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Allograft rejection in athymic nude rats by transferred T-cell subsets

I. THE RESPONSE OF NAIVE CD4$^+$ AND CD8$^+$ THORACIC DUCT LYMPHOCYTES TO COMPLETE ALLOGENEIC INCOMPATIBILITIES

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SUMMARY

PVG.rnu/rnu nude rats were pre-grafted with two allogeneic skin grafts, AO(RT1^u) and BN(RT1^n), 6–14 days in advance of cell transfer. Cellular requirements for rejection were established by transferring graded numbers of B cell-depleted (Ig$^-$) thoracic duct lymphocytes (TDL) or purified W3/25$^+$ (CD4$^+$) or OX8$^+$ (CD8$^+$) TDL subsets. Allografts were rejected by 10^5 to 5×10^6 Ig$^-$ TDL in a dose-dependent fashion. A similar dose–response relationship was found by transferring 5×10^5 to 5×10^6 Ig$^-$OX8$^-$ TDL (purified by depletion of B cells and OX8$^+$ cells). Larger numbers of Ig$^-$OX8$^-$ TDL ($10–30 \times 10^6$) did not significantly accelerate rejection. W3/25$^+$ TDL alone (10^5), highly purified by fluorescence-activated cell sorting (FACS), were sufficient to induce allograft rejection in this athymic nude rat model. In contrast, 10 times more FACS purified OX8$^+$ TDL (10^6) were unable to initiate skin graft rejection despite the complete class I and class II MHC incompatibilities. Furthermore, the addition of 10^6 OX8$^+$ cells did not accelerate or retard the rejection induced by 10^5 W3/25$^+$ cells alone. Pre-grafted nude recipients, irradiated (500 R) 2 hr before W3/25$^+$ TDL injection, in order to eliminate putative nude T cells, rejected allografts on the same day as unirradiated controls. We conclude that when confronted with complete MHC disparities, CD4$^+$ T cells are necessary and sufficient to induce skin allograft rejection whereas CD8$^+$ T cells do not appear to contribute.

INTRODUCTION

The mechanisms of allograft rejection are still incompletely understood. CD4 T-helper (Th) cells, CD8 T-cytotoxic (Tc) cells and inflammatory cells of the host have all been implicated in the rejection process (Mason *et al.*, 1984). In order to study these various components we wanted to establish an unambiguous model.

A number of different animal models have been used to investigate the activities of T-cell subsets in graft rejection. Immunologically crippled recipients were variously prepared by administering whole-body irradiation (Hall, Dorsch & Roser, 1978a,b; Lowry, Gurley & Forbes, 1983) or more recently by giving anti-T cell monoclonal antibodies (mAb) with or without thymectomy (Cobbold *et al.*, 1984; Cobbold & Waldman, 1986). These models allowed the study of the rejection process through a 'window' before the recipient recovered immunocompetency. Thymectomy followed by lethal irradiation and stem cell (bone marrow or foetal liver) replacement created recipients (TXBM) that could remain T-cell deficient indefinitely (Loveland *et al.*, 1981; Dallman, Mason & Webb, 1982). However, the unphysiological intervention required, and the potential for incomplete T-cell depletion, introduce a degree of uncertainty. The potential for a small number of T cells to expand *in vivo* in T-cell deficient animals is enormous (Bell *et al.*, 1987).

The complete failure of athymic nude animals to develop a functioning thymus during ontogeny (Wortis, Nehlsen & Owen, 1971; Van Vliet *et al.*, 1985) or to exhibit T-cell mediated responses, including allograft rejection, at any stage (Kindred, 1979; E. B. Bell *et al.*, submitted) recommends this model for the study of transplantation rejection mechanisms. We have recently shown that the progeny of mature T cells (CD4 and in some cases CD8) survive for at least 2 years after adoptive transfer to nude rats (Bell *et al.*, 1989) and that the nude recipient does not develop its own functioning T cells during this period (Drayson, Sparshott & Bell, 1989).

In view of conflicting reports in the literature (see reviews by Steinmuller, 1985; Mason & Morris, 1986) we aimed to establish definitively whether CD4$^+$ and/or CD8$^+$ T cells were autonomous with respect to skin allograft rejection. Using highly purified T-cell subsets from the thoracic duct (TDL) of syngeneic naive donors, and pre-grafted nude recipients, we show that grafts were rejected by T cells in a dose-dependent fashion;

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CD4+ TDL were necessary and sufficient to induce rejection, whereas highly purified CD8+ TDL alone were unable to destroy skin transplants bearing a full MHC mismatch.

MATERIALS AND METHODS

Rats

Inbred AO ($RT1^u$), DA($RT1^{av1}$), LEW($RT1^l$) and PVG.rnu/rnu ($RT1^c$) athymic nude rats were bred and maintained in a conventional environment by the Animal Unit, Manchester University Medical School, as previously detailed (Bell *et al.*, 1987). PVG ($RT1^c$) and BN ($RT1^n$) strains were bred in SPF isolator conditions until weaning and thereafter held under conventional animal laboratory conditions. AUG ($RT1^c$) rats were purchased from OLAC Ltd.

Thoracic duct lymphocytes (TDL)

These cells were obtained by cannulation of the thoracic duct as before (Bell *et al.*, 1987).

Skin grafting

Grafting was carried out as described previously (Bell *et al.*, 1987). Two different allogeneic skins were grafted on to nude recipients in advance of cell transfer, one on each lateral aspect of the thorax. Rejection was scored as the day when > 50% of a graft became necrotic.

Fluorescent staining

W3/25 (anti-CD4), FITC-W3/25, OX8 (anti-CD8), FITC-OX8, OX12 (anti-rat kappa light chain) and OX21 or W6/32 (anti-human, non-cross-reacting controls) mouse mAb were purchased (Serotec Ltd, Oxford) as ascites, diluted 1/100 and detected with either FITC–anti-mouse IgG or FITC–$F(ab')_2$–anti-mouse IgG (DAKO Ltd, High Wycombe, Bucks) diluted with 1% normal rat serum to block the cross-reaction with rat Ig. FITC–anti-rat Ig (Wellcome Reagents Ltd, Beckenham, Kent) was diluted 1/50 and used as a direct stain for B cells. Staining was scored by fluorescence microscopy and latterly by fluorescence-activated cell sorter (FACS) analysis.

Cell separation

A detailed description of cell separation by 'panning' and magnetic bead depletion is given elsewhere (Bell *et al.*, 1989). Briefly, B cells were depleted from TDL by adherence to plastic petri-dishes coated with immunopurified, sheep anti-rat-$F(ab')_2$ IgG. CD4+ or CD8+ cells were prepared by staining TDL with OX8 or W3/25, respectively, and allowing cells to adhere to plates coated with immunopurified sheep anti-mouse IgG. In later experiments anti-mouse IgG-coated magnetic Dynal beads (Dynal Ltd, New Ferry) or Biomag particles (Advanced Magnetics Inc., Piddington) were used for depletion of TDL stained with OX12, OX6 (anti-class II) and OX8 (or W3/25) instead of the panning method.

Irradiation

Rats were given whole-body irradiation from a linear accelerator.

RESULTS

Dose–response of Ig⁻ TDL

Athymic PVG nude rats were grafted 6–14 days in advance of cell transfer in order to allow skin transplants to establish a vascular supply before the onset of rejection. Cells for transfer were always obtained from TDL. Graded doses of PVG (+/+) TDL, depleted of B cells by panning (Ig⁻ TDL), were transferred into nude recipients each bearing two different allogeneic skin grafts, AO and BN. The results of seven experiments were pooled. The mean composition of the injected cells was as follows: 75·0% W3/25+, range 71·4–83·3%; 14·5% OX8+, range 11·9–18·5%; 3·85% Ig+, range 1·8–7·0%. Rejection of the allografts was dose-dependent (Fig. 1). BN skin was rejected 3–4 days faster than AO skin. As few as 10^5 Ig⁻ lymphocytes were sufficient to induce rejection; 5×10^6 Ig⁻ TDL, the largest dose tested in this series of experiments, rejected AO and BN allografts in 13·1 and 10·4 days, respectively.

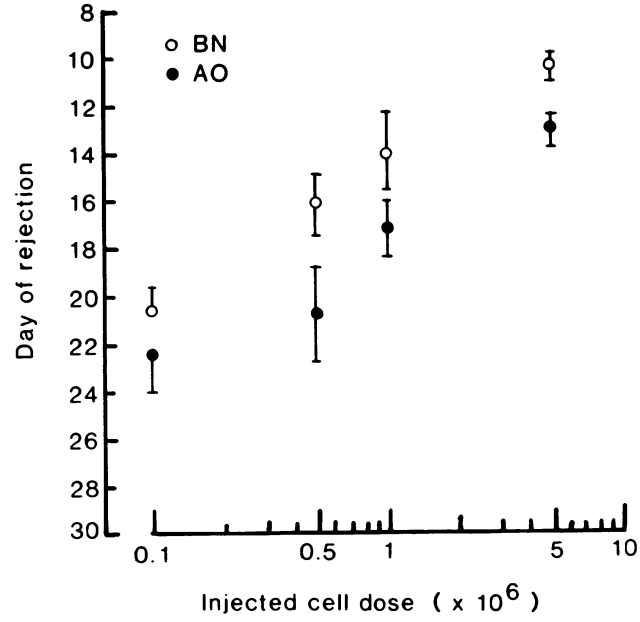

Figure 1. Skin allograft rejection by B cell-depleted (Ig⁻) TDL transferred to pre-grafted nude rats. Each point represents the mean day of rejection ± SE of 5–10 grafts. Nude recipients received AO and BN skin transplants 6–9 days before cell transfer. Donor TDL were depleted of B cells by 'panning'.

Dose–response of Ig⁻ OX8⁻ (CD4+) TDL

In order to establish the contribution of CD4 and CD8 lymphocytes to the rejection process, TDL were depleted of B cells and OX8+ cells by panning or by rosetting with magnetic beads. The level of OX8+ cell contamination was 1·1–2·7% by the panning method and < 0·5% by magnetic bead depletion. These highly enriched CD4+ TDL induced skin allograft rejection with similar dose–response kinetics (Fig. 2) to that found for Ig⁻ TDL (Fig. 1). The speed of rejection increased with dose between 5×10^5 and 5×10^6 Ig⁻OX8⁻ cells. Injection of $> 5 \times 10^6$ cells did not significantly accelerate the destruction of the transplant. These results suggest that CD4+ cells alone are sufficient to initiate rejection across a complete MHC incompatibility, in agreement with previous findings (Loveland *et al.*, 1981; Dallman *et al.*, 1982; Hall, de Saxe & Dorsch, 1983; Rosenberg *et al.*, 1987). They do not, however, rigorously exclude a requirement for CD8+ cells.

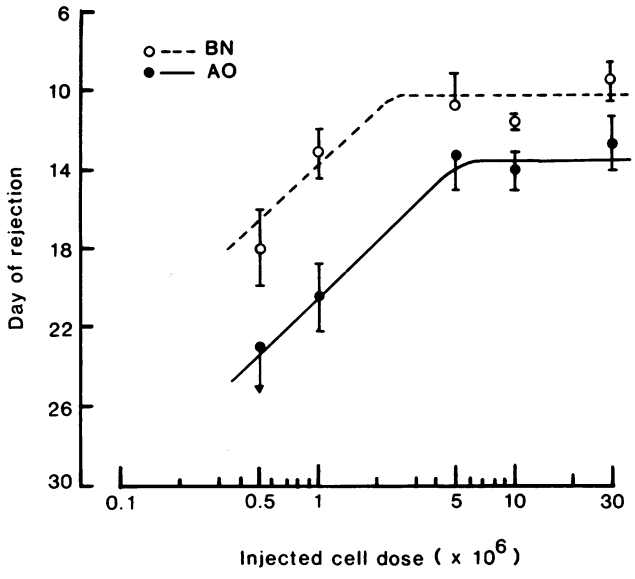

Figure 2. Skin allograft rejection by Ig^- $OX8^-$ ($CD4^+$) TDL transferred to pre-grafted (6–14 days) nude recipients. Each point represents the mean ±SE of 3–18 grafts; as the dose increases $n=5$, 18, 4, 4, 5, respectively. Rejection of AO skin with 5×10^5 cells was based on three recipients; two additional rats died on Days 27, 28 with AO skin intact. TDL were depleted of Ig^+ and $OX8^+$ cells by 'panning' or by magnetic beads before transfer.

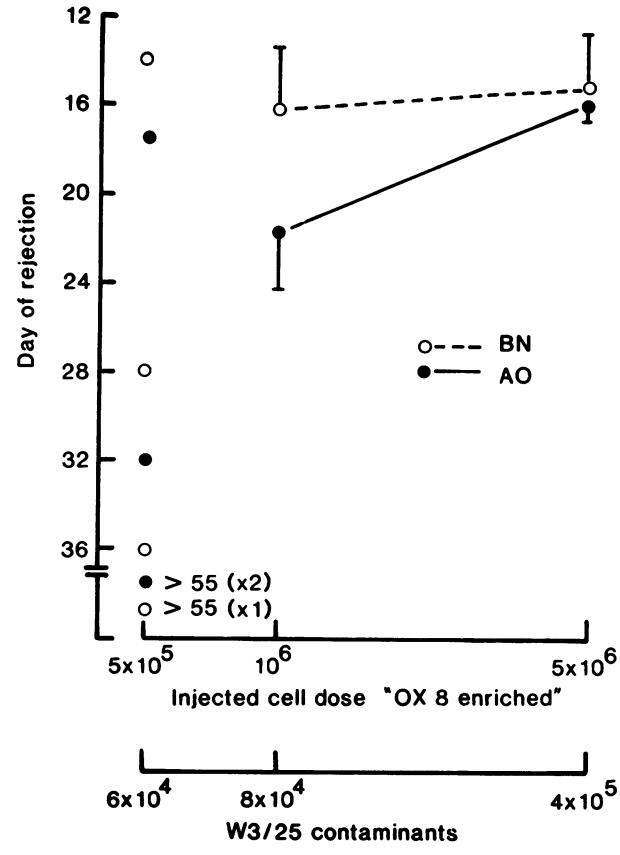

Figure 3. Skin allograft rejection by OX8-enriched TDL depleted of Ig^+ and $W3/25^+$ cells by 'panning' before transfer to pre-grafted nude recipients. Points are means ± SE of five grafts except for the lowest dose in which points represent individual grafts. '>' indicates grafts perfect at time of death. Lower scale gives the number of $W3/25^+$ cells co-injected with OX8-enriched TDL.

Dose–response of OX8-enriched TDL

Because $OX8^+$ cells represent only 7–10% of TDL in PVG rats, it proved difficult to obtain highly purified $OX8^+$ cells by panning alone. The effect of using TDL enriched for $OX8^+$ cells by panning on allograft rejection is illustrated in Fig. 3. An indication of the number of $W3/25^+$ cells contaminating the injected population is also included in Fig. 3. Pre-grafted nude recipients received 5×10^5, 10^6 or 5×10^6 OX8-enriched TDL. At the lowest dose, rejection times ranged from 14 to 36 days and two nudes died at Day 50; one animal had one skin graft still intact. At the two higher doses, all nude recipients destroyed the allografts, but there was not a clear dose–response relationship. Recipients who received 5×10^6 OX8-enriched TDL rejected AO and BN grafts on nearly the same day (15·9 and 15·0 days, respectively). At first glance, this suggests that $CD8^+$ T cells can induce rejection, but because of the low $CD4^+$ cell contamination, this experiment does not critically address the question of whether $OX8^+$ cells alone were sufficient and necessary to cause rejection.

Rejection by highly purified T-cell subsets

In an attempt to obtain uncontaminated T-cell subpopulations, TDL were initially depleted of B cells plus one or other T-cell subset, and subsequently sorted by FACS after staining with FITC-W3/25 or FITC-OX8. $Ig^-CD4^-CD8^-$ 'null' cells, which co-purify by conventional depletion strategies, were thus excluded. 10^5 $W3/25^+$ TDL (equivalent to $2{\cdot}5 \times 10^5$ unfractionated TDL) with virtually no $CD8^+$ cells (less than 200 $OX8^+$ cells would have been transferred), rejected AO and BN skin

Table 1. Allograft rejection in nude recipients injected with FACS purified $W3/25^+$ or $OX8^+$ TDL

	Cells injected*				
Group	$W3/25^+$	$OX8^+$	Skin	Day of rejection	Mean ± SD
1	10^5	None	AO	†, 23, 26·5, 23, 40	28·1 ± 8·1
			BN	20, 22, 16, 25, 33	23·2 ± 6·4
2	None	10^6	AO	>70‡ (×5)	NR§
			BN	>70 (×5)	NR
3	10^5	10^6	AO	21, 26, 33, 38, †, 40	31·6 ± 8·0
			BN	21, 17·5, 18, †, 26·5, 23	21·2 ± 3·7
4	10^5	None	LEW	26·5, 30, >67, >55	28·3
			DA	25, 23, 48, >55	32

* TDL were depleted of Ig^+ cells plus $OX8^+$ or $W3/25^+$ cells using anti-mouse Ig-coated magnetic beads, then stained with FITC-W3/25 or FITC-OX8 and sorted by FACS. Lymphocytes injected into pre-grafted PVG nude recipients were 99·8% $W3/25^+$ or 99·5% $OX8^+$.

† Technical failure, no result.

‡ > indicates grafts perfect when the rat died or was killed.

§ Not rejected.

Table 2. Increased numbers of $OX8^+$ cells and $W3/25^+$ cells in nude recipients after injection of FACS purified $W3/25^-$ or $W3/25^+$ passaged TDL

Dose	Injected cells‡	17 week LN analysis*			22 week LN analysis*			Day of rejection†	
		$W3/25^+$	$OX8^+$	Ig^+	$W3/25^+$	$OX8^+$	Ig^+	BN	AUG
10×10^6	$W3/25^+$	36·1§	2·7	51·0	—	—	—	11	17
10×10^6	$W3/25^+$	31·5	2·4	47·4	—	—	—	9·5	18·5
$4·4 \times 10^6$	$W3/25^-$	10·4	9·6	33·3	18·6	10·1	55·8	>25¶	>25¶
Euthymic (rnu/+)**		—	—	—	31·1	7·4	48·7	—	—
Nude**		—	—	—	10·1	3·5	66·4	—	—

* A cervical LN was removed 17 and 22 weeks after cell transfer for FACS analysis. Individual rats analysed.

† Recipients skin grafted 19 weeks after cell transfer. An additional rat, injected with 10×10^6 $W3/25^+$ cells, rejected BN and AUG grafts on Days 9 and 14, respectively.

‡ Syngeneic TDL were passaged from blood to lymph through 700 R irradiated, thoracic duct-cannnulated intermediate rats to enrich for T cells. Passaged TDL (89·9% $W3/25^+$, 6·6% $OX8^+$, 1·7% Ig^+) were stained and sorted into $W3/25^+$ (98·5%) and $W3/25^-$ (83·6% $OX8^+$) cells for injection.

§ Percentage positive.

¶ Grafts perfect at time of death, Day 25 after grafting.

** Age-matched with $W3/25^-$ TDL injected nude (6·5 months old).

grafts on Days 28 and 23 (Table 1, Group 1). These rejection times agree closely with those predicted by extrapolating the rejection curves of Fig. 2 (Days 31 and 23, respectively). In contrast, 10 times more $CD8^+$ cells (equivalent to 10^7 unfractionated TDL) failed to evoke any sign of rejection (Table 1, Group 2). We further asked whether $CD8^+$ cells could be activated by a small number of $CD4^+$ cells to accelerate rejection or alternatively whether a relative excess of $CD8^+$ cells could retard and suppress the rejection by $CD4^+$ cells. From the results in Table 1 (Group 3), it is clear that the addition of 10^6 $CD8^+$ cells did not change the rejection time induced by 10^5 $CD4^+$ cells alone. $CD8^+$ cells neither augmented nor suppressed graft destruction.

We also examined the ability of purified $CD4^+$ cells to reject skin grafts from two additional strains of rat: DA and LEW (Table 1, Group 4). Again, $CD4^+$ cells alone induced rejection, although the day of rejection varied widely. In the case of three allografts (two LEW, one DA), the recipient nudes died before rejection had occurred.

$CD8^+$ T cells in nude recipients

It could be argued that the failure of highly purified $OX8^+$ TDL to reject skin allografts was due to the lack of $OX8^+$ cell survival in nude recipients. The survival and expansion of $CD4^+$ cells has been amply demonstrated in long-term restored, TDL-injected nude rats (Bell *et al.*, 1987; Drayson *et al.*, 1989). Analysis of three nude rats, restored with bona fide recirculating lymphocytes (passaged from blood to lymph through a syngeneic, irradiated intermediate rat) separated by FACS into $W3/25^+$ (98·5%$^+$) and $W3/25^-$ (83·6% $OX8^+$, <0·2% $W3/25^+$) subsets is given in Table 2. There was no evidence of an increase in $OX8^+$ cells in nude recipients that received $W3/25^+$ cells. Unrestored nude rats have low levels of $W3/25^+$ cells, (Fossum *et al.*, 1980; Bell *et al.*, 1987) and $OX8^+$ NK cells (Ward, Argilan & Reynolds, 1983). However, injection of $4·4 \times 10^6$ $W3/25^-$ cells ($3·7 \times 10^6$ $OX8^+$) (Table 2) resulted in considerable $OX8^+$-cell expansion (10% $OX8^+$ cells, 17–22 weeks later). Based on previous estimates of the size of the recirculating pool in nude rats (Bell *et al.*, 1987) and allowing for $OX8^+$ cells of nude origin, this represents a 7–10-fold expansion in $CD8^+$ cells. The nude recipient given $W3/25^-$ cells did not reject either BN or AUG (minor histocompatibility antigenic differences only) skin grafts despite the presence of an estimated $50–60 \times 10^6$ $OX8^+$ cells.

Rejection in irradiated nude recipients

The presence of cells in nude mice and rats bearing T-cell markers including CD8, together with additional evidence (see Bell, 1989), has encouraged the belief that nude animals have T lymphocytes that have developed extrathymically. Others have observed that infiltrating cells during rejection contain $CD8^+$ cells even when T cell-deprived recipients were given $CD4^+$ cells alone (Dallman *et al.*, 1982; Bolton *et al.*, 1989). If $OX8^+$ nude-derived cells are equivalent to $OX8^+$ thymus-derived T cells, they could explain the apparent rejection by $CD4^+$ cells alone in the nude model. By taking advantage of the radiosensitivity of lymphocytes [more than 90% of lymphocytes are destroyed by 300 R (Everett, Caffrey & Rieke, 1964)], it was possible to test whether such extrathymic T cells were recruited into the allograft rejection process. Dallman & Mason (1983) previously reported that recipient irradiation (800 R) was effective in eliminating class II positive macrophages from rejecting skin allografts. In the present experiment pre-grafted nude recipients were irradiated (500 R) 2 hr before cell transfer of 10^6 Ig^-OX8^- ($CD4^+$) TDL, with or without 2×10^5 $Ig^-W3/25^-$ ($CD8^+$) TDL. The time of rejection by Ig^-OX8^- TDL was unaffected by prior irradiation (Table 3). There was no evidence from this experiment that a radiosensitive nude-derived lymphocyte contributed to rejection. The addition of 2×10^5 $CD8^+$ TDL did not significantly influence the rate of rejection of AO skin; BN skin was rejected on average 4 days faster when $CD8^+$ T cells were included in the inoculum, but was well within the range of rejection observed in both irradiated and non-irradiated $CD4^+$

Table 3. W3/25$^+$ cells reject skin allografts in nude and irradiated nude recipients equally

Dose	Cells*	Irradiation	Skin	Day of rejection	Mean ± SD
10^6	W3/25$^+$	None	AO	17, 19·5, 19·5, 14·5, 18, 39, 17, 17·5	20·2 ± 7·7
			BN	10, 14, 15, 17, 16·5, 20, 9, 10	13·9 ± 4·0
10^6	W3/25$^+$	500R	AO	20·5, 14, 21, 20·5, 10·5, 17, 15·5, 22·5, 28	18·8 ± 5·2
			BN	17, 20·5, 17, 15, 16, 17, 10, 10·5, 19	15·8 ± 3·5
10^6 $0·2 \times 10^6$	W3/25$^+$ OX8$^+$	500R	AO	19·5, 16, 13, 15	15·9 ± 2·7
			BN	12, 12·5, 11, 11·5	11·8 ± 0·6

* Purification by depletion with magnetic beads: W3/25$^+$ cells contained 0·94% OX8$^+$ cells; OX8$^+$ cells contained 2·0% W3/25$^+$ cells.

cell-injected recipients (Table 3). The small number of recipients in this group precludes any statement on the biological significance of the accelerated rejection.

DISCUSSION

The rejection of allografts is a complicated process. The expression of multiple target antigens (class I, class II, minor antigens) invites the participation of more than one inducer and/or effector mechanism. Destruction of the vascular supply or of cells within the graft could each lead to the demise of the transplant. In order to investigate these various alternatives, an attempt was made to define a model more carefully. Particular use was made of the athymic nude rat. In addition, allogeneic skin was grafted in advance in order that the rejection process should not compete with vascularization. The option of using naive TDL for transfer further defined the system; non-lymphoid cells do not form a significant component of lymph; sessile lymphocytes resident within LN were automatically excluded. Although similar, LN cells, spleen cells and TDL are not identical. For example, it has been shown that rat spleen and LN cells contain lymphocytes that transfer second-set rejection but that these 'memory' cells do not recirculate (Hall *et al.*, 1978b).

Although born without a thymus, circumstantial evidence has suggested that nude animals develop T cells extrathymically (Gillis *et al.* 1979; Hunig & Bevan, 1980; MacDonald *et al.*, 1981, 1987; Kishihara *et al.*, 1987) but that they do so inefficiently (Hunig, 1983). We must therefore enquire whether nude animals can be used as definitive recipients to study allograft rejection. The singular observation that full immunocompetence was restored permanently to nude rats by the once-only injection of mature thoracic duct T cells (Bell *et al.*, 1987), suggests that nude animals are complete except for the absence of cells processed by the thymus. CD4$^+$ donor T cells, bearing identifiable allotype markers survived, expanded many-fold in nude rats and were recovered 1–2 years later (Bell *et al.*, 1989); donor-derived but not nude-derived lymphocytes were active in graft-versus-host assays and were able to reject allografts. We also showed that the repertoire of donor T cells for alloantigens (in which a 'hole' was created) remained unchanged during this prolonged period (Drayson *et al.*, 1989). There was no evidence that the nude rat developed its own CD4$^+$ T cells that in any way (apart from phenotype) resembled the MHC class II-restricted products of the thymus. From our own work, and that of others (Kindred, 1979; Bell, 1989), there is no convincing evidence that nude-derived T lymphocytes function in allograft rejection, delayed-type hypersensitivity or in the induction of thymus-dependent antibody responses. Despite the increase with age in the number of nude cells bearing T-cell markers (MacDonald *et al.*, 1981; Vaessen *et al.*, 1986; Bell *et al.*, 1987; Lawetzky & Hunig, 1988) these animals never reject allografts. We have even grafted old (>6 months) athymic CBA mice; BALB/c skin survived in perfect condition until the death of the recipients >200 days later (the total age of each mouse was more than 400 days) (E. B. Bell *et al.*, unpublished observations). A recent study of purified CD4$^+$ and CD8$^+$ spleen cells from old nude mice found that these cells were intrinsically defective in their ability to divide despite their ability to produce IL-2 and express the IL-2 receptor (Kung, 1988; Kung & Thomas, 1988). Thus the nude animal has all the hallmarks of a definitive model in which to explore the T-cell requirements of allograft rejection.

The evidence which previously suggested that nude animals develop T cells extrathymically, particularly that derived from the induction of specific cytotoxic cells (Gillis *et al.*, 1979; Hunig & Bevan, 1980), has been considered in detail elsewhere (Bell, 1989). Recent information has shown that specific cytotoxicity is not an exclusive property confined to cells processed in the thymus (Rolstad *et al.*, 1985; Fossum, Ager & Rolstad, 1987; Ager *et al.*, 1988). Perhaps the true significance of the cytotoxic lymphocytes generated in nude mice has been overlooked. It seems that the studies by Gillis *et al.* (1979) demonstrated not that T cells develop extrathymically, but rather that there is a population of specific cytotoxic lymphocytes, stimulated by IL-2, which develops independently of the thymus. Note that this population would also be present in euthymic animals although it would have largely gone unnoticed. It may belong to the recently identified, non-thymus-derived Lyt-2$^+$ Ly 6C.2$^+$ population of spleen cells (Leo *et al.*, 1988).

The present experiments unambiguously demonstrated that highly purified naive recirculating CD4$^+$ T cells alone induced skin allograft rejection in a dose–response fashion. Although others have reported that CD4$^+$ T cells alone were sufficient to induce allograft destruction (Loveland *et al.*, 1981; Dallman *et al.*, 1982; Hall *et al.*, 1983; Cobbold *et al.*, 1984), their studies were unable rigorously to exclude a potential contribution by CD8$^+$ T cells (Steinmuller, 1985). The present results resolve any uncertainty and demonstrate that CD4$^+$ T cells are autonomous for inducing skin allograft rejection at least where there is a complete mismatch at MHC class I, II and minor loci. Bolton *et al.* (1989) reached a similar conclusion when studying the rejection of kidney allografts in nude rats. Our results are supported by investigations of murine skin allograft rejection using nude mice (Rosenberg *et al.*, 1987); unfortunately no dose–response data were provided in these latter studies and unusually large numbers (50×10^6) of L3T4$^+$ cells were transferred—500 times the number of cells used in the present experiments for recipients one-tenth the size.

It is interesting that in comparison with skin, rejection of

kidney allografts in PVG nude rats required 250 times as many CD4+ T cells ($2{\cdot}5 \times 10^7$) (Bolton *et al.*, 1989). What are the possible reasons for this difference? Vascular supply does not seem to be a critical issue; in our experiments the skin, like the kidney, would have been vascularized before the transfer to T cells. Perhaps rejection is a function of tissue mass and is also influenced by the organs own regenerative capacity. Thus when subcritical numbers of CD4+ T cells are transferred, the repair process simply outstrips the destructive components. If CD4+ T cell numbers are increased, damage accrues faster than repair and eventually rejection is recorded as kidney dysfunction or skin necrosis.

The role of CD8+ T cells in allograft rejection is a subject of some debate (Steinmuller, 1985; LeFrancois & Bevan, 1984; Rosenberg *et al.*, 1986, 1987; Mason & Morris, 1986; Sprent, Schaefer & Kerngold, 1986; Mason & Simmonds, 1988). Part of the confusion arises because of the diversity of technical options employed, which may have exposed different underlying mechanisms. These differences include the use of naive or sensitized donor cells, recirculating or non-recirculating cells, inadequately depleted or purified subsets, complete incompatibilities or isolated disparities at major and/or minor loci, or the use of discrete MHC class I or class II mutants. The present investigation shows that naive CD8+ T cells alone (obtained from the thoracic duct and therefore recirculating) were not capable of inducing skin allograft rejection despite the multiple histoincompatible target antigens, including MHC class I. The result agrees with the findings of other rat studies (Dallman *et al.*, 1982; Mason & Simmonds, 1988; Bolton *et al.*, 1989). The purity of the CD8+ population was of paramount importance; even a small CD4+ contamination (Fig. 3) was sufficient to induce rejection. Furthermore, the addition of 10 times as many CD8+ T cells to a minimal number (10^5) of CD4+ T cells did not influence the rejection time (Table 1). In cases where there was an isolated MHC class I disparity alone ($RT1A^a$), in our hands naive CD8+ T cells were required, but were unable to function without the addition of CD4+ T cells (E. B. Bell *et al.*, to be published). In contrast with our findings, Rosenberg *et al.* (1986, 1987) and Sprent *et al.* (1986) concluded that CD8+ T cells were autonomous. This was based on proliferation, IL-2 production and rejection of the MHC class I mutant bm1 skin (grafted onto nude mice) by the injection of very large doses of Lyt-2+ spleen cells (Rosenberg *et al.*, 1986, 1987). The bm1 mutant results may prove to be an exception to the rule. That CD8+ T cells are autonomous does not appear to be a valid generalization when non-mutant MHC class I disparities are examined.

Although CD8+ T cells were not required or indeed involved in the rejection of skin allografts in our nude model, this does not exclude a role for cytotoxic effector cells within the graft. Cytotoxic cells have been extracted from rejecting kidney allografts of nude rats restored with CD4+ cells alone (Bolton *et al.*, 1989). Using this model we have recently established that these kidneys contain CD4+ T cells of donor origin and CD8+ cells which are entirely of nude origin (J. A. Gracie *et al.*, unpublished observations). We do not yet know whether these non-thymus derived CD8+ cells are alloreactive, cytolytic or even contributing to the rejection process. It is important to recall that alloreactive specific cytotoxic cells need not be products of the thymus, as shown by the phenomenon of allogeneic lymphocyte cytotoxicity (Rolstad *et al.*, 1985; Ager *et al.*, 1988). However, if specific cytotoxic cells are required for allograft destruction, either CD4+ T cells are directly cytocidal within the graft or they evoke a population of host-derived specific effector cells. Present evidence would then demand that this host component must be able to escape sublethal irradiation or alternatively to differentiate rapidly from radio-resistant precursors.

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