Immunology 1989 **68** 547–556

The origin of T cells in permanently reconstituted old athymic nude rats. Analysis using chromosome or allotype markers

E. B. BELL, S. M. SPARSHOTT, M. T. DRAYSON & S. V. HUNT* *Immunology Group, Manchester University Medical School, Manchester and *Department of Pathology, Sir William Dunn School of Pathology, Oxford*

Accepted for publication 14 August 1989

SUMMARY

Athymic PVG-rnu/rnu rats receiving a single intravenous injection of syngeneic euthymic thoracic duct lymphocytes (TDL) develop normal levels of CD4$^+$ T lymphocytes and survive for more than 2 years in a conventional animal house. We investigated the origin of the T cells (and B cells) in reconstituted nude recipients by transferring TDL carrying either the 3T chromosome marker or the RT6^b+Igk-1^b allotype or the RT7^b (leucocyte-common) allotype markers. Karyotype analysis of spleen and lymph node (LN) cells from 1- to 2-year-old PVG-3T/3T-reconstituted nude recipients, stimulated *in vitro* with phytohaemagglutinin (PHA), unexpectedly revealed that a majority (79–97%) of dividing cells were of nude origin. However, extensive nude cell division was also recorded in PHA-stimulated cultures using mixtures of euthymic (PVG-3T/3T) and unreconstituted nude spleen cells; the assumption that only T cells divide in PHA-stimulated cultures thus appears to be erroneous. In contrast to the karyotype analysis, sIg$^-$ RT6b$^+$ LN cells obtained from nude recipients reconstituted 2 years earlier with PVG-RT6^b allotype-marked TDL, were all of donor origin with no indication of a nude-derived sIg$^-$ RT6a$^+$ population. Igk-1b$^+$ donor B cells were not found in these same recipients. Dual fluorescence analysis of TDL from 18- to 20-month RT7b-reconstituted nudes showed that 91–100% of CD4$^+$ cells were donor-derived. When tested functionally, sIg$^-$ RT7b$^+$ (donor) cells, but not sIg$^-$ RT7b$^-$ (nude-derived) cells, were able to reject skin allografts and induce local graft-versus-host (GVH) responses. Donor T cells, in contrast to CD4$^+$ cells of nude origin, divided extensively in nude recipients; FACS-purified RT7b$^+$ (donor) TDL retransferred from 17-month primary reconstituted nude rats, expanded further (60–100-fold) in secondary nude recipients. In conclusion, only the donor-derived CD4$^+$ cells in reconstituted nude rats displayed T-cell function; evidence to the contrary from karyotype analysis was flawed. At no stage in their life did uninjected or T-cell reconstituted nude rats develop endogenous cells that in any way resembled CD4$^+$ products of the thymus.

INTRODUCTION

Athymic nude rats receiving only small numbers of thoracic duct lymphocytes (TDL) from euthymic syngeneic donors have been shown to survive more than 2 years without special husbandry in a conventional animal house (Bell *et al.*, 1987). In these studies TDL-injected nude rats developed within 2 months a permanent expanded CD4$^+$ T-helper cell population which remained at normal levels. The classical T-dependent areas of peripheral lymphoid tissues became filled with lymphocytes. Typical T-cell function was restored: skin allografts were rejected promptly, GVH-reactive cells increased to near normal levels (Bell *et al.*, 1987; Drayson, Sparshott & Bell, 1989), and thymus-dependent antibodies could be stimulated (E.B. Bell and S. Matthews, unpublished data). The question arises whether the T cells were wholly derived from the donor population or whether endogenous, nude-derived cells made any contribution during the life of the nude recipient.

This is not simply a formal issue. There have been claims that T cells gradually appear in nude animals (reviewed by Hunig, 1983). Cell surface phenotyping (Fossum *et al.*, 1980; Brooks *et al.*, 1980; Chen *et al.*, 1984; Vassen *et al.*, 1986; MacDonald *et al.*, 1981, 1986; Lawetzky & Hunig, 1988), studies *in vitro* (Gillis *et al.*, 1979; Hunig & Bevan, 1980; Wagner *et al.*, 1980; Maryanski *et al.*, 1981) and observation of T-cell receptor gene rearrangement (MacDonald *et al.*, 1987; Maleckar & Sherman,

Abbreviations: GVH, graft-versus-host; LN lymph node; PBS-2-AZ, phosphate-buffered saline plus 2% fetal calf serum plus 0·02 M sodium azide; PE, phycoerythrin; PLN, popliteal lymph node; RT6a and RT6b, congenic strains PVG-RT6^a and PVG-RT6^b; RT7a and RT7b, congenic strains PVG-RT7^a and PVG-RT7^b; sIg, surface immunoglobulin; TDL, thoracic duct lymphocytes.

Correspondence: Dr E. B. Bell, Immunology Group, Manchester University Medical School, Manchester M13 9PT, U.K.

1987; Kishihara *et al.*, 1987) suggest that, given time, cells with normal T-cell characteristics can develop extrathymically in such a thymus-deprived environment. Are these cells indistinguishable from T cells apart from the lack of thymus-processing? If so, the thymus is not obligatory for T-cell differentiation. Because TDL-injected nude rats return to immunological near-normality and survive well into old age without a thymus implant, it was possible to ask whether nude-derived T cells could expand with time and contribute numerically or functionally to the T-cell pool.

In the present study by transferring histocompatible TDL from chromosome-marked or from congenic rats bearing distinctive surface alloantigen markers, the origin of T cells in restored nudes could be investigated. $CD4^+$ and $CD8^+$ populations in the host were quantified; donor and host fractions separated by flow cytometry (FACS) were assessed in allograft rejection and GVH induction assays.

MATERIALS AND METHODS

Rats

Inbred lines of PVG, AO and BN rats and selected F_1 hybrids were bred and maintained in the Animal Unit, Manchester University Medical School. Congenitally athymic nude rats (PVG-rnu/rnu), whose origin and derivation have been described in an earlier publication (Bell *et al.*, 1987), were also bred and maintained in the Animal Unit at Manchester University Medical School, but were given a continuously repeated programme of prophylactic antibiotics in the drinking water. The PVG-3T/3T strain (previously called HO.3T), a substrain developed by Howard (1971) is homozygous for the acrocentric morph of chromosome 3, designated 3T (3 terminal centromere).

PVG-$RT6^b$+Igk-1^b double-congenic rats were derived by two independent back-crossing programmes selecting the $RT6^b$ allele (from the DA strain) for 12 backcross generations and the Igk-1^b allele (from the DA strain) for 18 backcross generations, then intercrossing and extracting double homozygotes. RT6 controls the expression of a peripheral T-cell alloantigen, formerly called Pta (Howard & Scott, 1974), which is found on about 80% of mature T cells. PVG-$RT7^b$ was derived by selecting the $RT7^b$ allele from the LOU strain, backcrossing for 12 generations. The $RT7^b$ allele (formerly 'LC1.1') dictates a serological change in the leucocyte-common antigen (expressed on all leucocytes). Both these strains are fully histocompatible, as tested by skin allografting to PVG recipients. PVG and PVG-rnu/rnu are $RT6^a$, $RT7^a$, Igk-1^a. The congenic rat strains were bred and maintained under conventional animal house conditions at the Sir William Dunn School of Pathology, Oxford.

Cells

Thoracic duct lymphocytes. These were collected by the method of Gowans modified by Ford (1978).

Lymph node (LN) cells. Suspensions were prepared from a pool of LN including cervical, deep cervical, mesenteric, brachial and axillary LN. Inguinal LN from nude animals were also used.

The LN were cleaned and chopped coarsely then crushed gently with a perspex squasher to release the majority of the cells. Cell suspensions were filtered through gauze and held in Dulbecco's phosphate-buffered saline with mineral salts A and B (Oxoid Ltd, Basingstoke) containing 2% fetal calf serum (PBS-2) on ice until ready for use.

Chromosome analysis

Lymphocytes from spleen and LN were purified on Ficoll-Hypaque, washed and 2×10^6 cells distributed in 1·0 ml aliquots to round-bottomed culture tubes (Nunc Ltd, Paisley, Renfrewshire). Cells were grown in RPMI-1640 supplemented with 20% FCS (Flow Laboratories, Irvine, Ayrshire). Cultures containing 20 μl of reconstituted phytohaemagglutinin (9 mg/ml) (PHA, Code HA15, Wellcome Ltd, Beckenham, Kent), 4–10 for each animal studied, were maintained at 37° in an atmosphere of 5% CO_2 in air and gently resuspended twice daily. Sixty-six to 68 hr after initiation, colcemid (Demecolcine; Sigma) was added to a final concentration of 1 μg/ml and the cells, including those arrested in metaphase, harvested 4 hr later. Chromosome spreads were prepared by the standard method of hypotonic treatment (Ford, 1966). Pairs of culture tubes were pooled for chromosome preparations and routinely a minimum of 100 orcein-stained metaphase spreads was scored for a terminal (donor) or subterminal (host) centromere on chromosome pair 3. (In well spread preparations, both donor and host marker chromosomes can be positively identified with excellent consistency between scorers.) Only metaphase spreads in which the first four pairs of chromosomes could be clearly identified were considered for scoring. Identification of donor and host cells was assessed by three independent scorers at various stages of the investigation, with no difference in results.

Fluorescence staining

Lymphocytes ($1–3 \times 10^6$) washed in PBS-2 containing 0·02 M sodium azide (PBS-2-AZ) were incubated on ice with various monoclonal antibodies (mAb). The following mAb were purchased as ascitic fluid (Serotec Ltd, Oxford) and diluted 1/100: W3/25 (anti-CD4), MRC-OX8 (anti-CD8), MRC-OX19 (anti-CD5, pan T), MRC-OX12 (anti-rat kappa chain), W6/32 or MRC-OX21 (anti-human but no anti-rat activity, used as controls). mAb staining was detected by FITC-sheep anti-mouse IgG (Dako Ltd, High Wycombe, Bucks) diluted in PBS-2-AZ containing 1% normal rat serum to block cross-reactivity against rat Ig. In some instances direct staining was performed using FITC-sheep anti-rat Ig 1/20 (Wellcome Diagnostics, Beckenham, Kent), FITC-W3/25 (1/5) and FITC-OX8 (1/2) (Serotec). Alloantigens were detected with rat mAb: P4/16 (anti-RT6a), GY1/12 (anti-RT6b) (Butcher, 1987), gifts from Dr G. W. Butcher, Monoclonal Antibody Centre, AFRC Institute, Babraham, Cambridge; NDS58 (anti-RT7a) (Newton, Wood & Fabre, 1986) gift of Dr Mary Newton, Nuffield Department of Surgery, Oxford; 8G6.1 (anti-RT7b) (Mojcik *et al.*, 1987) gift of Dr Dale Greiner, Department of Pathology, University of Connecticut Health Center. Second-layer antibody for these was fluorescein-conjugated $F(ab')_2$ anti-rat IgG2 (gift of Dr A. F. Williams, MRC Cellular Immunology Unit, Oxford). Ig kappa allotypes were detected with fluorescein-conjugated polyclonal anti-Igk-1^b (Hunt & Fowler, 1981) and rat mAb MRC OX11 (anti-Igk-1^a) (Dyer & Hunt, 1981).

Fluorescence-activated cell sorting (FACS)

Cells were analysed or sorted by flow cytometry using the

Coulter Epics II, the Becton-Dickinson FACS IV (both maintained at the Paterson Laboratories, Christie Hospital, Manchester) or on a FACS II upgraded (MRC Cellular Immunology Unit, Oxford).

Aliquots of $3-5 \times 10^6$ cells were incubated first with the mAb NDS58 or 8G6.1 defining the leucocyte-common antigen RT7 variants, followed by FITC-anti-rat IgG2 antibody. The cells were washed five times in PBS-2-AZ then incubated with one of the standard mAb listed above. Phycoerythrin (PE)-conjugated anti-mouse Ig (Serotec Ltd) 1/5 in PBS-2-AZ, supplemented with 1% normal rat serum and 10% FCS as blocking agents, was used to detect this second-stage mAb staining.

The doubly fluorescent cells were sorted, into fluorescein (green) positive and negative fractions to isolate donor from host. Dead cells were excluded by forward angle light scatter. Cross-contamination of 'bright and dulls' was minimized by the provision of a generous 'buffer zone' (at least 20 channels) between the upper gate defining the negative population and the lower gate defining the positive population (Fig. 3).

The sorted fractions were then analysed for their red (PE) fluorescence so as to quantify the number of cells in each sorted donor or host population bearing a particular T- or B-cell phenotype. Final analysis required taking into account two different background (bk) subtractions; 'b' represents a conventional background of non-specific fluorescence (here determined with W6/32) deducted from the total number of positive cells (a); 'c' is deducted from the negative population. Both bks are subtracted from the total number (10^4) of cells analysed before calculating the net percentage positive (x) using the equation:

$$x = \frac{(a-b) \times 100}{10^4 - (b+c)}. \quad (1)$$

The need for a bk subtraction from the negative population became apparent on the reanalysis of cells that had been doubly stained for RT7b and OX12. Since 8G6.1 is a rat mAb bearing kappa light chains, subsequent staining with OX12 (anti-kappa) should result in all RT7b$^+$ cells being doubly stained. However, when FACS-purified RT7b$^+$ (green) cells were then analysed by FACS for OX12 (red), a small (1–2%) negative population was found (Fig. 3j). Since we were interested in quantifying both positive and negative fractions, this correction factor (c) was deducted from the total number of cells analysed and in a few instances accounts for values that just exceed 100% (Table 4).

Cell separation

Planning. Cells were separated by a variation of a published method (Mage, McHugh & Rothstein, 1977). Plastic 90 mm petri-dishes (Nunc Ltd) were coated with immunopurified sheep anti-rat F $(ab')_2$, 3 ml containing 20 μg/ml in Tris buffer, pH 9·5, overnight at room temperature. The plates were washed three times with cold PBS and coated to prevent non-specific binding by incubation for at least 5 min with PBS containing 5% FCS ready for use. Washed TDL (20×10^6) in 3·0 ml of RPMI-1640 + 20 mM HEPES were added and the plates incubated for 60 min at 4°. At 40 min they were gently agitated to redistribute any non-adherent cells. The non-adherent B-cell depleted population was recovered after gentle agitation of the petri-dishes. The frequency of B cells was reduced from 57% in the starting population to less than 8% after a single panning step. Recovery was 19% of the starting population.

T cells were removed by first incubating TDL with saturating concentrations of W3/25 or OX8 and allowing mAb-coated cells to adhere to plates coated with immunopurified sheep anti-mouse IgG absorbed against rat Ig.

Rosetting with magnetic beads

Cells coated with mouse mAb were depleted using magnetic polystyrene beads (Dynal Ltd, New Ferry) coated with sheep anti-mouse Ig. All washing and cell separation procedures were carried out in 25-ml sterile plastic disposable 'universal' containers (Sterilin) by magnetic adhesion. A row of four Samarium cobalt magnets (Magnetic Developments Ltd, Swindon, Wilts) ($10 \times 10 \times 3$ mm) was embedded and taped in place in an expanded polystyrene mould that supported the 'universal' container on three sides.

For B-cell depletion, magnetic beads, washed three times in RPMI-1640 with 20 mM HEPES, were used at the rate of 1 ml bead suspension (4×10^8 beads/ml) for every 100×10^6 lymphocytes. TDL were incubated with OX12 (ascites diluted 1:9) on ice, washed four times in PBS-2-AZ, resuspended in approximately 10 ml PBS-2-AZ and added to the anti-MIg-coated beads resuspended in approximately 10 ml RPMI/HEPES. The mixture was rosetted on a roller/rocker (Luckham Ltd, Burgess Hill) for 30 min at 4°, topped up with 5 ml of cold RPMI and placed on the bench in a vertical position. A row of magnets was applied to the side of the container allowing the cell-bead rosettes to be drawn horizontally from suspension. The universal with magnets in place was inverted, rotated and agitated to promote the removal of any remaining beads. The depleted cell population was recovered by decanting with magnets in place. The beads were regenerated in glycine-HCl (0·1 M, pH 2·8) after several hours of incubation at 37°, during which cells were released from the beads. Regenerated beads were stored in PBS-2-AZ and could be used three times with some reduction in cell purity. B cells in thoracic duct lymph were reduced from 40–50% in the starting population to 2–5% after depletion. Cell yield was 30% of the total starting population.

Rosetting with sheep red cells. This was as previously described (Hunt, 1987).

GVH assay

The GVH reactivity of individual cell populations was measured by the local popliteal LN (PLN) assay (Ford, Burr & Simonsen, 1970). Briefly, graded doses of cells obtained from PVG nude animals that had received RT7^b cells were injected into the hind footpad of (PVG × BN)F_1 hybrids and the resulting enlargement of the PLN measured 7 days later.

RESULTS

Reconstitution of nudes with PVG-3T/3T TDL

PVG nude rats injected up to 2 years earlier with 3T chromosome-marked TDL and permanently restored with W3/25$^+$ (CD4$^+$) cells (Bell *et al.*, 1987) were used to investigate the origin of these T cells. Spleen and LN cells were stimulated with the T-cell mitogen PHA for 3 days *in vitro* before the addition of colcemid to arrest dividing cells in metaphase. Lymphocytes from reconstituted animals were readily stimulated by PHA, as evidenced by the number of cells in metaphase. The frequency of host-derived cells in 1 to 2 year-restored nudes was unexpectedly high, ranging from 79% to 97% (Table 1), suggesting that nude

Table 1. Karyotype analysis of spleen and LN cells from PVG-3T/3T TDL-injected nude rats stimulated in culture with PHA

Time after reconstitution	No. of PVG-3T/3T TDL injected ($\times 10^6$)	No. of metaphases		% host
		Donor (3T)	Host	
3 weeks	20	37	83	69·2
	20	62	110	64·0
	20	37	103	73·6
2 months	20	88	137	60·9
	20	103	122	54·2
	20	104	116	52·7
4 months	100	53	68	56·2
11 months	2	31	119	79·3
	20	3	97	97·0
	200	11	89	89·0
23 months	20	6	101	94·4
	20	16	122	88·4

Table 2. Karyotype analysis of PHA-stimulated cultures of defined cell mixtures. Percentage of metaphases of recipient type

Culture	Chromosome marked cells of		Mixture ratio		
	Donor type*	: Recipient types†	3:1	1:1	1:3
A	None	Nu spleen		100‡	
B	Eu spleen	None		0	
C	Eu spleen	B cells (Nu)§		8	12
D	Eu spleen	B cells (R-Nu)¶		22	16
E	Eu spleen	B cells (rnu/+)**		13	10
F	Eu spleen	Nu spleen	10	22	42
G	T cells††	Nu spleen		4	40

*PVG-3T/3T euthymic (Eu).

†PVG-rnu/rnu nude (Nu).

‡50 metaphase spreads scored in all groups except culture A (only eight spreads) and culture G 1:3 (20 spreads).

§TDL from nude.

¶CD4$^-$ CD8$^-$ TDL from 2-month restored (R) nude: W3/25$^+$ and OX8$^+$ cells removed by panning.

**CD4$^-$ CD8$^-$ TDL from heterozygote (euthymic).

††sIg$^-$ TDL: B cells removed by panning.

rats were permanently capable of developing their own endogenous PHA-responsive T cells in the absence of thymic epithelium.

The percentage of dividing cells of the recipient karyotype 2 months and 4 months after reconstitution, a time when the frequency of CD4$^+$ cells has stabilized near maximum (Bell *et al.*, 1987) was also high, ranging from 53% to 60% (Table 1). Nude recipients injected only 3 weeks before with PVG-3T/3T TDL showed an even higher frequency (mean of 69%) of nude-derived metaphases (Table 1). This result was difficult to reconcile with our knowledge of T cell differentiation. Therefore, we considered whether the presence of PHA-stimulated euthymic T cells could be inducing cell divison of non-T cells *in vitro*.

To test this hypothesis, PHA-stimulated cultures were established using mixtures of cells from euthymic and nude donors. Nude spleen cells alone did not respond significantly to PHA in culture; metaphase spreads, although 100% of nude origin (Table 2, culture A) were extremely rare. Nor was there evidence of extensive B-cell proliferation in PHA cultures since PVG-3T/3T spleen cells, when mixed with B cells from thoracic duct lymph including nude TDL (culture C) and T cell-depleted TDL (CD4$^-$ CD8$^-$) from restored nudes (culture D) or euthymic rnu/+ heterozygotes (culture E), showed only modest numbers of metaphases of recipient karyotype. However, the addition of small numbers of euthymuc spleen cells (ratios of 1:3, euthymic:nude, culture F) or highly enriched T cells (Ig$^-$TDL) from euthymic donors (culture G) induced substantial (40–42%) nude cell division (Table 2, F and G). Both these observations undermine the assumption that only T cells were dividing in these PHA cultures.

Reconstitution of nudes with RT6b+ Igk-1^b TDL

As an alternative approach for establishing the provenance of T cells in reconstituted nudes, animals were injected with allotype-marked TDL, which allowed both dividing and non-dividing cells to be examined in the absence of deliberate stimulation. Ten PVG-rnu/rnu rats were reconstituted with 20×10^6 TDL from double congenic donors bearing both the RT6 (peripheral T-cell alloantigen) marker and the Ig kappa allotype marker (Hunt & Fowler, 1981). At 24 months, four rats remained healthy enough for analysis of their cervical LN or their TDL. From individual rats one portion of cells was stained for the donor- and host-type kappa allotypes, and another was depleted of B cells by sIg rosetting to stain for the donor- and host-type RT6 allotypes. Controls from rats homozygous for each marker were treated similarly. Table 3 and Fig. 1 summarize the results of FACS analysis.

In the sIg-depleted portion, two rats (nos 1 and 2, whose analysis was performed on cervical LN; Fig. 1) showed about 75% RT6$^+$ cells (Table 3), and these were entirely of donor origin. In the other two rats (nos 3 and 4, analysis on TDL) fluorescence was too weak for quantification, but no host-specific staining was seen. In some previous studies on the expression of the RT6 antigen, it has been shown that it is very sensitive to modulation by antibody (S. V. Hunt, unpublished data), but there was no trace of anti-RT6b alloantibody in these two rats to explain the low expression of antigen; since the rats were in good health, and no difference between TDL and LN is suspected, there is no satisfactory explanation for the paucity of RT6 expression. CD4$^+$ and CD5$^+$ cells were found in frequencies similar to those in Table 4 (43·1% and 43·4%, respectively, only one rat examined).

In none of the rats was there any convincing sign of residual donor-derived B cells, which would have been indicated by a separate peak of bright-staining kappa-allotype marked cells. In each case 50–60% of the sample was B cells, and these were essentially entirely of host (nude) origin.

Reconstitution of nudes with RT7$^+$ TDL: phenotypic analysis

The RT7 alloantigen system provides an alternative marker to study chimaeric nude rats. The a (host) and b (donor) alleles are expressed on all lymphocytes, are stable and are not modulated following congenic cell transfer by allele specific antibodies.

Table 3. Chimaerism in PVG-rnu/rnu recipients of 20×10^6 congenic (PVG-RT6b$^+$Igk-1^b) TDL 24 months after reconstitution

Rat	Cells	Undepleted			sIg-depleted		
		% Igk-1^b (anti-donor)	% Igk-1^a (anti-host)	% -ve* control	% RT6b (anti-donor)	% RT6a (anti-host)	% -ve* control
No. 1 TDL-Nude	LN	<4·0	NR	ND	79·7	3·1	5·5
No. 2 TDL-Nude	LN	3·5	49·8	ND	74·3	6·0	9·0
No. 3 TDL-Nude	TDL	2·5	56·1	3·6	Weak fluorescence; quantitative		
No. 4 TDL-Nude	TDL	1·5	61·6	1·4	analysis not possible		
DA (Igk-1^b) control	LN	22·8	2·3	2·1	NR	13·2	14·5
PVG (Igk-1^a) control	LN	2·1	42·3	1·5	2·7	80·1	4·3

NR, not recorded; ND, not done.
*Second-layer antibody alone.

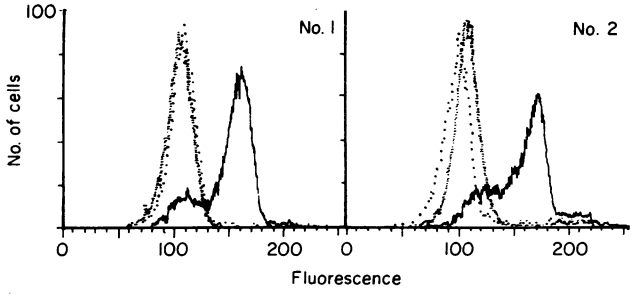

Figure 1. FACS profiles of sIg-depleted LN cells from 2-year chimaeric nude rats (Nos 1 and 2). Cells were stained for the RT6b allotype (donor, solid line), the RT6a allotype (host, dashed line) or with a control mAb (dotted line). Results shown in Table 3.

A summary of surface phenotypes of 10 RT7b TDL-injected nude rats is given in Table 4. CD4$^+$ cells (W3/25$^+$) comprised 39·5% of lymphocytes, values ranging from 30% to 59% in individual recipients. The remainder of the cells were CD8$^+$ (6·1%), sIg$^+$ (41·0%) or CD4$^-$CD8$^-$Ig$^-$ 'null cells' (13·4%). When populations were analysed for the RT7 allotype, a mean of 43·8% of cells were donor-derived (RT7b$^+$), values ranging from 32% to 68%. Cells from five recipients were also stained for the reciprocal allotype, RT7a; a mean of 44% of cells were RT7a$^+$. A proportion of cells stained weakly or not at all for either variant of the leucocyte common antigen.

Donor-derived cells in TDL collected from 17 months, RT7b TDL-injected nude recipients were purified by flow cytometry. $1·7 \times 10^6$ cells, representing 0·3% of the recirculating pool, and 97·6% RT7b$^+$ were transferred into each of two further nude recipients. Analysis of these secondary recipients 2 months later (Table 5) revealed a 60–100-fold expansion in CD4$^+$ cells. (This estimate was based on our previous quantitative analysis of the size of the recirculating pool in TDL-injected nude rats; Bell *et al.*, 1987.)

A linear regression analysis (Fig. 2) of the percentage of cells that were RT7b$^+$ and the percentage that were W3/25$^+$ was compiled using all recipients in Tables 4 and 5. There was an extremely close correlation ($r = 0·977$, slope = 1·26) between the frequency of RT7b$^+$ cells and CD4$^+$ lymphocytes in long-term restored nude recipients. This suggests (i) that of the T and B lymphocytes initially injected, CD4$^+$ cells survived and expanded preferentially, and (ii) that the CD4$^+$ cells in long-established nude recipients were donor- and not host-derived.

To prove whether CD4 cells in long-term reconstituted nude recipients were of donor or host origin, TDL or LN cells were examined after dual fluorescence staining by first sorting the cells on the basis of the RT7 allotype (using an FITC-labelled antibody) then analysing the sorted population by flow cytometry for T- and B-cell markers (PE-labelled antibody). Identifying donor and host cells required positive staining and sorting by 8G6.1 and NDS58 rat monoclonal alloantibodies, respectively. Although both positively and negatively sorted populations were evaluated, only the results for RT7a$^+$ and RT7b$^+$ cells are given. FACS profiles (rat 667.10) are illustrated in Fig. 3.

Between 76% and 99·5% of donor-derived RT7b$^+$ cells were also W3/25$^+$ (Table 6). Significant numbers of CD8$^+$ cells (11–12% were detected in two rats. In 6/7 rats the entire RT7b$^+$ donor-derived population could be accounted for by OX19 staining, a phenotype normally including all of CD4 and CD8. The only exception is rat 667.10 in which 3·7% of RT7b$^+$ donor cells could not be accounted for by the T-cell phenotypes CD4 or CD8 (Fig. 3). These results, together with those from the RT6b Igk-1^b allotype system, demonstrated that mature recirculating T cells, especially the CD4$^+$ population, expanded preferentially in nude recipients. There was no evidence that donor B cells survived for these prolonged periods.

Reconstitution of nudes with RT7b$^+$ TDL: functional analysis

There were a few CD4$^+$ (1–9%), CD5$^+$ (2–11%) and CD8$^+$ (0–4%) cells in the host-derived (RT7a$^+$) population (Table 6). Their function was tested in two ways.

Skin allograft rejection. Four months after nude recipients were reconstituted with RT7b lymphocytes, TDL were obtained from the first overnight collection and depleted of sIg-bearing cells by 'panning' on anti-rat F(ab')$_2$ IgG-coated plastic petri-dishes. The non-adherent sIg$^-$ cells were stained with 8G6.1 (anti-donor) and separated by FACS into RT7b$^+$ (88%) and RT7b$^-$ (12%) populations. FACS-purified cells were injected into PVG nude recipients bearing 7-day-old healthy skin allografts. As few as 10^5 highly purified CD4$^+$ cells alone are required to reject skin allografts in this model (Whitby,

Table 4. Phenotypic analysis of TDL or LN cells of nude rats (PVG-rnu/rnu, RT7a) injected 14–20 months earlier with 20×10^6 congenic (PVG-+/+, RT7b) TDL

		Percentage positive					
		(CD4) W3/25	(CD5) OX19	(CD8) OX8	sIg	RT7b	RT7a
Nude	*n*	10	3	8	10	10	5
	Mean ± SD	39·5 ± 8·3	41·2 ± 12·4	6·1 ± 2·5	41·0 ± 11·4	43·8 ± 10·6	44·0 ± 12·6
Euthymic (rnu/+)*		24·1	27·0	4·0	31·0	1·3	60·5

*A single 20-month-old PVG heterozygote (RT7a) was analysed for comparison.

Table 5. Phenotypic analysis of lymphocytes from nude rats (secondary recipients) injected 2 months previously with $1{\cdot}7 \times 10^6$ purified RT7b$^+$ TDL* obtained from nude rats (primary recipients) injected 17 months earlier with unfractionated congenic RT7b TDL

Secondary recipient (rnu/rnu)	% positive					
	W3/25	OX19	OX8	sIg	RT7b	RT7a
700·1†	22·8	23·3	3·7	58·7	21·9	70·3
700·2	27·1	36·7	7·5	43·6	28·2	60·4

*97·6% RT7b$^+$, purified by FACS after sIg depletion.

†At one month after cell transfer this rat received a BN skin graft which was rejected 11 days later.

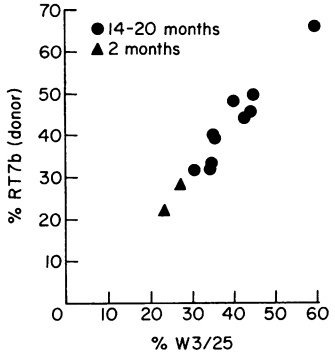

Figure 2. Correlation between the frequency of RT7b$^+$ and W3/25$^+$ lymphocytes obtained from reconstituted nude rats. ($r = 0{\cdot}977$, slope = 1·26). Cells obtained from primary nude recipients (●) injected with RT7b whole TDL or secondary nude recipients (▲) injected with FACS-purified RT7b$^+$ TDL from 17-month-old primary recipients.

Sparshott & Bell, 1990). Antibody-stained but unsorted cells and FACS-purified RT7b$^+$ cells rejected skin allografts in a dose-dependent fashion (Table 7). The RT7b$^-$ fraction, containing lymphocytes of nude origin, failed to reject skin allografts.

GVH activity. TDL from two 14-month, RT7b-reconstituted nude rats were incubated with OX12, depleted of sIg$^+$ cells using anti-mouse IgG-coated beads and stained subsequently with 8G6.1 mAb for FACS. Graded doses of FACS-purified cells were injected into the footpads of (PVG × BN)F$_1$ rats. As few as 3×10^5 RT7b$^+$ donor-derived cells induced a significant response (Table 8a). There were sufficient cells for only a single footpad injection of lymphocytes containing nude-derived cells, since only 2·4% of the sIg$^-$ population prepared for sorting were RT7b$^-$. The weight of the PLN draining the footpad injected with the RT7b$^-$ cells was not significantly greater than the PBS-injected control PLN weight. In a repeat experiment GVH activity was again demonstrable with low numbers (3×10^5) of sIg$^-$ RT7b$^+$ cells (Table 8b). Nude-derived, sIg$^-$ RT7b$^-$ cells were rare; 4×10^5 RT7b$^-$ cells injected into the footpad of (PVG × BN) F$_1$ rats induced a 3·76 mg net increase in LN weight compared with a 14·60 mg net increase using 25% fewer (3×10^5) RT7b$^+$ cells (Table 8b). The small increase in LN weight by RT7b$^-$ cells could be related to contamination with RT7b$^+$ cells (6·1%) in the sorted population.

DISCUSSION

The present experiments show that cells bearing the characteristic antigens of T cells (CD5, CD4 and CD8) appear and persist in the thoracic duct of athymic nude rats reconstituted intravenously with a few (2×10^7) normal TDL, and that these T cells come from the allotype-marked donor inoculum, not the host. They can underwrite graft rejection and GVH reactions. There is a glaring contrast between the potent proliferative and functional capacity of the donor-derived CD4$^+$ cells, which can retransfer into a secondary host, and the feeble inability of the rare host thoracic duct cells bearing CD4 or CD8 to do anything comparable.

The claim that the expanded population is of donor origin rests on the analysis of nude rats reconstituted with allotype marked TDL using two allotype marker systems, RT6 and RT7. In RT6 chimaeras, 75% of sIg$^-$ LN cells bore the donor RT6b alloantigen, and none bore host RT6a. Two other chimaeras in this series gave inadequate staining to prove that donor RT6b$^+$ cells were there but provided no contradictory sign of host RT6a$^+$ cells.

Conclusive evidence of donor origin came from seven chimaeras (Table 6), in which the RT7 alloantigen permitted the examination of essentially all CD4$^+$, CD5$^+$ and CD8$^+$ cells. Only a small proportion of cells (less than 15%) expressed RT7 weakly or not at all. The number of cells on which RT7 was weak correlated ($r = 0{\cdot}887$) with the number of CD4$^-$ CD8$^-$

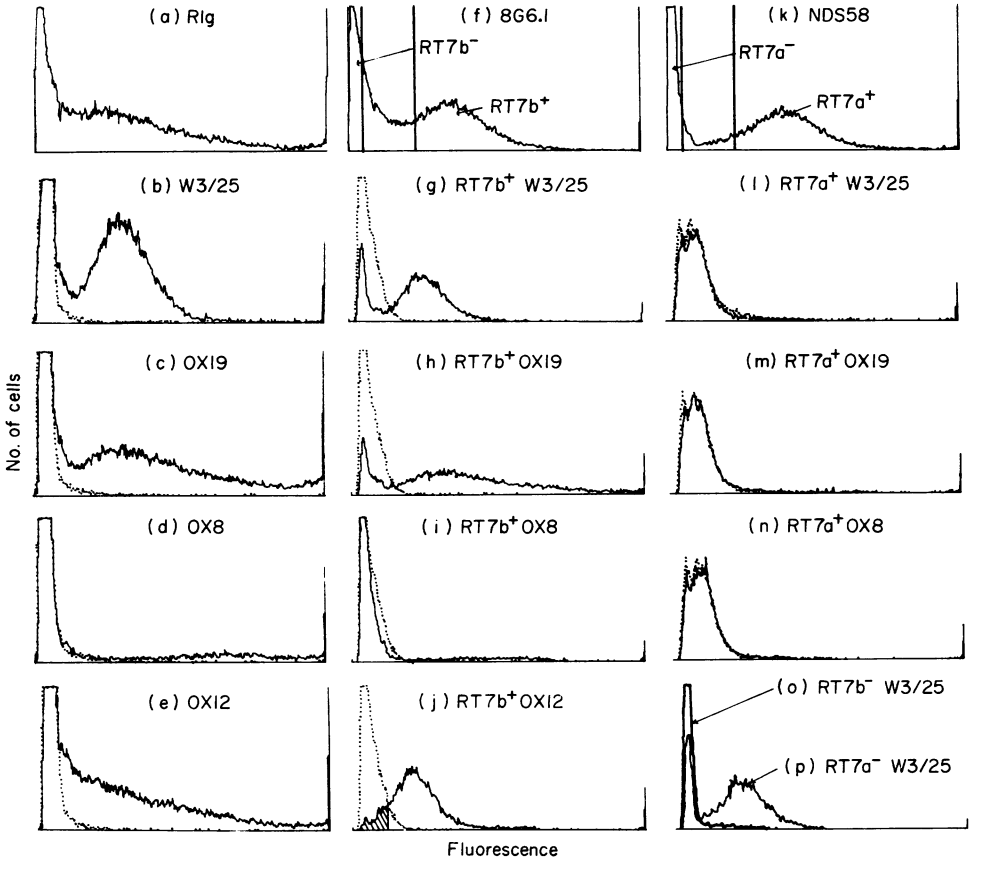

Figure 3. Dual fluorescence analysis (FACS profiles) of LN cells from an 18·5-month RT7b-reconstituted nude rat (667·10, Table 6). (a): FITC-anti-rat IgG, direct staining. (b–e) Single staining by indirect fluorescent (FITC). (f, k) Anti-RT7 allotype staining before cell sorting with gates for sorting indicated. (g–j) $RT7b^+$ positively sorted cells (donor-derived) analysed for the mAb indicated. (j) Hatched area represents the background subtraction value 'c' in equation (1) (see the Materials and Methods) for calculating 'net percentage positive'. (l–n) $RT7a^+$ positively sorted cells (nude-derived) analysed for the mAb indicated. (o, p) Profiles for W3/25 staining of negatively sorted $RT7b^-$ (nude-derived) and $RT7a^-$ (donor-derived) cells. Control profiles (W6/32) have been overlaid (dotted lines) where appropriate. 5×10^4 (a–e) or 10^4 (f–p) cells analysed after excluding red cells and dead cells by forward light scatter.

Table 6. Dual fluorescence analysis of lymphocytes from nude rats (RT7a) injected at various times earlier with RT7b TDL

Nude rat no.*	Month after RT7b TDL injection	$RT7b^+$ (donor)			$RT7a^+$ (nude)		
		CD4	CD5	CD8	CD4	CD5	CD8
667·9	18	94·9†	99·7	11·4	1·1	NC‡	0·2
667·10	18·5	84·2	89·9	12·1	9·4	2·5	4·0
667·11	18·5	76·0	102·7	NC	3·0	8·1	3·7
663·7	20	99·2	102·0	0	NC	NC	NC
663·8	20	99·5	100·2	<2·3	1·0	1·6	0
700·1¶	2(+17)	102·1	102·8	0·4	1·2	4·0	0
700·2¶	2(+17)	NC	107·2	0·7	5·9	11·4	2·1
Eu (rnu/+)	20 months old	NC	NC	NC	41·3	62·1	3·2

*Rats included in Tables 4 and 5.
†Values are net percent positive (see the Materials and Methods).
‡NC, no cells for analysis.
¶Secondary recipients (see Table 5 for details).

Table 7. Skin allograft rejection by nude rats given intravenous lymphocytes obtained from primary nude hosts injected 4 months earlier with RT7b TDL

Dose	Cells (sIg^-)	% $RT7b^+$*	Day of rejection†	Mean
10^6	Unsorted	88·0	17·5, 17·5, 18, 18	17·75
3×10^6	$RT7b^+$	95·8	14, 14·5	14·25
10^6	$RT7b^+$	95·8	15·5, 19·5, 19·5, 19·5	18·50
10^6	$RT7b^-$	>3·1	68, >93	>85‡

*TDL were depleted of sIg^+ cells and stained with anti-RT7b 8G6·1 mAb followed by FITC-anti-rat IgG2.

†AO ($RT1^u$) skin was grafted onto PVG-rnu/rnu recipients 7 days before cell transfer.

‡Rats died with perfect allografts and no sign of rejection.

Table 8. A comparison of GVH activity induced by sIg^- $RT7b^+$ (donor-derived) cells and sIg^- $RT7b^-$ (nude-derived) cells obtained from nude rats injected 14 months (a) or 17 months (b) earlier with RT7b TDL

Dose	Cells (sIg^-)	% $RT7b^+$*	*n*†	PLN (mg)‡	(±SD)
(a)					
10^6	Unsorted	97·6	3	21·96	(1·349)
$2·9 \times 10^5$	Unsorted	97·6	5	6·49	(1·020)
$9·4 \times 10^5$	$RT7b^+$	99·5	4	20·05	(1·253)
$3·3 \times 10^5$	$RT7b^+$	99·5	5	11·26	(1·767)
$2·9 \times 10^5$	$RT7b^-$	3·6	1	4·90	
None	PBS	—	4	3·11	(1·133)
None	Uninjected	—	2	3·14	(1·461)
(b)					
$9·8 \times 10^5$	$RT7b^+$§	97·6	5	29·34	(1·275)
$3·0 \times 10^5$	$RT7b^+$	97·6	5	18·98	(1·358)
$4·1 \times 10^5$	$RT7b^-$	6·1	2	8·14	(1·319)
None	PBS/uninjected	—	6	4·37	(1·333)

* TDL were depleted of sIg^+ cells and stained with 8G6.1 mAb followed by FITC-anti-rat IgG2. Values are before or after FACS.

† Number of PLN assayed.

‡ Geometric mean PLN weight 7 days after footpad injection: $(PVG \times BN)F_1$ recipients.

§ The sIg^- cells in this experiment were 91·8% $RT7b^+$ before cell sorting.

sIg^- null cells, which are prevalent in old nude rats (Bell *et al.*, 1987; Brooks *et al.*, 1980). The results from all RT7b chimaeric nudes examined indicated that functioning $CD4^+$ and $CD8^+$ peripheral T cells came from the donor, not the host. Three kinds of analysis showed this. First, there was an extremely high correlation ($r = 0·977$) between the $RT7b^+$ frequency and the $CD4^+$ frequency. Second, dual fluorescence analysis confirmed that in 4/5 chimaeras examined, 100% of the $RT7b^+$ cells were $CD4^+$, $CD8^+$ or $CD5^+$; in the fifth animal, all but 3·7% were phenotypically T cells. In contrast, the frequency of nude lymphocytes ($RT7a^+$) that stained for CD4, CD8 or CD5 remained low (0–11%). Third, the GVH activity and ability to reject a pre-existing skin allograft were tested. These studies deliberately used the *anti-donor* mAb 8G6.1 to avoid the possibility that surface binding of mAb could inhibit host (nude) cell function. Using TDL from 4-month chimaeric nudes only sIg^- $RT7b^+$ donor-derived cells were capable of eliciting skin allograft rejection (Table 7), while 10^6 sIg^- nude-derived cells ($RT7b^-$) did not. 10^6 T cells are at least 10-fold more than needed to evoke rejection (Whitby *et al.*, 1990). By allowing TDL-injected nude rats to age longer, neither the frequencies nor the activity of host cells increased. In two experiments no more than 2·4% and 9·1% of sIg^- cells were of non-donor origin ($RT7b^-$), and these had no GVH activity (Table 8).

In the transfer of congenic TDL, the donor population included B cells as well as T cells. In all but one of the RT7b TDL-injected nude recipients, the $RT7b^+$ cells could be entirely accounted for by T-cell phenotypes (Table 6). In the Igk-1^b restored nudes, there were no donor B cells bearing the Igk-1^b allotype (Table 3). B cells from TDL did not survive or self-replicate in the hosts, in accordance with previous competition experiments in euthymic radiation chimaeras (Hunt, 1979).

The conclusion that donor T cells alone repopulate the nude's T-cell compartment must be reconciled with the karyotype analysis. Chromsome markers have previously been used as a definitive means of establishing cell provenance. Only cells in division can be assessed and normally these are a small minority of the entire population. With lectins such as PHA or convanavalin A, which under standard conditions stimulate T cells but not B cells, karyotype analysis has determined the origin, kinetics and survival of murine T cells (Doenhoff & Davies, 1971). When analysing old nude rats replenished with chromosome-marked TDL, we consistently observed that the vast majority (79–97%) of metaphase spreads from PHA-stimulated spleen and LN cultures were unexpectedly host-derived (Table 1). In a similar study using TXBM mice (thymectomized, irradiated, bone marrow-restored) reconstituted with chromosome-marked (T6T6) LN cells, Piguet & Vassalli (1982) found that about 25% of cells in Con A-stimulated cultures were host-derived. Two observations from our subsequent studies seriously undermined the validity of the karyotype analysis. (i) Analysis of reconstituted nude rats at 3 weeks, a stage before extensive $CD4^+$ T-cell expansion occurs (Bell *et al.*, 1987), also showed many nude metaphase spreads (64–73%, Table 1). (ii) Small numbers of T cells from the spleen or thoracic duct of euthymic donors, when mixed *in vitro* with PHA and nude spleen cells, induced considerable division in the latter, accounting for more than 40% of dividing cells (Table 2). Therefore, either nude-derived T-cell progenitors simply differentiated very quickly in the presence of mature T cells (within 48 hr in tissue culture), which seems unlikely, or instead cells of nude origin were induced to divide in the milieu of mitogen-stimulated T cells. We are now trying to characterize the nude-derived cells that respond in these cultures.

In essence we have shown that the only truly accredited $CD4^+$ T cells in restored nude rats were donor-derived. Athymic animals failed to generate a comparable cell. Other experiments agree that $CD4^+$ nude-derived cells must be significantly different from those processed by the thymus. Thus, highly purified $CD4^+$ cells from nude mice were intrinsically defective (Kung & Thomas, 1988) in that they failed to proliferate *in vitro* in response to T-cell mitogens or to anti-CD3 or even when exposed to PMA plus ionomycin. The same was true of nude $CD8^+$ cells (Kung, 1988). Similarly $CD4^+$ cells in nude rats are

apparently non-functional *in vivo*. Even young unrestored nude rats, in which only 2% of TDL are W3/25$^+$, will contain approximately 10×10^6 'T cells'. This is 10–100 times more CD4$^+$ cells than are normally required to reject skin allografts (Table 7; Ford, Sparshott & Rolstad, 1983; Whitby *et al.*, 1990). This intrinsic difference between nude-derived and euthymic-derived peripheral CD4$^+$ cells is also powerfully demonstrated by the retransfer of RT7b$^+$ W3/25$^+$ cells obtained from the thoracic duct of 17-month RT7b chimaeric nudes. Two months later the donor CD4$^+$ population had expanded 60–100-fold in the secondary recipients. We recently reported that the alloreactive repertoire (in which a 'hole' was created by the passage of parental TDL through an F_1 hybrid) was not altered over 1 year during the expansion (Drayson *et al.*, 1989). Apparently the environment of the nude rat permits those CD4$^+$ cells processed by a thymus to proliferate and yet the endogenous CD4$^+$ cells of the athymic host do not flourish.

Both the present and previous studies (Bell *et al.*, 1987; Drayson *et al.*, 1989) focus attention on the regulation of the size of the T-cell pool. It evidently is controlled by a feedback mechanism which can give a powerful kick when the pool is small, and which operates independently and in the absence of a thymus but only on those cells that have been thymus-processed.

Our findings fit the simple orthodoxy that animals that lack a functioning thymic microenvironment (Kindred, 1979; Festing, 1981; Van Vliet *et al.*, 1985) are unable to generate their own T cells. This cannot be attributed to a genetic defect imprinted on nude stem cells (Pritchard & Micklem, 1973). Nor is it due to some kind of 'community effect' (Gurdon, 1988) by which a maturing CD4$^+$ nude population may never reach a critical size. The present experiments show that an environment saturated with mature T cells neither encourages nor discourages the nude-derived CD4$^+$ cells. Instead, nude cells developing extrathymically seem permanently crippled by not having experienced an appropriate maturation environment. Quite possibly they have not learned their MHC restriction specificity.

Were it not for a growing number of reports that favour an extrathymic pathway of T-cell development, our conclusions would be completely non-contentious. Surface phenotyping, TcR gene rearrangement/expression and the induction of specific cytotoxic cells form a substantial body of evidence that argues for exogenous athymic T-cell development. Recently, detection of $\gamma\delta$ T-cell receptor gene rearrangement (Yoshikai *et al.*, 1986, 1988, 1989) has also been used as evidence of nude-derived T cells (Matis *et al.*, 1987), but perhaps prematurely (Bell, 1989) since it has yet to be shown that $\delta\gamma^+$ cells in the periphery of euthymic animals were generated within the thymus and later emigrated out.

Is it possible to reconcile our failure to uncover an extrathymic pathway with this indirect but persuasive body of evidence to the contrary? The fact that specific alloreactive cytolytic cells can be generated from nude lymphoid cells (Gillis *et al.*, 1979; Hunig & Bevan, 1980; Wagner *et al.*, 1980; Maryanski *et al.*, 1981) is particularly revealing in the context of subsequent studies (Harel-Bellan *et al.*, 1987) which question the fidelity of the allospecificity. By the simplest interpretation, these results indicate that another cellular defence system must exist independently of the thymus. Accordingly, there is no need to press these nude-derived cells into a T-cell mould and there is no need to hypothesize an extrathymic pathway of development. These 'T-like' cells will exist in nudes (and presumably in euthymics but are as yet unrecognized) as a separate entity and may provide the limited defence against infection that nude animals certainly possess.

ACKNOWLEDGMENTS

The early studies were conceived and instigated by the late Professor W. L. Ford. Our thanks to Mike Hughes and Jeff Barry for running the FACS, to Jackie Jolley and Norma Haigh for typing the manuscript and to Bob Hallworth and Neil Yates for maintaining the nude rat colony. This work was supported by grants from the Medical Research Council.

REFERENCES

BELL E.B. (1989) Thymus-derived and non-thymus-derived T-like cells. The origin and function of cells bearing $\delta\gamma$ receptors. *Thymus* (in press).

BELL E.B., SPARSHOTT S.M., DRAYSON M.T. & FORD W.L. (1987) The stable and permanent expansion of functional T lymphocytes in athymic nude rats after a single injection of mature T cells. *J. Immunol.* **139,** 1379.

BROOKS C.G., WEBB P.J., ROBINS R.A., ROBINSON G., BALDWIN R.W. & FESTING M.F.W. (1980) Studies on the immunobiology of rnu/rnu 'nude' rats with congenital aplasia of the thymus. *Eur. J. Immunol.* **10,** 58.

BUTCHER G.W. (1987) A list of monoclonal alloantibodies specific for alloantigens of the rat. *J. Immunogenetics*, **14,** 163.

CHEN W.F., SCOLLAY R., SHORTMAN K., SKINNER M. & MARBROOK J. (1984) T-cell development in the absence of a thymus: the number, the phenotype, and the functional capacity of T lymphocytes in nude mice. *Am. J. Anat.* **170,** 339.

DOENHOFF M.J. & DAVIES A.J.S. (1971) Reconstitution of the T-cell pool after irradiation of mice. *Cell. Immunol.* **2,** 82.

DRAYSON M.T., SPARSHOTT S.M. & BELL E.B. (1989) Fidelity of the repertoire in T cell reconstituted athymuc nude rats. Preservation of a deficit in alloresponsiveness over one year. *J. exp. Med.* **170,** 691.

DYER M.J.S. & HUNT S.V. (1981) Committed T lymphocyte stem cells of rats. Characterisation by surface W3/13 antigen and radiosensitivity. *J. exp. Med.* **154,** 1164.

FESTING M.F.W. (1981) Athymic nude rats. In: *Immunologic Defects in Laboratory Animals* (eds M. E. Gershwin and B. Merchant), p. 267. Plenum Press, New York.

FORD C.E. (1966) The use of chromosome markers. Appendix to *Tissue Grafting and Radiation* (eds H. S. Micklem and J. F. Loutit). Academic Press Inc., New York.

FORD W.L. (1978) The preparation and labelling of lymphocytes. In: *Handbook of Experimental Immunology* (ed. D. M. Weir), 3rd edn, Chap. 23. Blackwell Scientific Publications, Oxford.

FORD W.L., BURR W. & SIMONSEN M. (1970) A lymph node weight assay for the graft-versus-host activity of rat lymphoid cells. *Transplantation*, **10,** 258.

FORD W.L., SPARSHOTT S.M. & ROLSTAD B. (1983) Do nude rats display any alloaggression? *Transplant. Proc.* **15,** 1658.

FOSSUM S., SMITH M.E., BELL E.B. & FORD W.L. (1980) The architecture of rat lymph nodes. III. The lymph nodes and lymph-borne cells of the congenitally athymic nude rat (rnu). *Scand. J. Immunol.* **12,** 421.

GILLIS S., UNION N.A., BAKER P.E. & SMITH K.A. (1979) The *in vitro* generation and sustained culture of nude mouse cytolotic T-lymphocytes. *J. exp. Med.* **149,** 1460.

GURDON J.B. (1988) A community effect in animal development. *Nature (Lond.)*, **336,** 772.

HAREL-BELLAN A., QUILLET A., MARCHIOL C., GERARD J.-P. & FRADELIZI D. (1987) Lack of reconstitution of nude mice alloreacti-

vity by purified interleukin 2 and induction of non-H-2-specific effector cells by crude supernatants. *Cell. Immunol.* **105,** 251.

HOWARD J.C. (1971) A histocompatible chromosome marker system in the laboratory rat *Rattus norvegicus*. *Transplantation*, **12,** 95.

HOWARD J.C. & SCOTT D.W. (1974) The identification of sera distinguishing marrow-derived and thymus-derived lymphocytes in the rat thoracic duct. *Immunology*, **27,** 903.

HUNIG T. (1983) T-cell function and specificity in athymic mice. *Immunol. Today*, **4,** 84.

HUNIG T. & BEVAN M.J. (1980) Specificity of cytotoxic T cells from athymic mice. *J. exp. Med.* **152,** 688.

HUNT S.V. (1979) Surface antigens characterising lymphopoietic stem cells in rats. In: *Immunobiology of Bone Marrow Transplantation* (eds S. Thierfelder, H. Rodt and H. J. Colb), p. 193. Springer Verlag, Berlin.

HUNT S.V. (1987) Preparation of lymphocytes and accessory cells. In: *Lymphocytes, a Practical Approach* (ed. G. G. B. Klaus), p. 43. IRL Press, Eynsham, Oxon.

HUNT S.V. & FOWLER M.H. (1981) A repopulation assay for B and T lymphocyte stem cells employing irradiation chimaeras. *Cell Tiss. Kinet.* **14,** 445.

KINDRED B. (1979) Nude mice in immunology. *Prog. Allergy*, **26,** 137.

KISHIHARA K., YOSHIKAI Y., MATSUZAKI G., MAK T.W. & NOMOTO K. (1987) Functional α and β T cell chain receptor messages can be detected in old but not in young athymic mice. *Eur. J. Immunol.* **17,** 477.

KUNG J.T. (1988) Impaired clonal expansion in athymic nude $CD8^+CD4^-$ T cells. *J. Immunol.* **140,** 3727.

KUNG J.T. & THOMAS C.A. (1988) Athymic nude $CD4^+8^-$ T cells produce IL-2 but fail to proliferate in response to mitogenic stimuli. *J. Immunol.* **141,** 3691.

LAWETZKY A. & HUNIG T. (1988) Analysis of CD3 and antigen receptor expression on T cell subpopulations of aged athymic mice. *Eur. J. Immunol.* **18,** 409.

MACDONALD H.R., BLANC C., LEES R.K. & SORDAT B. (1986) Abnormal distribution of T cell subsets in athymic mice. *J. Imunol.* **136,** 4337.

MACDONALD H.R., LEES R.K., BRON C., SORDAT B. & MIESCHER G. (1987) T cell antigen receptor expression in athymic (nu/nu) mice. Evidence for an oligoclonal β chain repertoire. *J. exp. Med.* **166,** 195.

MACDONALD H.R., LEES R.K., SORDAT B., ZAECH P., MARGANSKI J.L. & BRON C. (1981) Age-associated increase in expression of the T cell surface markers Thy-1, Lyt-1 and Lyt-2 in congenitally athymic (nu/nu) mice: analysis by flow microfluorometry. *J. Immunol.* **126,** 865.

MAGE M.G., MCHUGH L.L. & ROTHSTEIN T.L. (1977) Mouse lymphocytes with and without surface immunoglobulin: preparative scale separation in polystyrene tissue culture dishes coated with specifically purified anti-immunoglobulin. *J. immunol. Meth.* **15,** 47.

MALECKAR J.R. & SHERMAN L.A. (1987) The composition of the T cell receptor repertoire in nude mice. *J. Immunol.* **138,** 3873.

MARYANSKI J.L., MACDONALD H.R., SORDAT B. & CEROTTINI J.C. (1981) Cytolytic T lymphocyte precursor cells in congenitally athymic C57BL/6 nu/nu mice: quantitation, enrichment, and specificity. *J. Immunol.* **126,** 871.

MATIS L.A., CRON R. & BLUESTONE J.A. (1987) Major histocompatibility complex-linked specificity of $\gamma\delta$ receptor-bearing T lymphocytes. *Nature (Lond.)*, **330,** 262.

MOJCIK C.F., GREINER D.L., GOLDSCHNEIDER I. & LUBAROFF D.M. (1987) Monoclonal antibodies to RT7 and LCA antigens in the rat: cell distribution and segregation analysis. *Hybridoma*, **6,** 531.

NEWTON M.R., WOOD K.J. & FABRE J.W. (1986) A monoclonal alloantibody detecting a polymorphism of the rat leukocyte-common (LC) antigen. *J. Immunogenetics*, **13,** 41.

PIGUET P.-F. & VASSALLI P. (1982) Long term reconstitution of 'B' mice with post-thymic T cells: expansion of the post-thymic T cells and absence of extra-thymic maturation. *Behring. Inst. Mitt.* **70,** 96.

PRITCHARD H. & MICKLEM H.S. (1973) Haemopoietic stem cells and progenitors of functional T-lymphocytes in the bone marrow of 'nude' mice. *Clin. exp. Immunol.* **14,** 597.

VAESSEN L.M.B., BROCKHUIZEN R., ROZING J., VOS J.G. & SCHUURMAN H.J. (1986) T-cell development during ageing in congenitally athymic (nude) rats. *Scand. J. Immunol.* **24,** 223.

VAN VLIET E., JENKINSON E.J., KINGSTON R., OWEN J.J.T. & VAN EWIJK W. (1985) Stromal cell types in the developing thymus of the normal and nude mouse embryo. *Eur. J. Immunol.* **15,** 675.

WAGNER H., HARDT C., HEEG K., ROLLINGHOFF M. & PFIZENMAIER K. (1980) T-cell-derived helper factor allows *in vitro* induction of cytotoxic T cells in *nu/nu* mice. *Nature (Lond.)*, **284,** 278.

WHITBY E.H., SPARSHOTT S.M. & BELL E.B. (1990) 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. *Immunology* (in press).

YOSHIKAI Y., MATSUZAKI G., TAKEDA Y., OHGA S., KISHIHAVA K., YUUKIJ H. & NOMOTO K. (1988) Functional T cell receptor δ chain messages in athymic nude mice. *Eur. J. Immunol.* **18,** 1039.

YOSHIKAI Y., REIS M.D. & MAK T.W. (1986) Athymic mice express a high level of functional γ-chain but greatly reduce levels of α -and β-chain T-cell receptor messages. *Nature (Lond.)*, **324,** 482.

YOSHIKAI Y., TAKEDA Y., OHGA S., KISHIHARA K., MATSUZAKI G. & NOMOTO K. (1989) Rearrangement of T-cell antigen receptor γ and δ chain genes are detected in the long-term cultured bone marrow cells of athymic nude mice but not in those of euthymic mice. *Immunology*, **66,** 512.