor-specific tolerance in vivo and in vitro after limb transplantation

No clinical signs of rejection were observed in ACI limbs transplanted to [ACI → WF] chimeras, 3–6 months following BM reconstitution (groups 2 and 3). Histologic examination of skin (from CTA) and muscle biopsies performed at regular intervals during the experimental study confirmed the lack of rejection, which is further evidence of the tolerant state of these long surviving chimeras. In addition, prolonged survival of donor specific skin grafts in [ACI → WF] hosts (group 3) and vigorous rejection of third-party skin grafts from fully mismatched Fisher rats confirmed tolerance and immunocompetence in vivo (Fig. 3, upper panel).

Evidence of donor specific tolerance in vitro was established by the one-way MLR assay. Splenocytes from [ACI → WF] chimeras that received limbs without lymph nodes (group 3) showed hyporesponsiveness toward donor (ACI) with intact and significant reactivity toward third-party Fisher rat splenocytes ($p < 0.05$). The proliferation responses (expressed as CPM) from the MLR assays performed in groups 2 and 3 are summarized in Fig. 3, lower panel.

Location of LNs and enumeration of T and B cells in BM and LNs

Consistently, a single popliteal LN was found in each limb by dye injection. In our model, the inguinal fat pad flap is also transplanted to cover the anastomozed vessels. This fat contained three to four small inguinal LNs. With this information, all LNs could be easily removed prior to transplantation using microsurgical techniques (Fig. 4). The popliteal plus inguinal LNs of a single limb contained $117 \pm 16 \times 10^6$ cells and the BM from femoral and tibial bones $194 \pm 25 \times 10^6$ cells (group 4). The percentage of $\alpha\beta$-TCR$^+$ T cells and B cells within the LNs was $61.3 \pm 4.4\%$ and $20.6 \pm 3.6\%$ and in the BM $5.0 \pm 0.6\%$ and $31.2 \pm 2.2\%$ respectively.

Discussion

GVHD is a common phenomenon in allogeneic BM transplantation and represents a major cause of post

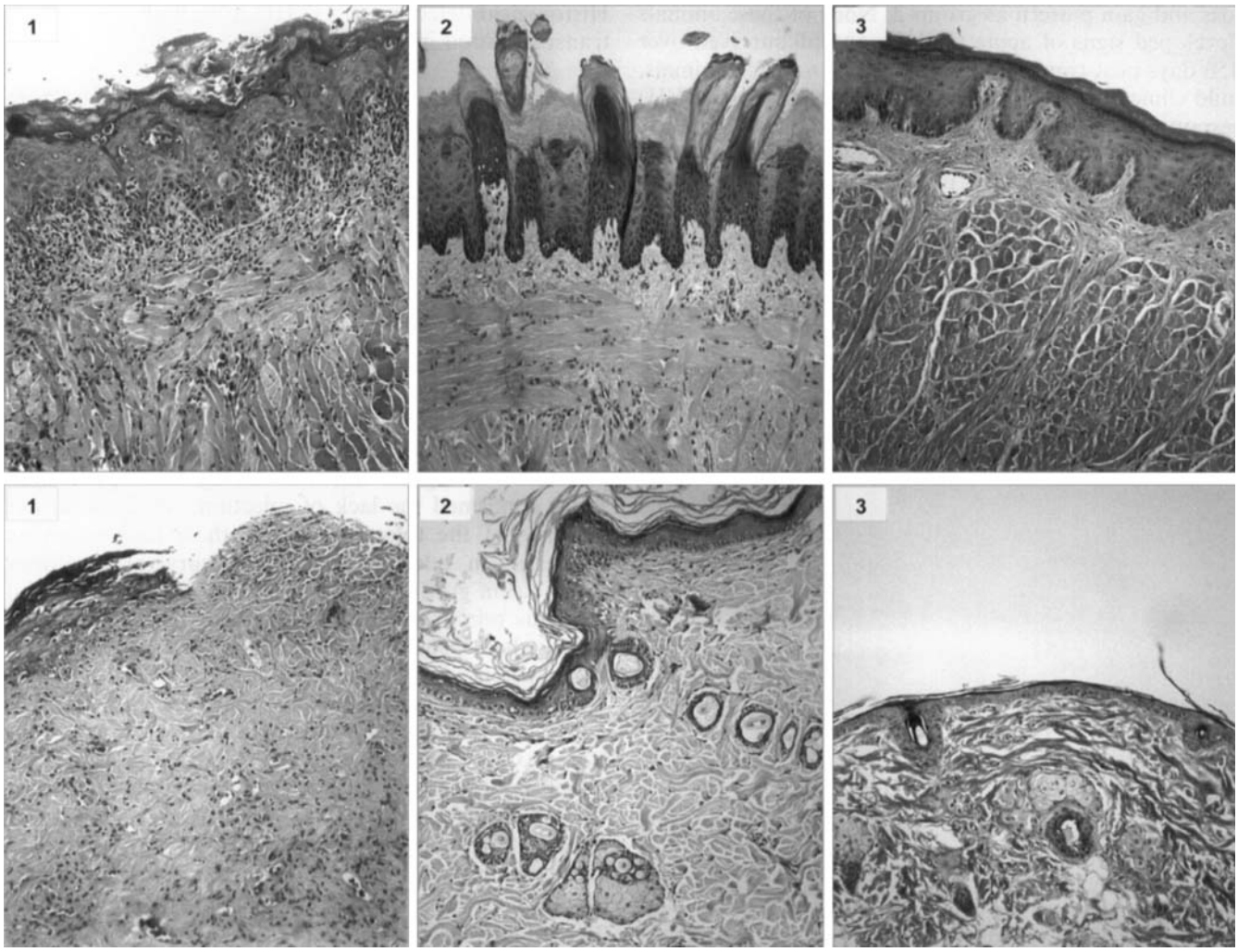

Fig. 2 Histopathologic assessment of GVHD. H&E sections of tongue and ear/skin of groups 1, 2, 3 are compared (×400). *Upper panel* tongue. In group 1 moderate to marked mononuclear cellular infiltration is noted in the epithelium and, particularly, the lamina propria. Chronic inflammatory infiltrates were also noted within the myocytes (myositis) with myocyte necrosis consistent with GVHD. Dyskeratosis and vacuoled epithelial cells were also noted. Similar to the skin sections, specimens from the remaining groups (*2 and 3*) demonstrated normal epithelium, lamina propria, and myofibrils. *Lower panel* skin. In group 1, specimens revealed scattered slight-to-moderate mononuclear cellular infiltrates within the dermis, necrosis and ulceration of the epidermis, dermal fibrosis, and loss of adnexa. Specimens shown from the remaining groups (*2 and 3*) demonstrate normal epidermis, dermis, and adnexal structures and lack of effects related to GVHD

BM transplant morbidity and mortality. Hosts of organ transplants are also at risk for GVHD, however, they are relatively immunocompetent, and the grafts usually do not contain large numbers of lymphocytes. As a result, the host-versus-graft (HVG) reaction is far stronger than the GVH component of this bi-directional reaction. Therefore, GVHD occurs only sporadically after solid organ transplantation, but has been reported following liver, kidney, heart-lung and multivisceral organ transplantation [18]. Transplantation of a lymphocyte rich organ such as the small intestine causes GVHD more frequently. The mortality rate, once GVHD occurs, is around 40% for solid organ transplantation recipients [18].

Introduction of composite tissue allotransplantation into the clinical arena could also come with the risk of GVHD due to the lymphocyte content in these grafts. In this new type of reconstructive procedure, multiple tissues are transplanted to reconstruct severe injuries and deformities of extremities, larynx or head and neck region. Even a small risk of GVHD in these reconstructive procedures would hamper its widespread clinical applicability. Current methods used clinically to prevent rejection of CTAs rely on toxic immunosuppressive drugs, which are a necessity to achieve prolongation of allograft survival. The ultimate goal is to replace these toxic immunosuppressive drugs with transplantation tolerance. However, if we do so, immunosuppression of the GVH reaction is also removed, and GVHD may

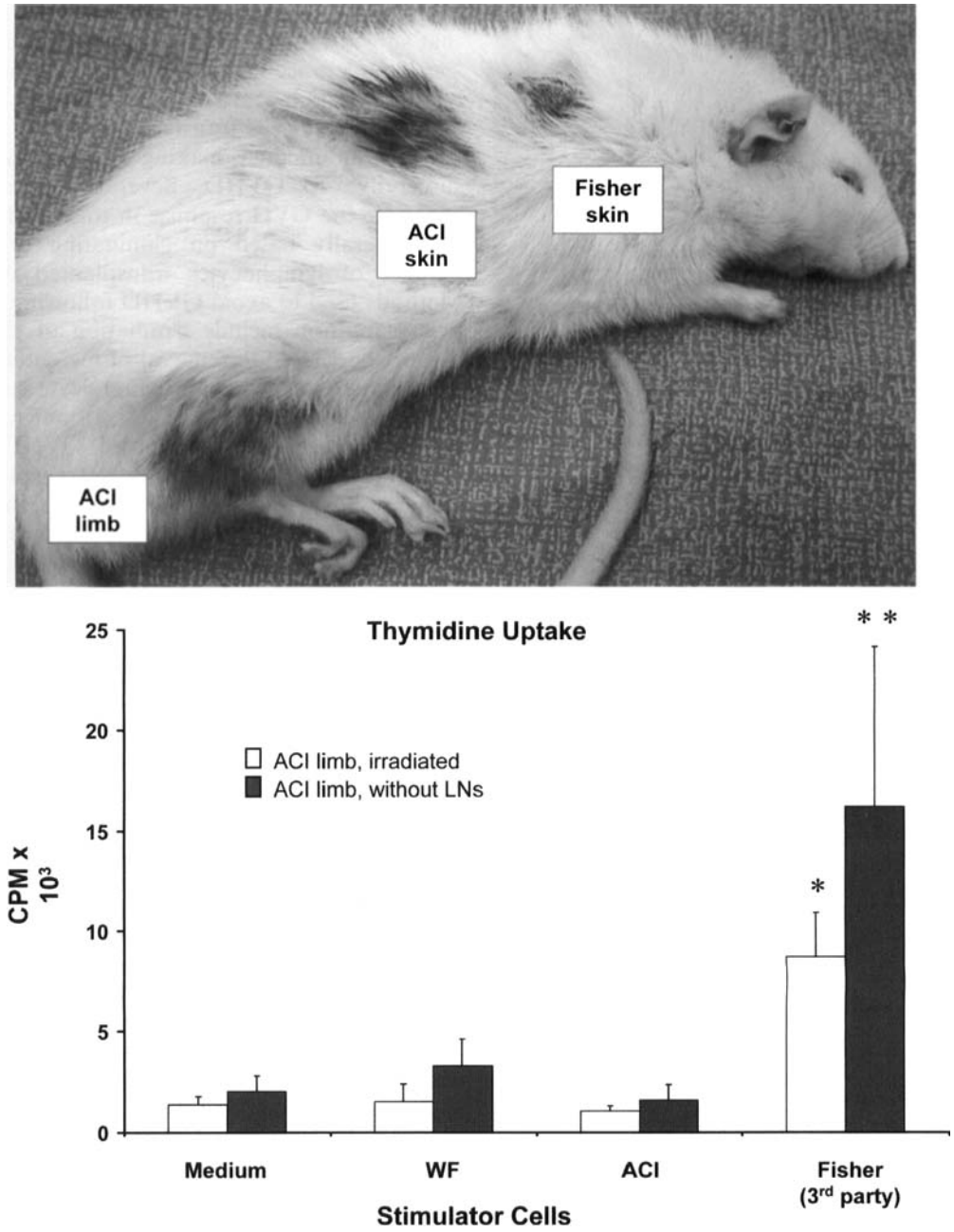

Fig. 3 Immunocompetence test of hosts. *Upper panel* shows chimeras [ACI → WF] that received a non-irradiated lymphadenectomized ACI limb (group 3). To test for donor-specific tolerance and third-party reactivity in vivo skin grafting was performed 150 days after limb transplantation in two animals. Fisher skin grafts were promptly rejected, while ACI skin grafts were accepted. Note the necrotic Fisher skin graft and the abundant hair growth of the ACI skin graft. The *lower panel* shows results of mixed lymphocyte reaction (MLR) assay from groups 2 and 3, quantified in counts per minute (CPM) + SEM. Chimeras that received irradiated or lymphadenectomized limbs demonstrated excellent reactivity towards third party antigens with donor specific (ACI) hyporesponsiveness. Baseline counts in cMLR medium are shown for comparison. GVHD in chimeras transplanted with non-irradiated limbs led to persistent and severe immuno-incompetence (not shown). * Indicates significant difference in proliferation comparing reactivity toward Fisher cells with reactivity toward ACI ($P < 0.05$) in group 2. ** Indicates significant difference in proliferation comparing reactivity toward Fisher cells with reactivity toward ACI cells ($P < 0.05$) in group 3. In both groups no difference was present in reactivity toward ACI cells when compared to reactivity toward WF cells, indicating donor specific tolerance towards ACI

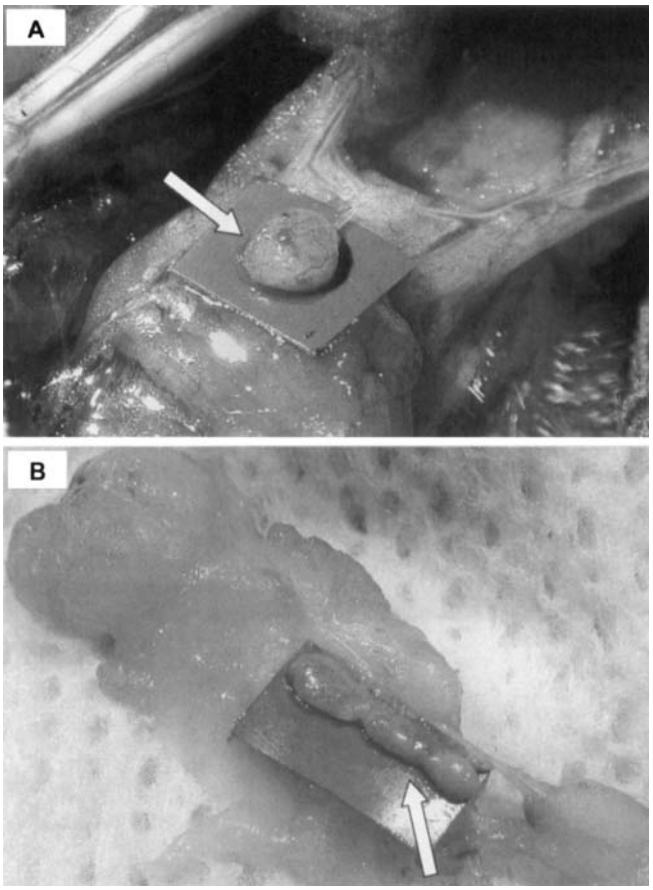

Fig. 4 Excision of lymph nodes prior to limb transplantation. **A** Single dissected LN in the popliteal space and **B** three dissected LNs in the inguinal fat pad are shown prior to excision (*arrow*). The vascular supply of the LNs and lymphatic vessels were carefully transected and the lymph nodes removed

occur and present a greater problem. Though GVHD does not occur in chimeras undergoing transplants such as skin [19], heart [20], lung [21], and kidney [22], it is well documented following transplantation of lymphocyte-rich grafts such as small intestine [11] and certain CTAs [8–10]. In previous studies we found that 10/10 chimeric hosts died of GVHD following hind limb transplantation [9]. In a similar study reported by Foster et al. [8] 1/9 animals developed lethal GVHD. Hewitt et al. [16] found that 37.5% of rat hind limb hosts developed lethal GVHD in a reverse, one-way parental to F1 hybrid model. In contrast, most studies using modern long term immunosuppressive regimens report the absence of (lethal) GVHD following rat hind limb transplantation non-chimeric hosts [17, 23, 24]. One study documented only transient GVHD in 33.5% of rat hind limb hosts maintained on FK506 [25], others noted non-lethal chronic GVHD in 30% of hosts receiving short term course of FK506 [26]. Lethal GVHD after limb transplantation was reported in one study with long term CsA therapy [27]. From this data it is clear that substitution of immunosuppressive drugs by donor specific tolerance may increase the risk of GVHD when lymphocyte-rich grafts are transplanted. Tolerant hosts allow donor T cells transferred with the graft to attack the host unhindered, making these animals particularly susceptible to GVHD. Several approaches exist to eliminate the GVH response in tolerant hosts, and they are generally based on eliminating or reducing the number of lymphocytes transplanted with the graft. Methods used to avoid GVHD following small intestine transplantation include irradiation of the graft [5] or mesenteric LNs [14], removal of mesenteric LNs [4] and donor treatment with antilymphocyte serum or anti-T cell monoclonal antibodies [15]. In our rat CTA model, we have successfully eliminated GVHD by radiating (1050 cGy) the hind limb prior to transplantation to the chimeric host [10]. We confirmed that the cells causing GVHD are radiosensitive and therefore eliminated by this procedure.

The tissues transplanted with the rat hind limb represent most tissues that would be included in many clinical CTA procedures. It has been speculated that of these tissues, BM [8] or the LNs [16] are responsible for GVHD seen in tolerant hosts. In the present study we developed a unique and clinically applicable alternative for graft radiation that could be used in certain types of CTAs. We investigated whether we could reduce the number of T cells transplanted with the limb and avoid GVHD using a selective surgical technique.

In the experiments described here we have demonstrated that removal of all LNs prior to transplantation decreases the amount of T cells transplanted with the limb dramatically, as is evidenced by the absence of GVHD in chimeric hosts. In addition, this indicates that the T cell content of the BM and other tissues of these lymphadenectomized limbs, such as skin, muscle and vascular bed, was not sufficient to cause GVHD, contradicting previous hypotheses. Two chimeric hosts that received limbs without LNs showed post-operative weight loss of approximately 15% during follow-up, combined with less abundant hair growth and a slight scruffy appearance. Though GVHD was not confirmed histologically, a mild GVH reaction could have been responsible for these signs. Since the severity of GVHD is closely related to the logarithm of the number of T cells in the graft [28], it is possible that more T cells remained in these transplanted hind limbs resulting in a mild GVH response.

To determine conclusively that in our limb model, the majority of mature T cells responsible for GVHD reside in the LNs and not the BM, we counted and enumerated the phenotype of cells from these respective tissues. The BM is mainly present in the femur and tibia, and typically one popliteal LN and 3–4 inguinal LNs are found. In rats, the majority of mature T cells are $\alpha\beta$-TCR$^+$ and

we showed that the percentage of $\alpha\beta$-TCR$^+$ T cells in LNs is approximately 12-fold of that in BM, whereas the total cellularity of BM is only 1.5–2 times greater than that of the LNs in one limb. These findings for the first time demonstrate that GVHD in tolerant hosts transplanted with hind limb allografts is caused by the large quantity of lymphocytes within the LNs that come with the graft. However, in this study we did not rule out the possibility that the microenvironment within the lymph nodes plays a role in the development of GVHD from BM derived lymphocytes. Future studies will need to explore the interaction between the lymph nodes microenvironment and lymphocytes in the pathogenesis of GVHD.

This study, like others, shows that induction of donor specific tolerance using BM chimerism can eliminate the need for toxic immunosuppressive drugs and prevent acute and chronic allograft rejection, while retaining third-party immunocompetence. All long-surviving chimeric hosts in our study, demonstrated donor specific tolerance and immunocompetence in both in vitro assays and in vivo assessment. In addition, histopathologic examination of tissue for signs of rejection or GVHD confirmed that these chimeras were tolerant and without GVHD. Donor specific skin grafts placed 150 days after limb transplantation were accepted indefinitely, while third party skin grafts were promptly rejected.

Prevention of GVHD in chimeric hosts using lymphadenectomized limbs turned out to be a straightforward procedure that provided a simple alternative to toxic graft irradiation which potentially could lead to tumor formation in CTAs. No long-term deleterious effect on lymphatic drainage of limbs were observed following lymphadenectomy. Although we did see edema early after transplantation, this edema resolved after 2 weeks. These observations were consistent with hind limb transplantation studies in which edema and lymphatic regeneration has been reported in non-lymphadenectomized limbs [29]. The present findings indicate that CTAs containing LNs could potentially induce GVHD in experimentally or, in future, clinically tolerized hosts. Though the percentage of mature T cells in human BM is higher than in rats, these findings demonstrate that CTAs without any LNs, such as a hand, almost certainly would not result in GVHD, especially when maintained on immunosuppressive drugs. This is also confirmed by clinical hand transplant recipients and indicates that irradiation of the donor hand, as performed by one clinical hand transplant team, was not necessary [30]. Furthermore, localization and removal of LNs from the graft could be aided by dye injection, which is clinically used to identify the sentinel LN of certain tumors. This technique could prove to be beneficial for the prospective transplantation of entire extremities and/or head and neck CTAs to individuals made tolerant for these grafts.

Acknowledgements This work was funded in part by grants from the Jewish Hospital Foundation in Louisville, Kentucky and the VSB, "De Drie Lichten" and "Prof. Van Vloten" foundations in The Netherlands. We would like to thank the animal facility staff for their outstanding animal care.

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