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Inhibition of skin xenograft rejection by depleting T-cell receptor αβ-bearing cells without T-cell receptor γδ-bearing cells or natural killer cells by monoclonal antibody

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SUMMARY

We compared the effects of *in vivo* administration of the anti-T-cell receptor (TCR) αβ monoclonal antibody (mAb) (H57-597) to those of the anti-CD3 mAb (145-2C11), with or without anti-NK1.1 mAb (PK136), on xenogeneic skin graft survival in mice. In anti-TCRαβ mAb-treated B6 mice, F344 rat skin grafts survived for about 54 days, whereas in anti-CD3 mAb-treated B6 mice with or without anti-NK1.1 mAb treatment grafts survived about 25 days. In anti-TCRαβ mAb-treated B6 mice, TCRαβ-bearing T-lymphocyte function was completely abrogated, although TCRγδ-bearing T-lymphocyte function was still intact on day 9. In the anti-CD3 mAb-treated mice, the functions of both types of T lymphocytes were completely abrogated. On day 32, when most of the skin xenografts had been rejected in the anti-CD3 mAb-treated mice, the functions of both T lymphocytes had recovered considerably, and could actually respond to F344 antigens. In contrast, the function of TCRαβ-bearing cells had only partially recovered in the anti-TCRαβ mAb-treated mice. Finally, natural killer (NK) activity in the anti-TCRαβ mAb-treated mice was intact on day 32, when rat skin grafts still survived. In contrast, NK activity in the anti-CD3 mAb plus anti-NK1.1 mAb-treated mice did not recover on day 32, when skin xenografts had already been rejected. These results suggest that TCRγδ-bearing T cells and NK cells by themselves, at least in the absence of TCRαβ-bearing T cells, do not mediate xenogeneic skin graft rejection in mouse/rat combinations.

INTRODUCTION

One of the most important problems in clinical transplantation is the shortage of supply of allogeneic organs.[1,2] Because practical xenogeneic transplantation would provide a solution to this problem, it is important to investigate the immunity to xenoantigens and the mechanism of xenograft rejection. Pierson *et al.*[3] reported that the cell-mediated response to xenogeneic antigens depends particularly upon $CD4^+$ lymphocytes, a feature shared by the response to allogeneic minor histocompatibility antigens. Furthermore, helper T cells recognize disparate xenoantigens as nominal antigens when presented in association with the self-class II major histocompatibility complex (MHC) molecule.[4,5] However, less is known about cell immunity to xenoantigens than to alloantigens.

Some authors have suggested that natural killer (NK) cells might mediate xenograft rejection.[1,6,7] In addition, Sharabi *et al.*[8] reported that depletion of NK 1.1^+ cells was necessary to induce donor-specific xenogeneic transplantation tolerance in a rat/mouse combination.[8] They suggested that $NK1.1^+$ cells might participate directly in xenogeneic skin graft rejection. However, it is still unclear whether NK cells by themselves directly contribute to xenogeneic skin graft rejection.

It has been reported that two distinct CD3-associated T-cell receptors, TCRαβ and TCRγδ, are expressed in a mutually exclusive fashion on separate subsets of T lymphocyte.[9] Some TCRγδ-bearing T lymphocytes are reported to have alloreactivity, cytolytic activity and the capacity to produce cytokines.[10–12] Recently, we found that TCRγδ-bearing T lymphocytes by themselves could not contribute to the allograft rejection *in vivo*, using the anti-TCRαβ monoclonal antibody (mAb) H57-597, in comparison with the anti-CD3 mAb 145-2C115.[13] However, it is still unclear whether TCRγδ-bearing T lymphocytes can mediate xenogeneic graft rejection.

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Abbreviations: Ab, antibody; CTL, cytotoxic T lymphocyte; FACS, fluorescence-activated cell sorter; i.p., intraperitoneal(ly); i.v., intravenous(ly); mAb, monoclonal antibody; MLR, mixed lymphocyte reaction; MST, mean survival time; PE, phycoerythrin; SD, standard deviation; TCR, T-cell receptor.

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Anti-CD3 mAb, such as OKT3, has been used clinically in the treatment of acute rejection,[14,15] and 145-2C11 has been used to study the immunosuppressive effects on allogeneic responses by *in vivo* administration in murine models.[16–19] In contrast, anti-TCR$\alpha\beta$ mAb has not yet been put into general use in either clinical or experimental allogeneic transplantation.[2,20–24] Moreover, the effects of this mAb on xenogeneic skin graft survival are still unknown.

In this report, using anti-TCR$\alpha\beta$ mAb, anti-CD3 mAb and anti-NK1.1 mAb, we investigated whether TCR$\gamma\delta$-bearing T cells and NK cells could contribute directly to the xenogeneic skin graft rejection in a mouse/rat combination. We also examined whether anti-TCR$\alpha\beta$ mAb could be as effective as anti-CD3 mAb in preventing xenogeneic skin graft rejection. Our results clearly demonstrate that the *in vivo* administration of anti-$\alpha\beta$ mAb is effective in preventing xenogeneic graft rejection. In addition, our results also suggest that TCR$\gamma\delta$-bearing T lymphocytes and NK cells by themselves, at least in the absence of TCR$\alpha\beta$-bearing T lymphocytes, cannot mediate the xenogeneic skin graft rejection.

MATERIALS AND METHODS

Animals

Female C57BL/6 Cr Slc (B6; H-2^b) mice were obtained from Japan SLC Inc. (Shizuoka, Japan). Female Fisher 344 (F344) rats were obtained from The Institute of Experimental Animals, Kyushu University (Fukuoka, Japan). Mice were used at 8–11 weeks of age, and rats were used at 4–8 weeks of age.

Monoclonal antibodies

Anti-TCR$\alpha\beta$ mAb (hybridoma H57-597, hamster IgG), anti-CD3 mAb (hybridoma 145-2C11, hamster IgG), and anti-NK1.1 mAb (hybridoma PK136, mouse IgG2a) were used in this study, and prepared as follows. The hybridomas were cultured in serum-free conditioned medium (SFM 101; Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) supplemented with sodium bicarbonate (1·4 g/l), insulin (10 mg/l), transferrin (10 mg/l), monoethanoleamine (20 μl/l) and antibiotics (25 mg/l toblamycin). Monoclonal antibodies were purified by ammonium sulphate, dialysed against phosphate-buffered saline, and quantified using a BCA protein assay reagent (Pierce, Rockford, IL). Monoclonal antibodies were used at a concentration of 4 mg/ml.

Treatment

Purified anti-TCR$\alpha\beta$ mAb and anti-CD3 mAb were administered i.v. on days -6 and -3, and i.p. on day 0, at a dose of 400 μg. Anti-NK1.1 mAb was administered at a dose of 400 μg on days -3 and 0, in combination with anti-CD3 mAb.

Skin grafting was carried out 9 days after the last injection of mAb, using a procedure we have reported previously.[25] Briefly, square, full thickness skin grafts (1 cm^2) were prepared from the trunk skin of donors. Graft beds, 1 cm^2, were prepared on the right lateral thoracic wall of the recipients. Grafts were fixed to the graft bed with eight interrupted sutures of 5–0 silk thread and were covered with protective tapes. The first inspection was carried out on the seventh day after grafting, followed by daily inspections thereafter. Grafts were defined as rejected at the time of complete sloughing or when they formed a dry scab. Survival was expressed as mean survival time (MST) ± standard deviation (SD).

FACS analysis

Spleen cells were obtained from mice treated with various mAb on days 9 and 32, and were doubly stained with anti-TCR$\alpha\beta$ mAb (H57-597) or anti-CD3 mAb (145-2C11) followed by fluorescein isothiocyanate (FITC)-conjugated anti-hamster IgG mAb (Meiji, Tokyo, Japan) and phycoerythrin (PE)-conjugated anti-Thy-1.2 mAb (Becton Dickinson & Co., Mountain View, CA), and in some experiments with FITC-conjugated anti-TCR$\gamma\delta$ mAb (PharMingen, San Diego, CA) and PE-conjugated anti-Thy-1.2 mAb. The effects of mAb on NK cells were evaluated by staining with FITC-conjugated anti-NK1.1 mAb (PharMingen). Fluorescence-positive cells were measured by a FACS (FACScan; Becton Dickinson, Sunny Vale, CA). The scatter was used to exclude dead cells. All data were displayed on a logarithmic scale of inverting green (FITC) and red (PE) fluorescence intensity. To obtain percentages of T-cell subpopulations, a total count was integrated in a scaled area of the counter plot.

Monoclonal antibody-induced proliferation

Various concentrations of purified anti-TCR$\alpha\beta$ mAb (H57-597), anti-TCR$\gamma\delta$ mAb (UC7-13D5) and anti-CD3 mAb (145-2C11) were added at a dose of 30 μl per round-bottomed microtitre well (25850; Corning, Corning, NY). Plates were incubated in an humidified atmosphere containing 5% CO_2 at 37° for 4–6 hr, and washed out three times with phosphate-buffered saline before use. The spleen cells were used as responders. Five hundred thousand of the responder cells in 200 μl of RPMI medium (Gibco, Grand Island, NY), which was supplemented with 10% Nu-serum (Collaborative Research, Lexington, MA), 5×10^{-5} M 2-mercaptoethanol and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), was added per well, and incubated in a humidified atmosphere containing 5% CO_2 at 37°for 48 hr as the optimal culture period, and pulsed for the last 6 hr with [^{3}H]thymidine (37 kBq/well) and then harvested. Results are expressed as the mean c.p.m./min of four samples.

Mixed lymphocyte reaction (MLR)

Spleen cells were used as both responders and stimulators in MLR. Responder cells (5×10^5 cells/100 μl) were cultured with irradiated (2500 rads) stimulator cells (5×10^5 cells/100 μl) in a flat-bottomed microplate (25860; Corning), in a humidified atmosphere containing 5% CO_2 at 37°, for various days, and were pulsed on the last day with [^{3}H]thymidine (37 kBq/well) followed by harvesting 8 hr later. Results are expressed as the mean c.p.m./min of four samples.

Assay of NK activity

The NK activity was evaluated by an ordinary ^{51}Cr-release method. The spleen cells were used as effector cells. YAC-1 lymphoma (originated in a A/Sn mouse) were labelled with ^{51}Cr (3.7 MBq) for 1 hr at 37° and used as the target blast cells. Mixtures of varied numbers of effector cells in 100 μl and 2×10^4 target cells in 100 μl were incubated in a round-bottomed microplate (25850; Corning) in a 5% CO_2 incubator for 4 hr at 37°. The complete RPMI medium described above was used for this assay. The amount of ^{51}Cr release in 100 μl

of supernatant was measured by a well-type gammacounter (Shimazu, Kyoto, Japan). Percentage of specific release was calculated as follows:

$$\% \text{ specific lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100.$$

The maximum release was obtained by adding 100 μl of 10% Triton-X to 100 μl of the target cells. The spontaneous release was the c.p.m. released from target cells incubated with the medium alone. The data were expressed as the mean value of six samples ± SD.

Statistics

The statistical significance of the data was determined by Student's *t*-test. A *P*-value of less than 0·05 was considered to be statistically significant.

RESULTS

The prolongation of skin xenograft survival in mice treated with anti-TCRαβ mAb, anti-CD3 mAb or anti-CD3 plus anti-NK1.1 mAb

To compare the effect of anti-TCRαβ mAb on the prolongation of skin xenograft survival with that of anti-CD3 mAb, and to observe the contribution of TCRγδ-bearing T lymphocytes to skin xenograft rejection, we grafted F344 skin to B6 mice treated with anti-TCRαβ mAb or anti-CD3 mAb. As shown in Fig. 1, we observed about 54 days of skin xenograft survival in anti-TCRαβ mAb-treated B6 mice, compared with 8–9 days in untreated B6 mice. The skin xenografts survived about 25 days in the anti-CD3-treated B6 mice. These results suggested that depletion of TCRγδ-bearing cells would not be able to prolong skin xenograft survival, and that anti-TCRαβ mAb would be more effective in prolonging skin xenograft survival. To investigate whether NK1.1$^+$ cells could mediate the rejection of the xenogeneic skin grafts in the anti-CD3 mAb-treated B6 mice, we also performed skin grafting to anti-CD3 mAb plus anti-NK1.1 mAb-treated B6 mice. We could not find any further prolongation of skin xenograft survival in these mice compared with that in the anti-CD3 mAb-treated B6 mice.

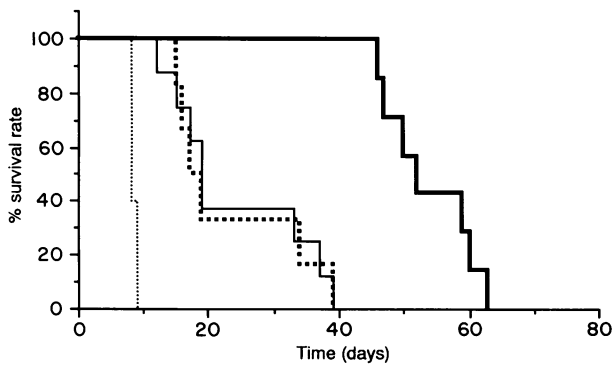

Figure 1. The effects of *in vivo* administration of anti-TCRαβ mAb, anti-CD3 mAb or anti-CD3 mAb plus anti-NK1.1 mAb on xenograft survival. B6 mice were injected i.v. with 400 μg of anti-TCRαβ mAb or anti-CD3 mAb on days −6 and −3, and i.p. on day 0. In some B6 mice, anti-NK1.1 mAb was injected i.v. at a dose of 400 μg on days −3 and 0, in combination with anti-CD3 mAb. Skin grafting with xenogeneic F344 skin was performed on day 9. Groups were as follows: bold solid line, anti-TCRαβ mAb-treated B6 mice; fine solid line, anti-CD3 mAb-treated mice; bold dotted line, anti-CD3 mAb plus anti-NK1.1 mAb-treated B6 mice; fine dotted line, untreated B6 mice. Mean survival times were 53·9 ± 6·3 (n = 7), 25·1 ± 10·1 (n = 8), 23·3 ± 9·5 (n = 6) and 8·4 ± 0·5 (n = 5) in the B6 mice treated with anti-TCRαβ mAb, anti-CD3 mAb, anti-CD3 mAb plus anti-NK1.1 mAb, and no mAb, respectively.

The *in vivo* effects of the mAb on the phenotype of T lymphocytes

To confirm whether these mAb could be effective *in vivo*, and what populations would mediate the skin xenograft rejection, we performed phenotypical and functional examinations on days 9 and 32. A representative immunofluorescence analysis is shown in Figs 2 and 3. On day 9, in the anti-TCRαβ mAb-treated mice, TCRαβ-bearing T lymphocytes completely disappeared, which was due not only to the depletion but also the down-modulation of the TCR–CD3 complex by anti-αβ mAb, because Thy-1.2$^+$αβ$^-$ cells and Thy-1.2$^+$CD3$^-$ cells increased in this group compared with those of the untreated group (Fig. 2a, b, d and e). Because TCRγδ-bearing T lymphocytes were not influenced, there were a few CD3$^+$ T lymphocytes which were thought to be TCRγδ-bearing T lymphocytes in this group (Fig. 2e and h). In the anti-CD3 mAb-treated mice, both types of TCR-bearing lymphocytes completely disappeared (Fig. 2c, f and i). However, there were more TCRαβ-bearing cells of which the TCR–CD3 complex was down-modulated by anti-CD3 mAb than those in the anti-TCRαβ mAb-treated group. In the anti-CD3 mAb-treated B6 mice, of which skin grafts had already been rejected, both T lymphocytes had considerably recovered on day 32, which was thought to be mainly due to recovery from down-modulation, partially due to new generation from the thymus (Fig. 3c, f and i). In the anti-TCRαβ mAb-treated mice on day 32, in contrast, TCRαβ-bearing T lymphocytes had not recovered, but TCRγδ-bearing T lymphocytes had slightly increased in the spleen (Fig. 3b, e and h). Results similar to those in anti-CD3 mAb-treated B6 mice were obtained in anti-CD3 mAb plus anti-NK1.1 mAb-treated B6 mice on both days 9 and 32 (data not shown).

The effects of *in vivo* administration of mAb on *in vitro* T lymphocyte proliferation in response to stimulation by mAb

We next evaluated the function of splenic T lymphocytes in various mAb-treated mice, using a mAb-induced *in vitro* proliferation assay. Spleen cells from untreated B6 mice responded equally well to both anti-CD3 mAb stimulation and anti-TCRαβ mAb stimulation, but only weakly to anti-TCRγδ mAb stimulation, throughout all the experiments (Fig. 4). On day 9, when we performed skin grafting, the response to anti-TCRαβ mAb stimulation of the spleen cells from the anti-TCRαβ mAb-treated B6 mice was completely abrogated (Fig. 4a). The response to the stimulation by anti-CD3 mAb in these mice partially remained (Fig. 4b). We considered that it reflected the responses of TCRγδ-bearing T lymphocytes, because the response to anti-TCRγδ mAb stimulation was not impaired compared with those of the untreated mice (Fig. 4c). In the anti-CD3 mAb-treated mice and anti-CD3 mAb plus anti-NK1.1-treated mice, the

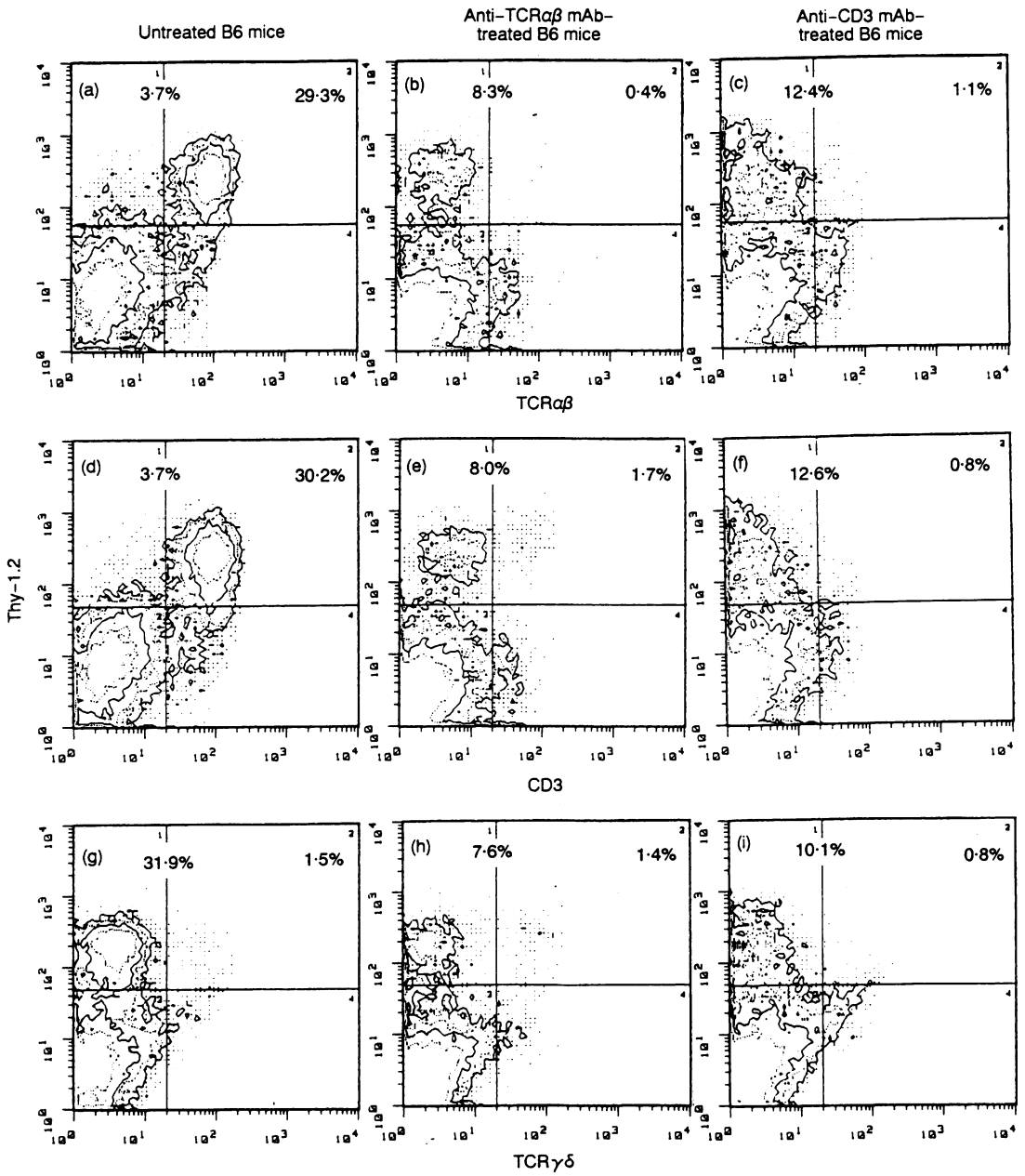

Figure 2. Effects of *in vivo* administration of mAb on T-cell subsets on day 9. In the spleen cells of the anti-TCR$\alpha\beta$ mAb-treated B6 mice, $\alpha\beta^+$ Thy-1.2$^+$ cells, but not $\gamma\delta^+$Thy-1.2$^+$ cells, almost completely disappeared, although both phenotypes disappeared in anti-CD3 mAb-treated B6 mice, by both depletion and down-modulation. Spleen cells were stained with anti-TCR$\alpha\beta$ mAb or anti-CD3 mAb, followed by FITC–goat anti-hamster IgG or FITC–anti-TCR$\gamma\delta$ mAb, and then stained with PE–anti-Thy-1.2 mAb. Spleen cells were obtained from untreated B6 mice (a, d and g); anti-TCR$\alpha\beta$ mAb-treated B6 mice (b, e and h); and anti-CD3 mAb-treated B6 mice (c, f and i). These mice were not xenografted. Data represent one of three animals tested. The other two showed similar staining patterns to each panel.

responses to these three mAb stimulations were completely abrogated (Fig. 4a, b and c).

On day 32, in anti-TCR$\alpha\beta$ mAb-treated mice, the response to anti-TCR$\alpha\beta$ mAb stimulation recovered to only 17% of the normal level. In anti-CD3 mAb-treated mice and anti-CD3 plus anti-NK1.1 mAb-treated mice, however, the responses to anti-TCR$\alpha\beta$ mAb stimulation recovered to 57% and 78% of the normal level, respectively (Fig. 4d). The responses to anti-TCR$\gamma\delta$ mAb stimulation were completely recovered in these two groups (Fig. 4f). Furthermore, in anti-TCR$\alpha\beta$-treated mice, the responses to anti-TCR$\gamma\delta$ mAb stimulation were augmented rather than recovered, which was considered to make the response to anti-CD3 mAb stimulation rise up to 40% of the level of untreated mice (Fig. 4e and f). These results demonstrated that both anti-TCR$\alpha\beta$ mAb and anti-CD3 mAb were equally effective to abrogate the function of

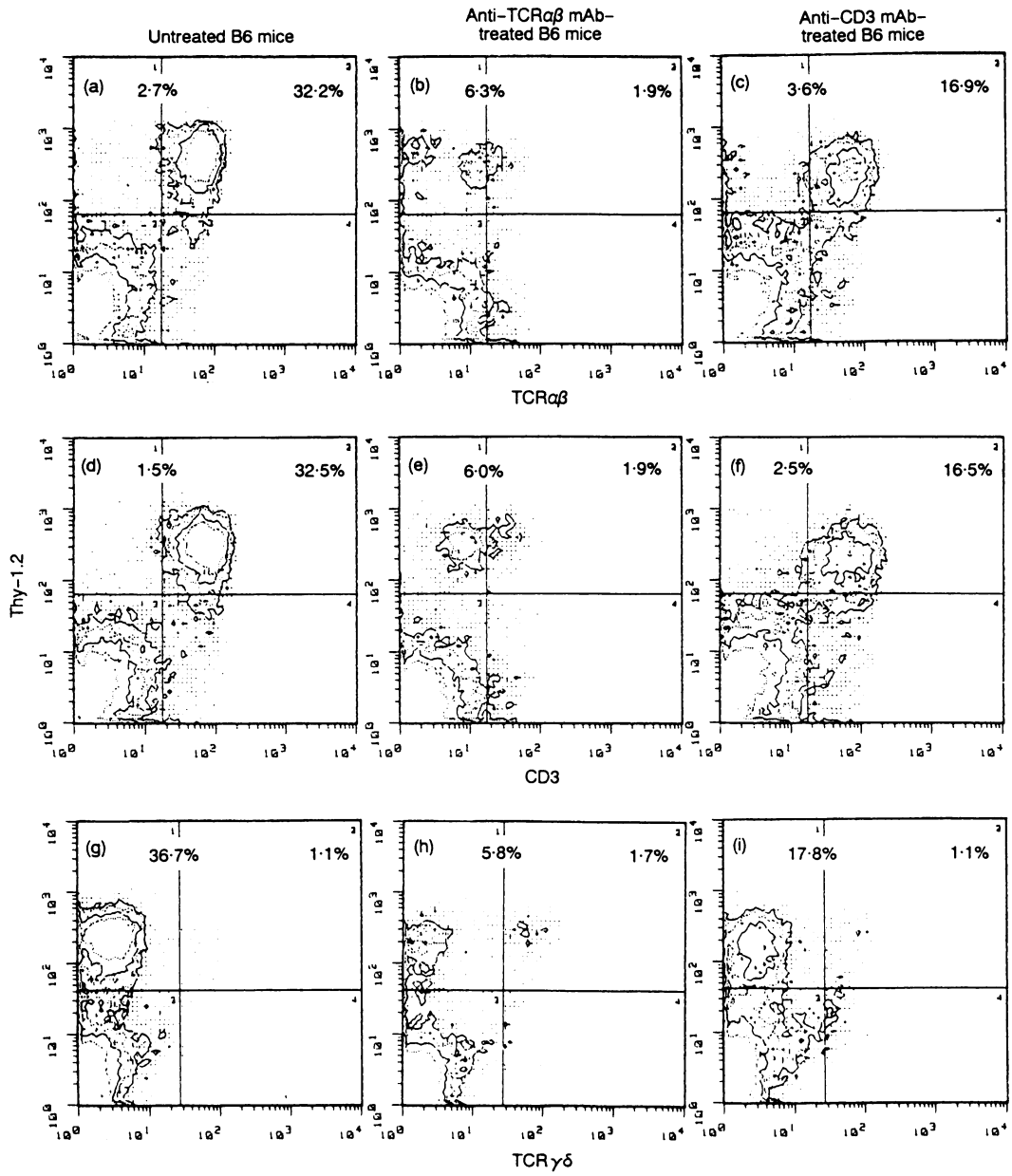

Figure 3. Effects of *in vivo* administration of mAb on T-cell subsets on day 32 using the mice grafted on day 9. In the spleen cells of the anti-TCR$\alpha\beta$ mAb-treated B6 mice, TCR$\alpha\beta$-bearing T lymphocytes had not recovered from down-modulation, whereas both types of T lymphocytes had already recovered in those of anti-CD3 mAb-treated B6 mice. Spleen cells were obtained from untreated B6 mice (a, d and g); anti-TCR$\alpha\beta$ mAb-treated B6 mice in which xenogeneic skin grafts survived (b, e and h); and anti-CD3 mAb-treated B6 mice that had already rejected xenogeneic skin grafts (c, f and i).

TCR$\alpha\beta$-bearing cells by *in vivo* administration, but recoveries from their effects were not equal, probably due to the difference in proportion of down-modulation by each mAb. In contrast, TCR$\gamma\delta$-bearing T cells were intact phenotypically and functionally in anti-TCR$\alpha\beta$ mAb-treated B6 mice, on both day 9 and 32. It is suggested that TCR$\gamma\delta$-bearing cells by themselves could not directly mediate skin xenograft rejection.

MLR in mice treated with these mAb

The responses to anti-TCR$\alpha\beta$ mAb stimulation had considerably but not completely recovered in anti-CD3 mAb-treated mice and anti-CD3 mAb plus anti-NK1.1 mAb-treated mice on day 32. Moreover, even in anti-TCR$\alpha\beta$ mAb-treated mice, a low but appreciable response to anti-TCR$\alpha\beta$ mAb stimulation was detected at that time. So, it was unclear whether or not these T lymphocytes could actually respond to xenogeneic F344 antigens. We next tested MLR to xenogeneic F344 antigens to investigate whether spleen cells of the mice in each group could actually respond to F344 antigens on day 32. In the anti-TCR$\alpha\beta$ mAb-treated B6 mice, of which skin xenografts survived, the responses to both F344 and allogeneic AKR completely disappeared (Fig. 5b). In the anti-CD3 mAb- or anti-CD3 plus anti-NK1.1 mAb-treated B6 mice, of which skin

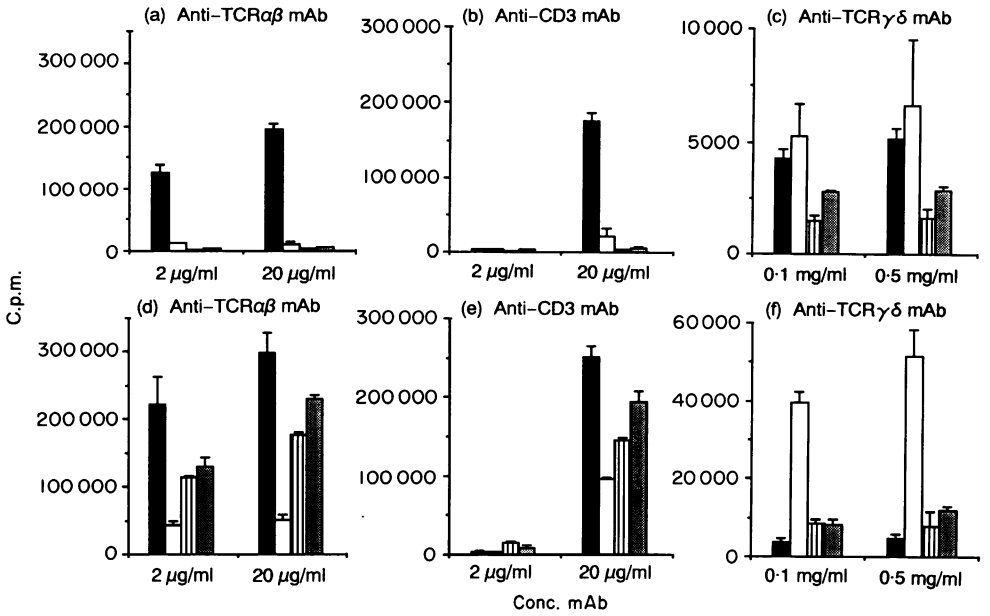

Figure 4. Effects of *in vivo* administration of mAb on a mAb-induced proliferation assay on day 9 (a, b and c) and day 32 (d, e and f), using ungrafted mice and grafted mice, respectively. The purified anti-TCRαβ mAb (H57-597), anti-CD3 mAb (145-2C11) and anti-TCRγδ mAb (UC7-13D5) were diluted to the indicated concentration with phosphate-buffered saline, and 30 μl was added per round-bottomed microtitre well. Plates were incubated for 4–6 hr 37° and then washed three times before use. Five hundred thousand spleen cells in 200 μl was added per well, and plates were cultured for 48 hr, pulsed for the last 6 hr with 37 kBq of [^{3}H]thymidine, and then harvested. Groups were as follows: (■), untreated B6 mice; (□), anti-TCRαβ mAb-treated B6 mice; (▥), anti-CD3 mAb-treated B6 mice; (▨), anti-CD3 mAb plus anti-NK1.1 mAb-treated B6 mice. On day 32, when most of the xenogeneic skin grafts had been rejected in the B6 mice given anti-CD3 mAb with or without anti-NK1.1 mAb, spleen cells were obtained from mice which had already rejected skin xenografts in these groups.

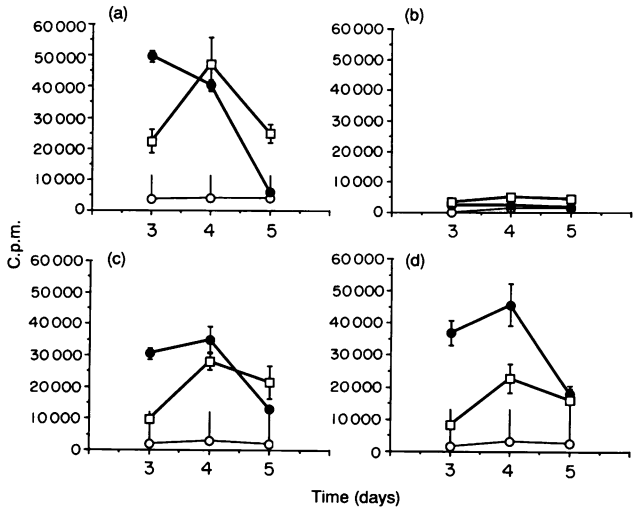

Figure 5. Effects of *in vivo* administration of mAb on the proliferative response by *in vitro* stimulation with xenogeneic F344 antigens on day 32. Spleen cells from anti-TCRαβ mAb-treated B6 mice were actually unresponsive. Five hundred thousand spleen cells from each B6 mouse was incubated with 5×10^5 2500 rads-irradiated F344 (●), AKR (□) and B6 (○) spleen cells for the given number of days. Spleen cells were obtained from (a) untreated B6 mice, (b) B6 mice that had been treated with anti-TCRαβ mAb, (c) anti-CD3 mAb, and (d) anti-CD3 plus anti-NK1.1 mAb.

grafts had been already rejected, their responses to F344 could be clearly detected (Fig. 5c and d).

The *in vivo* effects of the mAb on NK activity in the spleen cells

The *in vivo* administration of anti-NK1.1 mAb was not effective in prolonging skin xenograft survival further in anti-CD3 mAb-treated mice, as shown in Fig. 1. To investigate whether NK activity had actually been abrogated, we tested NK activity on days 9 and 32. On day 9, in the anti-CD3 mAb plus anti-NK1.1 mAb-treated mice, NK activity was completely abrogated. In the anti-TCRαβ mAb- or anti-CD3 mAb-treated mice, NK activities were slightly reduced at that time (Fig. 6a). On day 32, in the anti-CD3 mAb plus anti-NK1.1 mAb-treated B6 mice NK activity had not recovered yet, although skin grafts had already been rejected. In contrast, NK activity completely recovered in the anti-TCRαβ mAb-treated B6 mice, although skin xenografts survived in these mice (Fig. 6b).

The disappearance of NK1.1$^+$ cells in the spleen of mice treated with the mAb

The *in vivo* effects of the mAb on NK1.1$^+$ cells in the spleen were phenotypically examined by immunofluorescence analysis. These results were considered to be compatible with the NK activity assay. Namely, NK1.1$^+$ cells were slightly decreased in the anti-TCRαβ mAb- or anti-CD3 mAb-treated B6 mice, and completely disappeared in the anti-CD3 mAb plus anti-NK1.1 mAb-treated B6 mice on day 9. On day 32, these cells remained

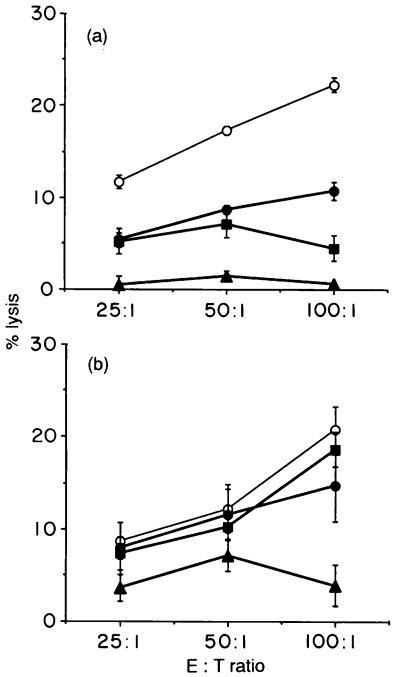

Figure 6. NK activity on days (a) 9 and (b) 32. NK activity was evaluated by a standard ^{51}Cr-release method. Spleen cells were used as effector cells, and ^{51}Cr-labelled YAC-1 cells were used as target cells. Spleen cells, obtained from anti-TCR$\alpha\beta$ mAb-treated B6 mice (●), anti-CD3 mAb-treated B6 mice (■), anti-CD3 mAb plus anti-NK1.1 mAb-treated B6 mice (▲), and untreated B6 mice (○), were incubated with 1×10^4 target cells for 4 hr. Data were calculated as described in the Materials and Methods.

at an undetectable level in the anti-CD3 mAb plus anti-NK1.1 mAb-treated B6 mice, although completely recovered in the anti-TCR$\alpha\beta$ mAb- and anti-CD3 mAb-treated B6 mice (Table 1). These results suggested that skin xenograft survival would not be parallel to the kinetics of NK1.1$^+$ cells and NK activity.

Table 1. Percentage of NK1.1$^+$ cells in the spleen cells derived from various mAb-treated mice on days 9 and 32

Group	Treatment*	% NK1.1$^+$ cells	
		Day 9	Day 32
1	—	3·09 ± 0·68	2·22 ± 0·50
2	Anti-TCR$\alpha\beta$ mAb	2·01 ± 0·30†	2·32 ± 0·48¶
3	Anti-CD3 mAb	1·51 ± 0·25‡	1·91 ± 0·44¶
4	Anti-CD3 mAb Anti-NK1.1 mAb	0·23 ± 0·11§	0·83 ± 0·19**

* Purified anti-TCR$\alpha\beta$ mAb and anti-CD3 mAb were administered i.v. on days −6 and −3, and i.p. on day 0 at a dose of 400 μg. In some mice, anti-NK1.1 mAb was administered at a dose of 400 μg on days −3 and 0, in combination with anti-CD3 mAb.

† Not significant compared with group 1.

‡ $P < 0.05$ compared with group 1.

§ $P < 0.01$ compared with group 1.

¶ Not significant compared with group 1.

** $P < 0.05$ compared with group 1.

DISCUSSION

In this report, we set up three groups, one depleted of TCR$\alpha\beta$-bearing cells, one depleted of TCR$\alpha\beta$-bearing and TCR$\gamma\delta$-bearing cells, and one depleted of TCR$\alpha\beta$-bearing, TCR$\gamma\delta$-bearing and NK1.1$^+$ cells, by *in vivo* administration of anti-TCR$\alpha\beta$ mAb, anti-CD3 mAb or anti-CD3 mAb plus anti-NK1.1 mAb, respectively. In the phenotypical examination, we could not find complete depletion by these mAb, because some of the cells were only down-modulated on day 9 (Fig. 2). However, we could confirm that *in vivo* administration of these mAb was effective in abrogating cell function, by the mAb stimulation assay and NK activity (Figs 4 and 6). We observed the prolongation of skin xenograft survival in each group, in order to examine the role of TCR$\gamma\delta$-bearing cells and NK cells for skin xenograft rejection. We found that xenogeneic skin graft survival in these three groups was prolonged compared with that of untreated mice, and that skin xenograft survival was no more prolonged by abrogation of either TCR$\gamma\delta$-bearing cell function or NK activity in addition to the function of TCR$\alpha\beta$-bearing cells (Fig. 1). These results suggest that it would be sufficient to abrogate the function of TCR$\alpha\beta$-bearing cells in order to obtain the prolongation of skin xenograft survival, but that it would not be necessary to abrogate either the TCR$\gamma\delta$-bearing cell function or NK activity.

We also found that the *in vivo* administration of anti-TCR$\alpha\beta$ mAb was more effective in preventing the xenogeneic skin graft rejection than that of anti-CD3 mAb, and that three administrations of 400 μg anti-TCR$\alpha\beta$ mAb prior to skin grafting resulted in about 54 days of skin xenograft survival. To determine what populations could mediate xenograft rejection, we also performed phenotypical and functional examinations on day 32. In anti-TCR$\alpha\beta$ mAb-treated mice, of which all skin xenografts survived, the TCR$\alpha\beta$-bearing cell function remained at a low level, whereas the TCR$\gamma\delta$-bearing cell function was augmented on day 32 (Fig. 4). In contrast, the function of TCR$\alpha\beta$-bearing cells and TCR$\gamma\delta$-bearing cells had already recovered in anti-CD3 mAb-treated mice, of which skin xenografts had already been rejected (Fig. 4d, e and f). These results clearly demonstrate that TCR$\gamma\delta$-bearing T lymphocytes by themselves, at least in the absence of TCR$\alpha\beta$-bearing cells, do not contribute to xenograft rejection in the rat/mouse combination. Furthermore, it is also suggested that the different effects in prolonging skin xenograft survival between anti-CD3 mAb and anti-TCR$\alpha\beta$ mAb would be merely due to the differences of recovery from down-modulation of TCR$\alpha\beta$-bearing cells. It is probably because there were more down-modulated TCR$\alpha\beta$-bearing cells in the anti-CD3 mAb-treated mice than in the anti-TCR$\alpha\beta$ mAb-treated mice, and because such down-modulated cells recovered faster in the former group than in the latter group. However, it is still unclear why there were such differences between anti-TCR$\alpha\beta$ mAb-treated mice and anti-CD3 mAb-treated mice. It may be due to a difference in immunoglobulin subclass, because the IgG subclass of hamster antibodies, to which anti-TCR$\alpha\beta$ mAb and anti-CD3 mAb belong, have not been identified yet. Alternatively, it may reflect the differences of signal transduction caused by each mAb. However, it is clear that the *in vivo* administration of anti-TCR$\alpha\beta$ mAb was more effective in prolongation of skin xenograft survival than that of anti-CD3 mAb, as far as the materials used here are concerned.

Furthermore, as we and others have reported previously, TCR$\gamma\delta$-bearing T cells that we can preserve by anti-TCR$\alpha\beta$ mAb treatment play a crucial role *in vivo* in some bacterial infections.[13,26–30] So, we consider that there would be some advantage of anti-TCR$\alpha\beta$ mAb in clinical transplantation.

In this study, xenogeneic skin grafts could survive in the anti-TCR$\alpha\beta$ mAb-treated mice, in which we could confirm that TCR$\gamma\delta$-bearing T lymphocytes existed both functionally and phenotypically on days 9 and 32. However, in spite of undetectable MLR to xenoantigens of the spleen cells from the anti-TCR$\alpha\beta$ mAb-treated mice (Fig. 5), we could not determine directly whether or not TCR$\gamma\delta$-bearing T lymphocytes had xenoreactivity, because the percentage of TCR$\gamma\delta$-bearing T lymphocytes of the responder cells in MLR was very low (about 1·7%). It has been reported that some TCR$\gamma\delta$-bearing T lymphocytes have alloreactivity, cytolytic activity, and the capacity to produce cytokines.[10–12] Despite these alloreactive TCR$\gamma\delta$-bearing cells, TCR$\gamma\delta$-bearing cells by themselves could not contribute to allograft rejection.[13] Therefore, there might be some TCR$\gamma\delta$-bearing cells which could react to xenoantigens, but could not mediate skin xenograft rejection. Attempts to determine whether or not TCR$\gamma\delta$-bearing T lymphocytes have xenoreactivity are currently in progress.

In the anti-TCR$\alpha\beta$ mAb-treated mice and anti-CD3 mAb-treated mice, NK activity was partially reduced and NK1.1$^+$ cells were also decreased on day 9 (Fig. 6). NK cells were relatively decreased in number in the spleen cells from these mice (Table 1), probably due to extramedullary haematopoiesis induced not only by anti-CD3 mAb, as reported elsewhere,[31] but also by anti-TCR$\alpha\beta$ mAb. NK activity per a definite number of spleen cells from anti-CD3 mAb-treated mice was more reduced than that of anti-TCR$\alpha\beta$ mAb-treated mice, probably because anti-CD3 mAb could induce extramedullary haematopoiesis more strongly than anti-TCR$\alpha\beta$ mAb. On day 32, however, NK activity had already completely recovered in the anti-TCR$\alpha\beta$ mAb-treated mice in which xenogeneic skin grafts survived (Fig. 6 and Table 1). In anti-CD3 mAb plus anti-NK1.1 mAb-treated mice, in contrast, NK activity and NK1.1$^+$ cells did not recover by day 32, though their skin xenografts had been already rejected. These results suggest that NK cells cannot contribute to skin xenograft rejection directly.

Recently, unusual TCR$\alpha\beta^+$ NK1.1$^+$ cells have been reported to be able to mediate allogeneic bone marrow cell rejection, to play a role in the regulation of lymphohaematopoiesis,[8,32–35] and probably to contribute to xenogeneic graft rejection. We investigated this phenotypically in bone marrow (data not shown). However, because the kinetics of these cells in bone marrow was parallel with that of TCR$\alpha\beta$-bearing T lymphocytes in the anti-TCR$\alpha\beta$ mAb-treated mice, we could not distinguish the effects of this population on xenograft rejection from those of TCR$\alpha\beta$-bearing T lymphocytes. Because this population could be depleted by the administration of anti-CD3 mAb or anti-NK1.1 mAb, it is suggested that the differences of the prolongation of skin xenograft survival between the anti-TCR$\alpha\beta$ mAb-treated mice and in the anti-CD3 and anti-NK1.1 mAb-treated mice would not depend upon the presence of this population. Further studies are necessary to determine whether these cells would mediate skin xenograft rejection. Even if these cells could directly mediate xenograft rejection, it would be sufficient for obtaining prolongation of skin xenograft survival to deplete TCR$\alpha\beta$-bearing cells.

In this study, though we used anti-CD3 mAb at a dose of 1·2 mg, none of the mice which received anti-CD3 mAb died. Hirsch *et al.*[16] have previously reported that 40% of mice which had received 400 μg anti-CD3 mAb 145-2C11 died early in the course of their experiments. We consider that the anti-CD3 mAb which we used in this study is as effective as theirs, because in our anti-CD3 mAb-treated mice, such side-effects as diarrhoea, body weight loss, hyperactivity and massive piloerection were observed. Even if our anti-CD3 mAb might be less effective, we think that 1·2 mg of anti-CD3 mAb was sufficient for this experiment because 1·2 mg of anti-CD3 mAb is an excessive dose to abrogate the function of CD3$^+$ cells completely on day 9 (Fig. 4a, b and c). The differences of mortality between our experiment and their study[16] may be due to the environment in which the mice were kept. Namely, we have used mice free from exposure to mouse hepatitis virus.

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