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Monoclonal antibodies to promote marrow engraftment and tissue graft tolerance

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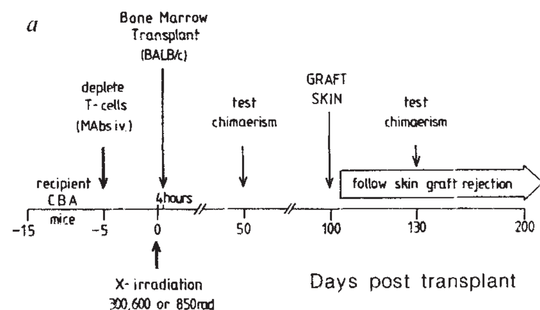
Allogeneic reactions are the major limitation to organ transplantation. These are manifested as rejection of the grafted tissue, and also, in the case of bone marrow transplantation (BMT), graft-versus-host disease (GVHD)[1]. Recent methods of avoiding GVHD, by depleting T cells from donor marrow, have led to an increased incidence of marrow graft rejection[2-5]. Current recipient conditioning protocols involving drugs or irradiation cannot safely be increased, so alternatives must be found. Monoclonal antibodies can be used to control immune responses *in vivo*[6,7], and would be useful in this context if we could define and deplete the cells responsible for marrow rejection. We show here that elimination of residual L3T4$^+$ and Lyt-2$^+$ cells from mice receiving fully mismatched bone marrow abrogates rejection and promotes tolerance to donor-type skin grafts, even in sub-lethally irradiated recipients.

The rejection of allogeneic marrow is a serious limiting factor in the clinical application of bone marrow transplantation (particularly if there is no matched sibling donor[3]), as graft-versus-host disease can now be avoided by removing T cells from the donor marrow[2,4,8-10]. It is clear that the recipients' 'resistance' to marrow grafting must be further reduced, but it is unlikely that current conditioning regimens could be increased as these are already near the limit of nonspecific toxicity. If marrow rejection could be prevented by a more specific and less toxic method, then BMT could be used more widely in, for example, the correction of inherited disorders, and perhaps to induce specific tolerance to other organ transplants. A number of mechanisms have been proposed to explain the resistance to marrow grafting, including natural killer (NK) cells[11,12], and antibody-dependent cell-mediated cytotoxicity (ADCC)[13]. Using mouse models of T-cell purged, mismatched BMT, we have recently shown that lethally irradiated recipients depleted of all T cells with monoclonal antibodies were much improved in long-term survival and chimaerism when compared to undepleted recipients[14]. It therefore became important to establish which T-cell subsets are responsible for rejecting marrow, and to determine whether monoclonal serotherapy to eliminate these subsets might supplement any sub-optimal conditioning of the transplant recipient.

Recipient CBA mice were given different doses of X irradiation, up to the lethal dose of 850 rad, either with or without pretreatment with monoclonal antibodies to deplete L3T4$^+$ and Lyt-2$^+$ T cells *in vivo*. The details of the protocol are shown in Fig. 1*a*. Acceptance or rejection of the T-cell depleted allogeneic marrow was determined by measuring both the level of chimaerism, and the degree of either unresponsiveness or priming to donor antigens as assessed by skin graft survival (Fig. 1*b*). Third



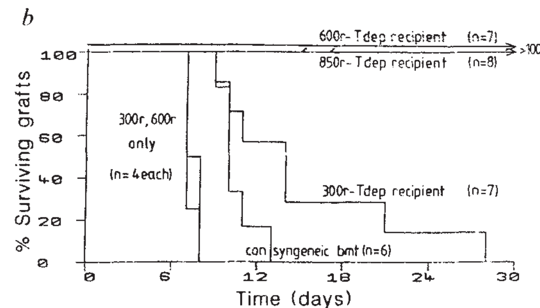


Fig. 1 Recipient T-cell depletion avoids marrow rejection and assists tolerance induction, even with sub-lethal irradiation. ***a***, Experimental protocol. All animals were bred and maintained in the conventional animal facility of the Department of Pathology, Cambridge University. Groups of CBA/Ca mice (16-20 weeks old, 8-20 per group), some of which had received 50 μg monoclonal antibodies YTS 169.4+YTS 191.1 intravenously (sufficient to deplete all T cells by >90%[6]), were given different doses of X irradiation (38 rad min^{-1}) on day zero. These were inoculated 4 h later with a mixture (10^7 of each) of bone marrow and spleen cells (to be comparable to the blood-contaminated marrow used in human BMT) from BALB/c mice which had been adult thymectomized (ATX) and depleted of all T cells wth antibodies to L3T4 and Lyt-2 (YTS 191.1 and YTS 169.4, 2×500 μg intravenously, at least 10 days previously[6,14]. The recipient mice were given oral metronidazole (days −15 to −5), Terramycin (oxytetracycline, days −5 to 0), followed by *p*H 2 water (days 0 to 100) and then Terramycin again from day 100. The test for chimaerism is detailed in Fig. 2. Randomly selected 100-day survivors (4-8 per group) were simultaneously grafted with donor-type (BALB/c) and third party (C57/B10) tail skin grafts (~10×5 mm) in the same thoracic graft bed, separated by a syngeneic CBA/Ca graft. Dressings were removed on day 7 and the grafts observed for rejection for at least 100 days. Control animals were syngeneic mice transplanted with CBA/Ca bone marrow and equivalent in all other respects to the 850 rad allogeneic group. Overall survival for each group at 100 days was: 100% (300 rad), 88% (600 rad) and 40% (850 rad, T-depleted), and at 200 days: 100% (300 rad), 76% (600 rad) and 30% (850 rad, T-depleted). ***b***, Responses to donor-type skin grafts. Analysis of graft survival was by the 'log-rank' method[26]. Control syngeneic (CBA→CBA, 850 rad) rejection of BALB/c; median = 10 days. T-depleted donor only (300 or 600 rad with no recipient T depletion): median = 7 days, $P \leq 0.02$. T-depleted 300 rad recipients: median = 14 days, $P \leq 0.12$ versus controls. T-depleted 600 or 850 rad recipients: no rejection at 100+ days ($P \leq 0.001$), but two 850 rad animals died with grafts intact on days 14 and 17.

party skin was also grafted to establish the specificity of any unresponsive (tolerant) state. Recipients which had been T-cell depleted and lethally irradiated (850 rad) before marrow transplantation became specifically tolerant of donor-type antigens, as evidenced by complete skin graft acceptance. In contrast, similar recipients without T-cell depletion did not survive until day 100, as previously reported[14]. The majority of animals given 600 rad total body irradiation (TBI) survived, but with a remarkable difference between the T-cell depleted and untreated recipient groups: the former showed specific acceptance of donor-type skin grafts, while the latter rejected donor grafts

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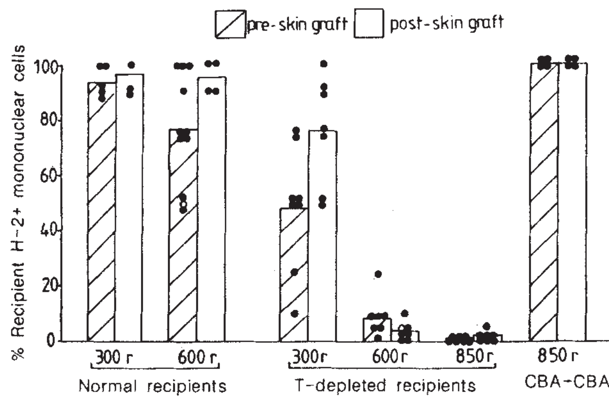

Fig. 2 Chimaerism in T-cell-depleted allogeneic bone marrow recipients. The levels of chimaerism were tested in the animals of Fig. 1 by immunofluorescence on whole blood smears using a rat monoclonal anti-mouse H-2 which binds to H-2K (recipient-type) but not H-2D[27]. (This method has previously been shown to correlate well with full H-2 phenotyping by microcytotoxicity[14].) Individual animals are shown as closed circles, while those with open circles had low blood white cell counts ($<5.0\times10^6$ ml^{-1} mostly neutrophils; normals were $6–8\times10^6$ ml^{-1} with approximately 60% lymphocytes). Where possible, 200+ mononuclear cells were counted when slides had <10% H-2K$^+$ cells. Mean % recipient-type cells is indicated by the blocks. Polymorphs showed the same trend but were generally less positive for H-2K, even in control CBA mice (data not shown).

more rapidly than controls (that had received a syngeneic marrow graft). Third party grafts were rejected (data not shown) either at the normal rate or, in the group rejecting the marrow, at an accelerated rate (probably due to priming against cross-reactive transplantation antigens). A similar pattern was seen using animals irradiated with 300 rad, except that the antibody-treated recipients were now only partially unresponsive to donor-type skin grafts (Fig. 1*b*). The animals in this group were found to be mixed chimaeras, albeit predominantly recipient-type (Fig. 2), suggesting that this particular conditioning regime did not give the donor marrow sufficient advantage to compete effectively with an autologous recovery. In the case of T-cell-depleted mice given 600 rad or 850 rad, the peripheral blood cells reconstituted mainly to donor type (negative for recipient H-2; Fig. 2), and have remained so (>300 days), while the equivalent untreated (600 rad) group had an autologous reconstitution of the blood system. We conclude that monoclonal antibody depletion of recipient T cells prevents marrow graft rejection, leading instead to acceptance and specific tolerance of donor-type bone marrow and skin. Perhaps the most striking finding is that the autologous recovery expected after sub-lethal irradiation is reduced when the marrow is not rejected. This probably reflects a natural haemopoietic competition between the donor stem cells and the irradiated recipient cells.

What, then, is the mechanism of rejection, and which T cells are responsible? There are two major subsets of peripheral T cells in both man and rodents, which differ in the way they recognize foreign antigens[15]. The first of these responds to antigens in the context of class II (Ia) major histocompatibility complex (MHC) molecules[16], and expresses the differentiation antigen L3T4 (ref. 17) (CD4 in man[18]). This subset can provide 'help' for other effector functions, and generate MHC class II-restricted effector cells. The second subset recognizes antigen in the context of class I MHC molecules[19] (for example, H-2K, H-2D) and expresses the Lyt-2 differentiation antigen[20] (CD8 in man[18]). This subset is classically linked with the generation of cytotoxic T cells. To what extent, then, are each of these subsets capable of rejecting tissue grafts?

We have previously shown that L3T4$^+$ T cells can rapidly reject either H-2 or minor antigen mismatched skin, while Lyt-2$^+$

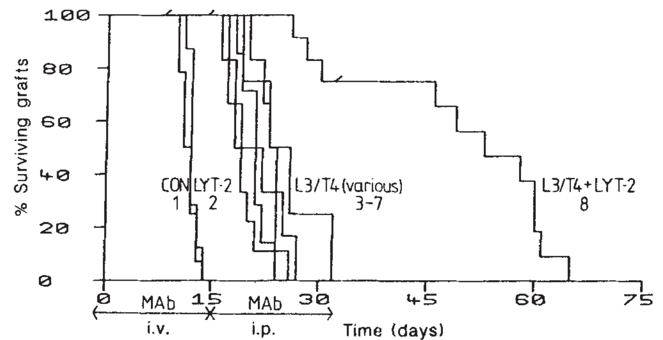

Fig. 3 Both L3T4$^+$ and Lyt-2$^+$ cells can reject allogeneic skin grafts. Adult CBA/Ca mice were grafted on day 0 with BALB/c tail skin on the lateral thoracic wall, by the method of Billingham *et al.*[28]. The following schedules of monoclonal antibody treatment were used: (1) controls received no antibody ($n=14$): (2) YTS 169.4 (anti-Lyt-2)- approximately 500 μg intravenously on days −1, +2, +8, and intraperitoneally on day +12 ($n=8$, one animal died of anaphylaxis on day 8 with graft intact): (3) YTS 191.1 (anti-L3T4: epitope P), approximately 500 μg intravenously on days −1, +7, +12 and +19 ($n=6$): (4) YTA 3.1 (anti-L3T4: epitope Q), approximately 500 μg intravenously on days −1, +7, +12 and +19 ($n=6$): (5) YTS 191.1 + YTA 3.1 (250 μg of each) as in 3 and 4 ($n=9$): (6) YTS 191.1 + YTA 3.1 (25 μg of each) intravenously on days −1, +1 and three times per week thereafter, switching to intraperitoneal injection on day +15 until day +33 ($n=8$): (7) YTS 191.1 alone, doses as in 6 ($n=5$, one animal died on day 14 with graft intact): (8) YTS 191.1 + YTS 3.1 (10 μg of each synergistic anti-L3T4) and also YTS 169.4 + YTS 156.7 (10 μg of each synergistic anti-Lyt-2: S.Q. *et al.*, in preparation) as in 6 ($n=13$, one animal died with graft intact on day 32). All mice were given oxytetracycline (50 mg l^{-1} in the drinking water, Terramycin, Pfizer) and the antibody was 0.2 μm sterile filtered in PBS with penicillin (Crystapen; Glaxo; 500 U per injection) in groups 6, 7 and 8. Dressings were removed on day 10 and the grafts were observed daily, with the day of rejection recorded when no viable graft remained. All animals were shown to have no detectable (<0.5%) cells positive for the depleted antigen on days 15 and 35 (control and anti-Lyt-2-treated day 15 only), by immunofluorescence on whole blood smears[14] using the synergistic pairs and FITC-NORIG 7.16.2 (anti-rat IgG2b[29]). Analysis of the survival curves by the log-rank method[26] gave the following P values of statistical significance: controls versus Lyt-2, not significant; Lyt-2 versus all L3T4, $P\leq0.0001$; various L3T4 compared, $0.13\leq P\leq1.0$; L3T4 + Lyt-2 versus L3T4, $P\leq0.0001$.

cells dominate the secondary graft response[21]. It was not clear, however, whether the Lyt-2$^+$ cells could reject such grafts independently, or whether they needed to interact with L3T4$^+$ 'helpers'. Recent data show that purified Lyt-2$^+$ cells can respond *in vitro* to class I MHC differences without any help from L3T4$^+$ cells[22]. The experiment in Fig. 3 shows that both subsets can indeed reject skin grafts relatively independently. Long-term survival of a fully mismatched skin graft was only obtained if both L3T4$^+$ and Lyt-2$^+$ cells were kept depleted. The fact that Lyt-2 depletion alone did not cause any delay confirms that L3T4$^+$ cells can reject rapidly on their own. However, maintaining depletion of the L3T4$^+$ cells, using a variety of aggressive protocols (including pairs of non-competing, rat IgG2b monoclonal antibodies which synergize for cell destruction *in vitro* and *in vivo*; refs 23, 24 and S.Q., unpublished) could not delay graft rejection beyond 35 days. The considerable extra suppression gained by eliminating both subsets demonstrates that the Lyt-2$^+$ cells can also reject grafts without detectable L3T4$^+$ cells, but only after some delay. It should be noted that antibody treatment alone did not impair the animals' ability to eventually reject their grafts, and was thus insufficient to impose a state of tolerance to this major antigenic challenge.

Bone marrow rejection, it seems, can also be elicited by either of the L3T4$^+$ or Lyt-2$^+$ subsets. In Fig. 4, it can be seen that,

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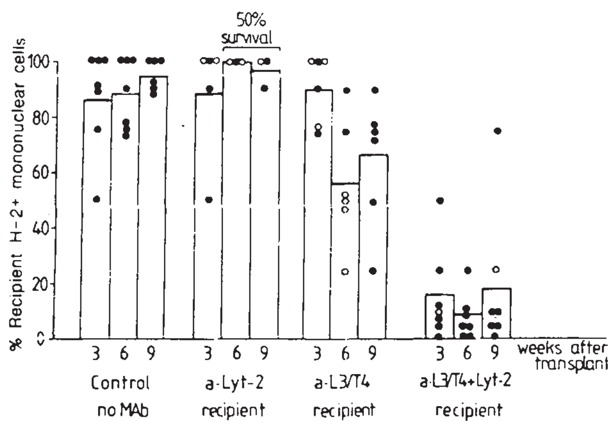

Fig. 4 Both L3T4$^+$ and Lyt-2$^+$ cells reject allogeneic marrow. Groups of CBA/Ca mice (14–18 weeks old) were treated according to the 600 rad protocol of Fig. 1, except that two new groups were included—separate recipient depletion of L3T4 (YTS 191.1, 50 μg intravenously on day −5) or Lyt-2 (YTS 169.4, 50 μg intravenously on day −5) with T-cell-depleted donor ATX BALB/c bone marrow and spleen cells, as before. Chimaerism was determined as described above, 3, 6 and 9 weeks post-transplant. Individual mice are shown as closed circles, while open circles depict a low white cell count (mostly polymorphs) as in Fig. 3 (except anti-Lyt-2 group on week 3 were mainly lymphocytes of recipient type). Mean % recipient-type cells is indicated by the blocks.

after sub-lethal irradiation (600 rad TBI), histoincompatible marrow transplants were only accepted by mice depleted of both the T-cell subsets. Recipients depleted of just one subset rejected the donor marrow and reconstituted instead with autologous blood cells, as did untreated controls. This shows that both subsets have an independent capacity to reject allogeneic marrow. None of the animals depleted of Lyt-2 cells had any evidence of significant donor cells in the blood, and 3 out of 6 mice died before day 28, suggesting some inhibition of autologous reconstitution. The L3T4$^+$ cells seemed more effective than Lyt-2$^+$ cells for marrow rejection, because there was evidence of partial chimaerism in the L3T4-depleted group. However, these mice rejected donor-type skin grafts after only 8 days (median: data not shown), indicating that they had become primed to the donor marrow antigens. This is in contrast to the tolerance-inducing effects of anti-L3T4 treatment as recently described for antibody responses to soluble proteins[25] It is likely that tolerance to donor transplantation antigens is due to the continuous presence of donor haematopoietic cells, which is only possible after acute marrow rejection has been avoided by removing the recipients' T cells (which can be achieved with either anti-L3T4 + Lyt-2 as described above, or with anti-Thy-1[14]).

In practical terms, it is possible that the ability to improve the conditioning of bone marrow transplant recipients with appropriate monoclonal antibodies will be directly applicable to man. This would simplify mismatched marrow transplantation, and perhaps allow nonspecific ablative conditioning to be reduced to a level where BMT might be used to induce tolerance to other donor organ grafts.

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Isolation of cell lines possessing functional and serological properties resembling those of thymocyte precursors

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Thymocytes develop from a committed haematopoietic progenitor, referred to as a prothymocyte[1–4]. They are uniquely capable of migrating to and restoring the thymus of a lethally irradiated host, a property which has been exploited as a specific assay for these cells[4–6]. Like other committed haematopoietic progenitors, prothymocytes are found only in small numbers in even the richest sources (0.05–1.0% of the nucleated cells in bone marrow). Purification has proved difficult both in terms of finding a suitable starting material and in the degree of enrichment achieved[7,8]. We now report the isolation of cloned lines of cells with some of the serological and functional properties of prothymocytes. One of these lines has been in continuous culture for almost 2 years. When injected into irradiated recipients, cells from this line migrate to the thymus and there develop into cells which resemble normal cortical thymocytes.

Attempts have been made to culture thymocyte precursors from a variety of haematopoietic tissues. Our first isolate was obtained from a mixed leukocyte culture (MLC) in which spleen cells from BALB/c mice were cultured with irradiated (2,000 rad) C57BL/6 spleen cells in Iscove's modified Dulbecco's minimal essential medium supplemented with 10% (v/v) conditioned medium from rat spleen cells stimulated by concanavalin A (Rat T-cell monoclone; Collaborative Research, Lexington) and 10% fetal bovine serum. One week after the initial burst of proliferation of allogeneic T cells had subsided, clusters of large blasts appeared. These were collected by aspiration and transferred into fresh medium as above. When the cultures reached a density of 500,000 cells ml^{-1} they were diluted 1 : 5. After 5 weeks, they were stained for surface Thy-1.2 antigen and the apparently negative population (95% of the total) was isolated by sorting. This population was cloned using a single-cell deposition device. The initial cloning efficiency was 5–10%. The cells were recloned twice and one of these clones (now designated pT-D8) was selected for further study.

After 6 months in culture, supplementation with conditioned medium became unnecessary. Recent isolates of similar clones

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