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Lymphocyte changes associated with prolongation of cardiac allograft survival in adult mice using anti-CD4 monoclonal antibody

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SUMMARY

This study investigated the effect of anti-CD4 MoAb treatment on lymphocyte phenotype and function and correlated these changes with the prolongation of cardiac allograft survival in adult mice. Indefinite survival of heterotopic cardiac allografts was obtained in several fully allogeneic strain combinations when two doses of the anti-CD4 MoAb, YTS 191.1, were given at the time of transplantation. A dose response analysis in the C57BL/10 to C3H/He strain combination showed that very low doses of YTS 191.1 (25 μg/dose) were able to induce prolonged allograft survival when administered perioperatively. At the time of transplantation the immunosuppression induced by administration of the anti-CD4 MoAb is not antigen-specific, as heart grafts from different donor strains, mismatched for both major and minor histocompatibility antigens, showed prolonged survival in treated recipients. Immunocompetence was restored by 6 weeks after MoAb treatment, as recipients regained the ability to reject a cardiac allograft transplanted at this time point. However, while recovery of immunocompetence could be demonstrated *in vivo*, leucocytes isolated from the peripheral lymphoid organs of treated mice continued to be hyporesponsive in mixed leucocyte culture (MLC). Phenotypic analysis of the peripheral lymphoid tissues showed that C3H/He recipients treated with 25 μg/dose of YTS 191.1 had a marked, but not complete, elimination of the $CD4^+$ subset at the time of transplantation, which was gradually restored to 50% of normal by 6 weeks after treatment. Thus, complete elimination of the $CD4^+$ subset was not required to achieve indefinite allograft survival, and immunocompetence, as assessed *in vivo*, returned even when the $CD4^+$ subset was present at half the normal level. Low doses of anti-CD4 MoAb (25 μg) had no effect on the expression of the CD4 molecule by thymocytes, and yet thymocytes were hyporesponsive to alloantigen *in vitro*. At higher doses of YTS 191.1, immature $CD4^+8^+$ thymocytes were selectively depleted. These results suggest that anti-CD4 MoAb therapy may modulate the intrathymic T cell selection process. These studies provide further insight into the mechanism of action of low dose, depleting anti-CD4 MoAb therapy in allograft rejection, and form a basis from which rational modifications to therapeutic protocols in transplantation models can be made.

Keywords CD4 monoclonal antibody transplantation

INTRODUCTION

The $CD4^+$ T cell subset plays a central role in the allograft rejection response [1–4] and therefore is a logical target for immunosuppressive therapy. Selective inactivation of these cells may allow not only more effective, but also immunologically specific suppression of the immune response in comparison with the use of other MoAb [5,6] or drug therapies at present used in clinical transplantation. Increased immunological specificity is a highly desirable goal because of the significant side effects associated with the non-specific state of immunosuppression currently required to maintain allograft survival [7]. The immunosuppressive ability of anti-CD4 MoAbs has been demonstrated in a variety of experimental models, including inhibition of the primary humoral response to protein antigens [8–12], alloantigens [13,14] and in some rodent transplantation models [8,15–22]. In addition, in the non-human primate model, Jonker *et al.* [23] and more recently Cosimi *et al.* [24] have shown the ability of anti-CD4 MoAb to prolong the survival of renal allografts. These results suggest that anti-CD4 MoAbs have the potential to be very powerful immunosuppressive agents, and the clinical benefit of anti-CD4 MoAb has been shown in several patients with autoimmune disease [25,26]. Initial studies in

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human transplant recipients have begun [27], but optimal therapeutic benefit in clinical use will only be obtained when the effects of anti-CD4 MoAbs *in vivo* are better understood. To this end, the current studies extend the initial observations made in this laboratory [22] on the ability of anti-CD4 MoAb to prolong the survival of vascularized mouse cardiac allografts. The effect of a short course of treatment given at the time of transplantation was examined in several fully allogeneic strain combinations. Purification and accurate quantification of the MoAb has allowed the determination of the critical parameters which determine the effectiveness of the anti-CD4 MoAb *in vivo* and has permitted these factors to be correlated with the phenotypic and functional changes that occur in the recipient's lymphocyte populations following MoAb treatment.

PATIENTS AND METHODS

Mice

C3H/He(H-2^k) and BALB/c(H-2^d) mice were purchased from Harlan Olac, Ltd (Bicester, UK). C57BL/10(H-2^b) and SJL (H-2^s) mice were obtained from either Harlan Olac, or from the NIMR (Mill Hill, UK). All mice were housed in conventional facilities at the Biomedical Services Unit, John Radcliffe Hospital, Oxford, UK, and were males between 8 and 14 weeks old at the time of first treatment.

Anti-CD4 MoAb

The YTS 191.1 hybridoma ([8], the kind gift of Dr Herman Waldmann, University of Cambridge, UK) was grown in 2,6,10,14-tetramethylpentadecane (Pristane) (Koch-Light Ltd., Haverhill, UK) primed (DA X LOU) F_1 rats. The antibody was purified from ascites by precipitation with 50% (w/v) ammonium sulphate followed by DEAE Sephacel (Pharmacia, Uppsala, Sweden) ion-exchange chromatography, and the selected effluent was dialysed in PBS. The protein content was measured by the absorbance at 280 nm. The purity of each MoAb preparation was at least 85% as determined by densitometric analysis of a 12·5% SDS–PAGE gel. The purified YTS 191.1 MoAb was stored in aliquots at −20°C, and diluted in sterile PBS prior to use *in vivo*.

Treatment protocols

Heart transplant recipients were treated intravenously with YTS 191.1 the day before transplantation and with a second dose immediately upon completion of the procedure. Recipients transplanted at intervals after treatment were given a dose of YTS 191.1 on two consecutive days, with the day of transplant being recorded as the number of days after the second dose of MoAb. Mice killed for analysis of their lymphocytes *in vitro* were treated intravenously with a dose of YTS 191.1 on two consecutive days, with the day of analysis being recorded as the time after the second dose. All doses of YTS 191.1 were administered in 0·5 ml of sterile PBS. Control mice received no treatment.

Heart transplantation

Fully vascularized heterotopic heart transplantation was performed using microsurgical techniques essentially as described by Corry *et al.* [28]. Rejection was defined by the loss of palpable cardiac contractions or the cessation of all electrical activity on the electrocardiogram [29].

Phenotypic analysis of lymphoid compartments after YTS 191.1 treatment

Lymphocytes were isolated from spleen, lymph node (peri-abdominal aorta, mesenteric, axillary and mediastinal locations) and thymus by centrifugation over a Ficoll/sodium diatrizoate (Histopaque 1083, Sigma Chemical Co., Poole, UK) gradient. Cells (10^7) were incubated in conditions of antibody excess for 45 min at 4°C. For single-colour analysis this consisted of 25 μl of spent tissue culture medium followed by FITC-conjugated goat anti-rat IgG (Cat. no. F-6258, Sigma). Two-colour analysis was performed using a combination of directly FITC-conjugated and biotinylated antibodies. Biotinylated antibodies were followed with a second stage of streptavidin-PE (Cat. no. P-162, Southern Biotechnology Associates, Birmingham, AL). MoAbs used in both types of analysis were: (i) anti-CD3: KT3 [30]; (ii) anti-CD4: YTS 191.1 [8]; (iii) anti-CD8: YTS 169 [8]. Cells were stored in PBS with 2% formaldehyde before flow cytometric analysis using an Orthocytofluorograf System 50 (Ortho Diagnostic Systems).

Mixed lymphocyte culture

Responder lymphocytes from spleen, thymus and lymph node were isolated from the interface of a Ficoll/sodium diatrizoate gradient. Splenocytes were further purified by selecting the non-adherent population after incubation for 2 h at 37°C in a 10-cm diameter tissue culture Petri dish (Nuclon, Roskilde, Denmark). Stimulator splenocytes were prepared by water lysis and irradiated (25 Gy) before use. All cells were resuspended at $2{\cdot}5 \times 10^6$/ml in RPMI supplemented with 5% fetal calf serum (FCS), 2 mM glutamine, 2-mercaptoethanol, streptomycin (45 μg/ml), penicillin (45 μg/ml) and kanamycin (90 μg/ml). All cultures were performed using a 1:1 stimulator-to-responder ratio in triplicate in 96-well U-bottomed plates with 200 μl per well at 37°C and 5% CO_2 in a humidified incubator. On days 3, 4, 5 and 6 of culture, 1 μl of ^{3}H-thymidine (1 μCi/μl, Amersham Corp., Amersham, UK) was added to each well and the cells harvested on to filter paper 16–20 h later. The incorporated ^{3}H-thymidine was measured using a 1205 BetaPlate Counter (Pharmacia Wallac, Turku, Finland).

Statistical analysis

Allograft survival between all groups within an experiment was compared by the Kruskal–Wallis test and two groups compared with each other using the Mann–Whitney–Wilcoxon test [31]. The significance of differences between the lymphocyte phenotypic expression of two experimental groups was assessed using the unpaired Student's *t*-test, while the relative effect of treatment on two different strains of mice was compared by regression analysis [32]. Statistical analysis was performed using Minitab 6.2 software (Minitab Inc. State College, PA).

RESULTS

Successful prolongation of allograft survival

Treatment of recipient mice with YTS 191.1 in the perioperative period produced significant prolongation ($P \leq 0{\cdot}05$) of cardiac allograft survival in all of the fully allogeneic (entire H-2 plus multiple minor histocompatibility antigens) strain combinations tested (Table 1).

Table 1. YTS 191.1 prolongs cardiac allograft survival

Donor strain	Recipient strain	Treatment	*n*	Survival time (days)	MST (days)	*P* value‡ *versus* control
C57BL/10	C3H/He	Control	4	8, 8, 11, 12	9	
C57BL/10	C3H/He	YTS 191.1*	3	100, 100, 100	>100	0·05
BALB/c	C3H/He	Control	5	7, 7, 7, 7, 8	7	
BALB/c	C3H/He	YTS 191.1†	5	24, 34, 34, 93, 100	35	0·01
C57BL/10	BALB/c	Control	4	8, 9, 11, 13	10	
C57BL/10	BALB/c	YTS 191.1*	5	37, (100)×4	>100	0·02
C3H/He	BALB/c	Control	4	6, 8, 8, 13	8	
C3H/He	BALB/c	YTS 191.1*	6	8, 37, (100)×4	93	0·04

* Recipients were transplanted on day 0 after treatment with 150 μg of YTS 191.1 on day −1 and day 0.

† Recipients were transplanted on day 0 after treatment with 100 μg of YTS 191.1 on day −1 and day 0.

‡ Significance value is for treated *versus* control.

MST, Median survival time.

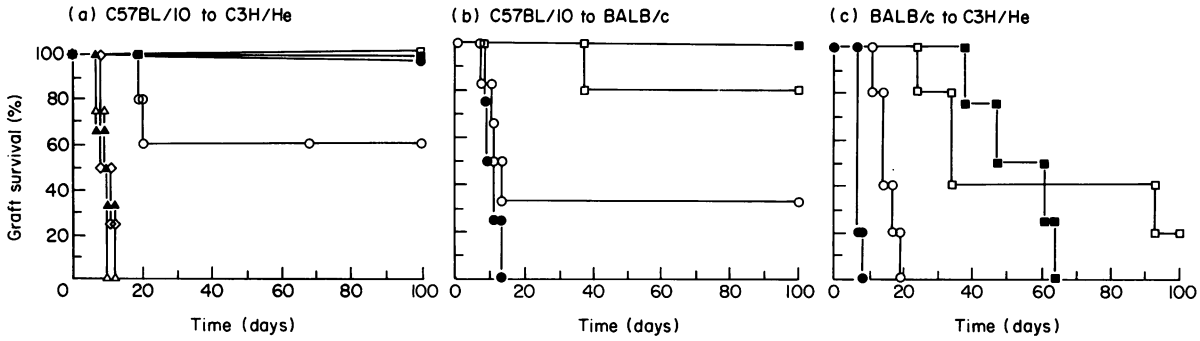

Fig. 1. Prolongation of allograft survival is dependent on the dose of YTS 191.1 (a) Groups of C3H/He mice were treated with decreasing dosages of YTS 191.1 on day −1 and day 0 as follows: □, 150 μg/dose (mean survival time (MST) >100 days, $n=3$, $P=0·05$); ■, 100 μg/dose (MST >100 days, $n=5$, $P=0·02$); ○, 50 μg/dose (MST >100 days, $n=5$, $P=0·02$); ●, 25 μg/dose (MST >100 days, $n=4$, $P=0·03$); △, 10 μg/dose (MST=9 days, $n=4$, $P=0·66$); ▲, 5 μg/dose (MST=10 days, $n=6$, $P=0·59$); ◇, no treatment (control, MST=9 days, $n=4$). Heterotopic transplantation of a C57BL/10 heart was on day 0. Significance values are *versus* control. (b) Groups of BALB/c mice were treated with decreasing dosages of YTS 191.1 on day −1 and day 0 as follows: □, 150 μg/dose (MST >100 days, $n=5$, $P=0·02$); ■, 50 μg/dose (MST >100 days, $n=4$, $P=0·03$); ○, 10 μg/dose (MST=12 days, $n=6$, $P=0·45$); ●, no treatment (control, MST=10 days, $n=4$). Heterotopic transplantation of a C57BL/10 heart was on day 0. Significance values are *versus* control. (c) Groups of C3H/He mice were treated with decreasing dosages of YTS 191.1 on day −1 and day 0 as follows: □, 100 μg/dose (MST=35 days, $n=5$, $P=0·01$); ■, 50 μg/dose (MST=54 days, $n=4$, $P=0·02$); ○, 25 μg/dose (MST=17 days, $n=5$, $P=0·01$); ●, no treatment (control, MST=7 days, $n=5$). Heterotopic transplantation of a BALB/c heart was on day 0. Significance values are *versus* control.

Definition of the minimal effective dose of YTS 191.1

The effect of the MoAb dose on the prolongation of graft survival was examined in three strain combinations. When C57BL/10 hearts were transplanted into either C3H/He (Fig. 1a) or BALB/c (Fig. 1b) mice, a dose of 25 μg and 50 μg respectively was found to produce indefinite graft survival in greater than 80% of recipients, while 10 μg/dose or less had little effect (Fig. 1a,b). However, in the BALB/c to C3H/He combination treatment with 25 μg or 50 μg of MoAb per dose did not induce indefinite graft survival, and even when higher doses of MoAb were used (100 μg/dose) only 20% of the grafts survived indefinitely (Fig. 1c). Thus, small amounts of YTS 191.1 prolonged cardiac allograft survival in all of the strain combinations tested, but the minimal effective dose varied.

YTS 191.1 produces prolonged non-specific immunosuppression

The duration of non-specific immunosuppression resulting from treatment with YTS 191.1 was investigated in the C57BL/10 to C3H/He strain combination. Heart grafts were transplanted at weekly intervals after a dose of 25 μg of YTS 191.1 on two successive days (Table 2). Indefinite survival was obtained from 100% of grafts transplanted 14 days after treatment, but if transplantation was delayed until 42 days after treatment, none of the hearts survived indefinitely. In a second experiment, a

Table 2. Prolonged but finite duration of immunosuppression

Treatment*	Interval from treatment to transplant (days)	*n*	Survival time (days)	MST (days)	*P* value‡ *versus* control
Control§	NA	4	8, 8, 11, 12	9	NA
YTS 191.1	0	4	63, 91†, >100, >100	95	0·03
YTS 191.1	7	4	38, (>100)×3	>100	0·03
YTS 191.1	14	4	(>100)×4	>100	0·03
YTS 191.1	21	4	18, (>100)×3	>100	0·03
YTS 191.1	28	8	(17)×3, 20, 42, 95, 87†, >100	20	0·01
YTS 191.1	42	10	8, 9, 11, 18, 19, 20, 20, 42†, 77, 82	19	0·09

* Groups of C3H/He mice were treated with 25 μg of YTS 191.1 on day −1 and day 0 and transplanted with a C57BL/10 cardiac allograft at intervals after treatment as indicated.

† Recipient found dead with a surviving allograft at last assessment.

‡ Significance value is for treated *versus* control, which were C3H/He mice transplanted with a C57BL/10 heart without treatment.

§ This group is a representation of the control data presented for this strain combination in Table 1.

MST, Mean survival time.

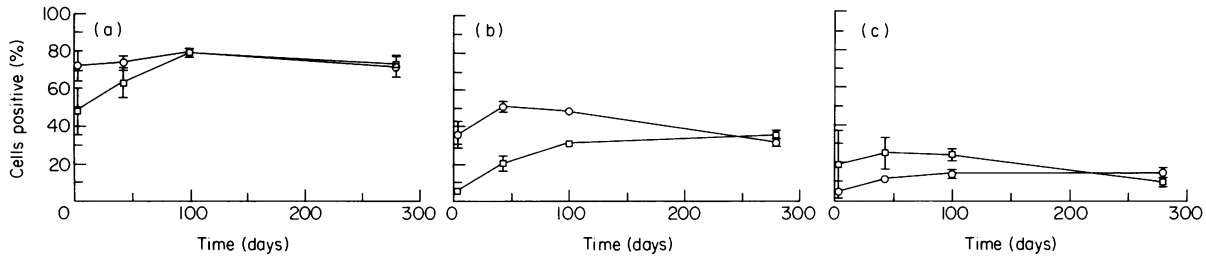

Fig. 2. Persistent T cell phenotypic changes in the peripheral lymph nodes after YTS 191.1 treatment. Groups of C3H/He mice were treated with a dose of 25 μg of YTS 191.1 on day −1 and day 0, and the peripheral lymph nodes harvested for analysis on days 3, 42, 100 and 280. Naive controls received no treatment. Results are expressed as the mean ± s.d. of three animals analysed individually at each point for the per cent of lymphocytes expressing CD3 (a), CD4 (b), and CD8 (c). The initial decrease in the percent $CD3^+$ cells in the treated mice ($P=0·06$) has resolved by 100 days after treatment ($P=0·75$). In contrast, the decrease in the percentage of cells that are $CD4^+$ at 3 days after treatment ($P=0·02$) remains at 100 days ($P=0·0003$), but does return to normal by 280 days ($P=0·12$). Likewise, the per cent of $CD8^+$ cells remains increased at 100 days after treatment ($P=0·03$), but is the same as in naive mice by 280 days ($P=0·15$). ○, Naive; □, YTS 191.1.

total of 12 C3H/He mice were transplanted 42 days after treatment with two 25-μg doses of YTS 191.1; 11 of these recipients rejected the C57BL/10 allograft within 11 days, while one graft survived for 31 days. Thus, by 42 days after MoAb treatment these mice are immunocompetent, as judged by their ability to reject a cardiac allograft.

YTS 191 MoAb produces marked and prolonged depletion of peripheral $CD4^+$ cells

Three days after two 25-μg doses of YTS 191.1 there was a marked (>80%), but incomplete elimination of the $CD4^+$ cells in the peripheral lymph nodes (Fig. 2b). This was followed by a gradual repletion of $CD4^+$ cells, so that by 42 days after treatment the percentage of peripheral lymphocytes expressing the CD4 molecule was approximately 50% of that in naive mice. The initial decrease in the percentage of lymph node cells expressing CD3 (Fig. 2a) was not as great as that for CD4, and thus early after MoAb treatment there was a compensatory increase in the percentage of cells expressing CD8 (Fig. 2c). The percentage of $CD3^+$ cells in YTS 191.1-treated mice had returned to naive levels by 100 days after treatment (Fig. 2a). However, the ratio of $CD4^+$ to $CD8^+$ cells was still decreased (Fig. 2b,c). By 280 days after treatment the percentage of lymphocytes in each subset had returned to normal in the treated mice (Fig. 2). Increasing the dose of YTS 191.1 to 250 μg did not result in further significant depletion of $CD4^+$ cells in the lymph nodes at 3 days after treatment (250 μg, 3·0 ± 0·4% *versus* 25 μg, 4·9 ± 0·7%, mean ± s.d. of three mice analysed individually). However, only at the high dose was residual MoAb detectable on the surface of lymph node leucocytes at 3 days after treatment. Furthermore, the few $CD4^+$ cells remaining after both the 25-μg and 250-μg dose treatments showed significantly decreased fluorescence intensity compared with cells from naive mice, suggesting modulation of CD4 can occur when a depleting anti-CD4 MoAb is used. Similar phenotypic changes were also found in the leucocytes isolated from the spleen when analysed at 3–42 days after treatment. In the thymus, no phenotype changes were observed at 3 days after two

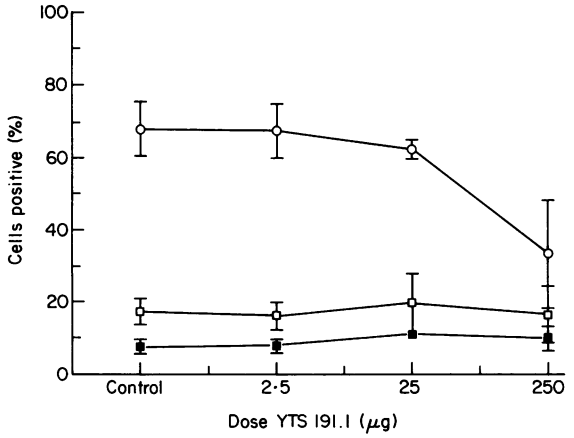

Fig. 3. Dose response analysis of T cell phenotypic changes in the thymus after YTS 191.1 treatment. Groups of C3H/He mice were treated with a dose of 250 μg, 25 μg or 2·5 μg of YTS 191.1 on day −1 and day 0, and the thymocytes isolated for analysis on day 3. Results are expressed as the mean ± s.d. of three animals analysed individually at each point, except for the 250 μg/dose group which had two mice. Two-colour analysis documents no change in the percentage of thymocytes that are $CD3^+4^+$ or $CD3^+8^+$ after any treatment dose. After the 250 μg/dose treatment there was a decrease in the percentage of thymocytes expressing $CD4^+8^+$, but this change did not reach statistical significance ($P = 0{\cdot}21$). □, CD3/CD4; ■, CD3/CD8; ○, CD4/CD8.

25-μg doses of YTS 191.1, whereas when 250 μg/dose was administered there was a decrease in the immature, $CD4^+8^+$ population (Fig. 3).

The ability of YTS 191.1 to deplete peripheral $CD4^+$ cells is not strain-dependent. Equivalent elimination of $CD4^+$ cells was found in C3H/He (48·9 ± 4·5% to 3·8 ± 2·5%) and BALB/c (49·1 ± 6·0% to 3·2 ± 0·9%) mice 3 days after YTS 191.1 treatment (50 μg/dose on 2 consecutive days). These results are the mean ± s.d. of four mice analysed individually.

A persistent decrease in proliferation to alloantigen in vitro

The proliferative response to BALB/c, SJL and C57/BL10 alloantigen *in vitro* of lymphocytes from thymus, lymph node and spleen was compared between that of lymphocytes from naive C3H/He mice and those at various time intervals after treatment with YTS 191.1 (25 μg/dose). There was a marked and persistent decrease in proliferation when the responding cells were from the peripheral lymph nodes of YTS 191.1-treated mice (Table 3). Leucocytes from treated recipients remained hyporesponsive in the mixed leucocyte culture (MLR) even after immunocompetence had returned *in vivo*, 42 days after treatment (Tables 2 and 3).

Table 3. Persistent non-specific hyporesponsiveness in the mixed leucocyte culture (MLC)

		Stimulator strain					
		C57BL/10		BALB/c		SJL	
Day	Group*	ct/min†	RR‡	ct/min†	RR‡	ct/min†	RR‡
3	Naive	222 453		229 381		293 837	
	Treated	48 261	0·22	20 112	0·08	70 316	0·24
7	Naive	178 685		211 183		195 472	
	Treated	24 545	0·14	39 228	0·19	65 085	0·33
14	Naive	151 704		108 749		124 275	
	Treated	91 315	0·60	65 642	0·60	77 472	0·62
21	Naive	222 759		152 772		150 073	
	Treated	98 489	0·44	74 686	0·49	70 493	0·47
28	Naive	113 423		102 324		208 577	
	Treated	61 895	0·55	67 346	0·66	107 986	0·52
42	Naive	117 234		140 421		164 312	
	Treated	71 222	0·61	57 623	0·41	101 691	0·62

* Treated C3H/He mice were given 25 μg of YTS 191.1 on two consecutive days. Lymphocytes from the lymph nodes (pooled from mesenteric, peri-abdominal aorta, axillary and mediastinal nodes) were pooled from four treated and four naive mice at each time point for analysis at 3, 7, 14, 28 and 42 days after the first day of treatment and proliferation of C3H/He lymphocytes in response to C57BL/10, BALB/c and SJL splenocyte stimulators measured at days 3, 4, 5 and 6 in culture.

† Proliferation (counts per minute of incorporated ^{3}H-thymidine) in the allogeneic minus that in the syngeneic MLC. Results are shown from the day of culture of maximal naive proliferation.

‡ Relative response. Results are expressed as the response in treated mice relative to that of cells from naive mice on the day of culture of maximal naive proliferation. This was calculated using the following formula:

$$\text{Relative response} = \frac{(\text{treated allogeneic}) - (\text{treated syngeneic})}{(\text{naive allogeneic}) - (\text{naive syngeneic})}.$$

DISCUSSION

The anti-CD4 MoAb, YTS 191.1, is a potent immunosuppressive agent. Given alone in the peri-operative period this MoAb prolonged cardiac graft survival in all of the full H-2 and miH antigen mismatched strain combinations tested (Table 1). The potency of YTS 191.1 is also attested to by the minimal doses required to produce indefinite graft survival (Fig. 1) and the duration of the immunosuppressive effect (Table 2). While a similar dose response analysis with anti-CD4 MoAb has not been carried out in other transplantation models, the minimal effective total dose (approximately 2 mg/kg body weight) determined in this study for the C57BL/10 to C3H/He strain combination is significantly lower than has been used successfully in other vascularized organ transplantation models testing anti-CD4 MoAb [18–20,33], but similar to the doses of these MoAbs which have been shown to inhibit humoral responsiveness in the mouse [10,11].

Complete elimination of $CD4^+$ cells is not essential for cardiac allograft survival to be prolonged (Figs 1a and 2) [18–20], but on the other hand partial depletion of $CD4^+$ cells does not necessarily result in prolonged graft survival (Fig. 1). For example, a dose of 2·5μg of YTS 191.1 produces significant depletion (51%) of $CD4^+$ cells in the periphery (18·1 ± 2·3% positive *versus* 35·4 ± 6·8% positive in controls, mean ± s.d., $P = 0{\cdot}05$), but this dose does not produce any prolongation of graft survival (Fig. 1). Furthermore, C3H/He mice regain the ability to reject C57/BL10 grafts, if transplantation is delayed until 42 days after treatment with YTS 191.1 (Table 2), and yet at this time point the $CD4^+$ subset has returned to only approximately 50% of normal (Fig. 2). While transplantation at either of these points fails to produce indefinite graft survival, there is some prolongation of graft survival in the group

transplanted 42 days after treatment (Table 2) and thus the level of depletion of the $CD4^+$ population at the time of transplantation is not a reliable predictor of graft survival.

The effects of a depleting MoAb on the immune response are not necessarily limited to target cell elimination. The dose response analysis with YTS 191.1 provides considerable insight into the actions of this bivalent rat IgG2b MoAb on peripheral lymphocytes, and indicates that a combination of $CD4^+$ cell elimination, modulation of the cell surface antigen, and MoAb coating of the CD4 molecule can all occur. All of these changes may influence the efficiency of MoAb therapy.

An examination of the phenotypic changes in the thymus after anti-CD4 MoAb treatment may also be important, as Herbert & Roser found that prior thymectomy abrogated the ability of anti-CD4 MoAb to induce long-term survival of rat neonatal cardiac grafts in a high responder strain combination [33]. In our study, YTS 191.1 treatment had little effect on the phenotype of thymocytes at the two lower doses of MoAb, but with a 250-μg dose there was a selective decrease in the $CD4^+8^+$ population (Fig. 3) and MoAb coating of a small percentage of the $CD4^+$ cells (data not shown). This therefore indicates that the MoAb delivered intravenously can reach the thymic cortex where the majority of $CD4^+8^+$ cells reside. Although this finding is in disagreement with the long-standing concept of a blood–thymus barrier [34], there is now very convincing evidence that MoAbs do have access to the thymic cortex, perhaps by the transcapsular route [35]. This route of entry would deliver MoAb directly to the cortex where $CD4^+8^+$ cells constitute the vast majority of the thymocytes, but it does not, however, explain cell elimination, as the thymus is thought to be devoid of the effector mechanisms required for MoAb-facilitated cell death [36].

How then does anti-CD4 MoAb treatment result in the selective elimination of $CD4^+8^+$ thymocytes in the adult mouse? While our studies cannot exclude a role for complement-mediated lysis of $CD4^+$ thymocytes, further understanding may be gained by an analysis of the critical role of the CD4 molecule in the intrathymic selection process of thymocyte development [37–39]. Anti-CD4 MoAb may alter this process as a result of inhibiting the binding of CD4 to class II MHC [40] or via a direct effect on the CD4 signalling mechanism [41]. Another intriguing possibility is that the anti-CD4 MoAb may modulate the selection process by augmenting the T cell receptor (TCR) expression of the developing thymocytes. This hypothesis is supported by recent work which shows that the anti-CD4 MoAb GK1.5, when given *in vivo* to neonatal mice, selectively increases the level of TCR expression on $CD4^+8^+$, but not on mature $CD4^+8^-$ thymocytes [42]. The relevance of these thymic changes to cardiac allograft survival in our model is not clear.

The persistent decrease in the proliferative response to alloantigen by spleen and lymph node leucocytes from anti-CD4-treated mice (Table 3) is readily explained by the persistent decrease in the per cent of $CD4^+$ cells in these tissues after treatment (Fig. 2). However, the significance of these findings to the allograft model must be questioned because in this study, as has been shown by other investigators [18,43–45], this *in vitro* measure of T cell function does not accurately predict allograft survival *in vivo*. Thus, although proliferation to C57/BL10 stimulators *in vitro* is reduced 42 days after treatment with anti-CD4 MoAb, at this time point recipients are able to reject a C57BL/10 cardiac allograft (Table 2).

This study has demonstrated a marked depletion, but not complete elimination of peripheral $CD4^+$ cells after treatment with anti-CD4 MoAb that results in prolonged cardiac allograft survival. These changes are of long duration, are dependent on MoAb dose and the tissue of origin of the target cell, but are not strain-specific. This study provides further insight into the complex effects of anti-CD4 MoAb on the murine immune system and forms a basis from which rational modifications to treatment protocols in transplantation models can be made.

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