T cell regeneration after allogeneic bone marrow transplantation

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SUMMARY

Various T cell subsets were characterized by double immunofluorescent staining using monoclonal antibodies (MoAb) in blood, bone marrow (BM) and tissues of 29 patients after allogeneic BM transplantation (BMT). In an attempt to prevent graft versus host disease (GvHD), 15 patients received cyclosporin A (Cy A). In the remaining 14 patients the BM was pre-incubated with a MoAb, OKT3. The regeneration of T4$^+$ subset was delayed and the level of T8$^+$ cells was abnormally high even 1 year after engraftment. This did not have any predictive value for the appearance of complications such as GvHD or severe viral infections. The number of T8$^+$ cells was lower in the group of patients who received Cy A than in the OKT3 group (0.7 ± 0.2 *vs* $1.5 \pm 0.3 \times 10^9$/l at day 90). In contrast to normal individuals, the T4/T8 ratio in both blood and regenerating BM of BMT patients was < 1. A sizeable subset of circulating T cells expressed the phenotype T8$^+$,T10$^+$,HNK-1$^+$,DR$^+$. Circulating cells of this phenotype were transiently very high (up to 50%) in patients with active GvHD or suffering from severe viral infection. This subpopulation of lymphocytes was not found in the epidermal infiltrate that accompanied GvHD where the predominant phenotype was T8$^+$,T1$^-$,T10$^-$,HNK-1$^-$,DR$^-$. We conclude therefore that after BMT the number and phenotype of circulating T cells reflects the T cell distribution seen in the regenerating BM.

Keywords bone marrow transplantation graft versus host disease cyclosporin A T cell development T cell subsets

INTRODUCTION

After uncomplicated allogeneic and autologous bone marrow (BM) transplantation (BMT) in man the regeneration of T lymphocytes of suppressor/cytotoxic type (T8$^+$, Leu-2$^+$) is quicker than that of the inducer type subset (T4$^+$, Leu-3$^+$) (De Bruin *et al.*, 1981; Friedrich *et al.*, 1982; Atkinson *et al.*, 1982). The more rapid regeneration of the T8$^+$ subset cannot be directly attributed to either the effects of graft versus host disease (GvHD) or to those of overt viral infections. In one group of patients, predominantly children, a gradual recovery of Leu-3$^+$ (T4 equivalent) cells with normalization of subset profiles was observed within a 3–6 month period (Friedrich *et al.*, 1982). In

a larger group of adult patients treated for leukaemia, the absolute number of $T4^+$ cells remained depressed for a much longer period, i.e. of up to 2 years (Atkinson *et al.*, 1982).

Markers as OKT10 (De Bruin *et al.*, 1981; Atkinson *et al.*, 1982) and binding of peanut agglutinin (PNA; Van der Griend *et al.*, 1981) appear on circulating T cells after BMT, and have been interpreted as putative markers of immaturity and perhaps even thymocyte markers (Van der Griend *et al.*, 1981). This phenomenon requires close attention because the educative role of thymus after BMT in adults has been assumed but not proven.

In the present study a double colour immunofluorescence (IF) analysis of the various T cell subsets has been carried out in two groups of patients after allogeneic BMT. In group A (14 patients) the BM had been pre-treated, prior to re-infusion, with a monoclonal antibody (MoAb) to peripheral T cells (OKT3 of IgG2 class; Kung *et al.*, 1980) in an attempt to prevent GvHD (Prentice *et al.*, 1982). Individuals in group B (15 patients) had been given cyclosporin A (Cy A) from 0–180 days after BMT (Powles *et al.*, 1980). Neither of these measures prevents GvHD fully (Prentice *et al.*, 1982; Powles *et al.*, 1980) and the following questions have been investigated:

(1) What is the speed of 'uncomplicated' recovery of blood borne $T8^+$ and $T4^+$ subpopulations in groups A and B?
(2) Does either GvHD or overt viral infection influence this recovery pattern?
(3) What are the exact phenotypic features of the regenerating T cell subsets when analysed by a panel of MoAbs (Reinherz *et al.*, 1980a; Janossy *et al.*, 1981; Tidman *et al.*, 1981)? Selected combinations of antibodies were applied in order to determine the co-expression T8, HLA-DR, T10 and T1 antigens (Tidman *et al.*, 1981) as well as the expression of HNK-1, an antigen associated with cells expressing natural killer (NK) activity (Abo, Cooper & Balch, 1982).
(4) Finally, how do the characteristics of circulating T lymphocytes following BMT compare with those of normal thymocytes (Reinherz *et al.*, 1980a; Tidman *et al.*, 1981) and with the features of T cells seen in the patients' BM and skin during the development of GvHD?

PATIENTS

The patients were in remission from leukaemia. Thirty cases were aged 17–50 years and two children aged 4 and 12 years. All received a conditioning regimen of high dose cyclophosphamide and total body irradiation followed by intravenous injection of donor BM containing $1\cdot2$–2 (mean $1\cdot9$) $\times 10^8$ cells/kg unseparated nucleated BM cells or $3\cdot6$–$7\cdot4$ (mean $5\cdot0$) $\times 10^5$ cells/kg separated mononuclear BM cells. The transplants were matched for HLA-A,B,C as well as for D and Dr antigens. In group A (14 patients) the BM (concentrated in 50–70 ml Hanks solution) was pre-incubated with 1 mg OKT3 antibody for 30 min at $20°C$. After one wash patients received these 'coated' T cells. In addition, 12 of the patients received intravenous methotrexate (10 mg) at week 15. In group B the 15 recipients were given Cy A therapy ($12\cdot5$ mg/kg/day from day 0 to 6 months). In both groups patients developing GvHD received prednisone therapy.

Patients were studied at monthly intervals from 3 weeks to 6 months. In addition, in group A 10 patients were studied between 1 and 3 weeks. In group B nine patients were followed for 6–8 months, i.e. up to 2 months after the cessation of Cy A therapy. In both groups, few patients followed for a shorter period of 3–4 months have been included because patients with similar clinical courses were studied at complementary intervals.

The clinical course was regarded as uncomplicated if (a) the patient had no GvHD or developed only a transient skin rash (grade 1) which resolved without treatment and (b) showed no evidence of a severe viral infection (eight patients in group A and 11 in group B). Most of the patients developed transient local herpes infection with a good response to acyclovir. The complicated cases included three patients (two in group A; one in group B) presenting only CMV infection (assessed on positive culture and rise of antibody titres), 10 patients (six in group A, four in group B) with a severe grade II-IV GvHD, and, finally, four patients (three in group A, one in group B) with CMV infection and severe GvHD. The clinical assessment and gradation of GvHD were based on involvement of gut, liver, and skin as reported by Thomas *et al.* (1980).

MATERIALS AND METHODS

Reagents and their combinations (Table 1). The proportion of T4$^+$ and T8$^+$ cells was routinely determined by double staining using fluorescein isothiocyanate (FITC; green label) for T4 and tetraethylrhodamine isothiocyanate (TRITC; red label) for T8 (Tidman *et al.*, 1981). This convenient combination detects T cells of inducer (T4$^+$,T8$^-$) and suppressor/cytotoxic type (T8$^+$,T4$^-$) as well as thymocyte like cells with immature features (T4$^+$,T8$^+$) and non-T cells (T4$^-$,T8$^-$). The presence of T3$^+$ cells (pan-T reagent) indicated the number of mature T lymphocytes. In 13 samples from eight patients (in group A) cells were stained for T6 human thymocyte antigen in suspension (FITC), smeared and restained for nuclear terminal transferase (TdT; TRITC). T6$^+$, TdT$^+$ cells were regarded as immature thymocytes (Janossy *et al.*, 1980; Tidman *et al.*, 1981).

In the next stage of the study the T8$^+$ population was studied in more detail in combination with the following reagents: anti-HLA-DR detecting human Ia like antigen (Reinherz *et al.*, 1980b), OKT10, a monoclonal antibody to immature lympho-haemopoietic cells and activated T cells (Reinherz *et al.*, 1980a; Janossy *et al.*, 1981), RFT-1 (an OKT1-like, Leu-1 like reagent; Janossy *et al.*, 1981; Martin *et al.*, 1981) detecting the Lyt-1 equivalent antigen of 69 dalton on human lymphocytes and, finally, with HNK-1 (Leu-7) antibody which labels 'large granular lymphocytes' including natural killer (NK) cells (Abo *et al.*, 1982). HNK-1$^+$ populations expressing HLA-DR antigens were also studied with double staining (Table 2 and Fig. 2).

The quality controls and absorption procedure of the heterologous antisera have been published previously (Janossy *et al.*, 1981; Tidman *et al.*, 1981).

Cells and tissues. Red cells and the majority of granulocytes were removed from the blood and bone marrow samples on a Ficoll-Hypaque gradient. Cells were resuspended at a 10^7/ml concentration and stained in indirect immunofluorescence test (Janossy *et al.*, 1980, 1981). Tissue biopsies were snap frozen in cooled isopentane and cut in a cryostat. The sections were dried, fixed in acetone, and stored at $-70°C$ in lyophilized form until use (Lampert *et al.*, 1982). The indirect immunofluorescence staining was performed using the same final antibody concentrations as for cell suspensions.

The expression of results and controls. The T4/T8 ratio in the samples have been directly determined by the double staining combination (Table 2). The absolute numbers of T4$^+$ and T8$^+$ cells in the samples were calculated on the basis of (1) blood lymphocyte count, (2) the proportion of T4$^+$ and T8$^+$ cells within the lymphoid population (corresponding to the proportion of T3$^+$ cells; 70–75% of lymphoid cells) and (3) the proportion of T3$^-$ non-T cells (25–30% within the lymphoid population representing a variable mixture of SmIg$^+$ B cells, OKM1$^+$ cells of lymphoid appearance and non-T, HNK-1$^+$ cells). The latter values (25–30%) were established on eight samples of blood. Parameters 1 and 2 were routinely performed in each sample. The two sets of control blood values were normal volunteers ('c') and treated patients in haematological leukaemia (pre-transplant controls, 'p').

RESULTS

Analysis of T4$^+$ and T8$^+$ cells in blood during BM regeneration

The results have been evaluated in both group A ('OKT3') and group B ('Cy A') patients with no serious post-transplant complications (full symbols in Fig. 1). The regeneration of T8$^+$ cells was rapid in both groups and reached normal levels within 25 days (Fig. 1). The regeneration of the T4$^+$ population was slower and reached normal levels around 90–120 days post-transplant in both groups. By that time the absolute numbers of T8$^+$ cells had further increased. In the 'OKT3' group the mean of T8$^+$ cells were two–four times higher than control levels, while in the Cy A group the means of T8$^+$ cells were one and a half–three times higher than control levels. The only statistically significant difference between the two groups was the higher absolute numbers of T8$^+$ cells in the 'OKT3' as compared to the 'Cy A' group among the patients with an uncomplicated course. As a result, the T4/T8 ratios remained severely depressed throughout the whole follow-up period in both

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Table 1. Double reagent combinations used in this study*†

Combination	1st layer	2nd layer	1st layer	2nd layer	Comment about use
1	OKT4γ2	G anti-γ (FITC)	T8μ	G anti-μ (TRITC)	T4/T8 ratio
2	OKT6γ2	G anti-M (FITC) (membrane antigen)	R anti-TdT (nuclear antigen)	G anti-R (TRITC)	Cortical thymocytes (TdT is detected in smears)
3	OKT8γ2	G anti-γ (FITC)	HLA-DRμ	G anti-μ (TRITC)	'activated' DR$^+$ T8 cells
4	OKT8γ2	G anti-γ2 (FITC)	OKT10γ1	G anti-γ1 (TRITC)	T10$^+$ thymocytes and 'activated' T8 cells
5	T8μ	G anti-μ (TRITC)	T1γ1	G anti-γ (FITC)	T1 is the homologue of murine Lyt-1
6	OKT8γ2	G anti-γ (FITC)	HNK-1	G anti-1μ (TRITC)	HNK labels large granular lymphocytes with NK activity
7	HLA-DRγ1	G anti-γ (FITC)	HNK-1	G anti-μ (TRITC)	

* G = goat; M = mouse Ig; R = rabbit Ig; C = chicken Ig. G anti-γ, γ1, γ2 and μ refer to goat anti-mouse (sub)class specific reagents purchased from Miles Laboratories and further absorbed when necessary with human rabbit or mouse Ig (sub)class (Tidman et al., 1981). TdT = terminal transferase.

† OKT reagents are the available ORTHO range. Monoclonal anti-HLA-DRμ, HLA-DRγ1, T8μ and T1γ1 antibodies have been made in the Royal Free Hospital. T8 (RFT8) and T1 (RFT1) bind to the same epitopes as OKT8 and OKT1 respectively. HNK-1 is marketed by Becton-Dickinson as Leu-7.

Table 2. Phenotypic characteristics of T8+ cells in normal blood, BM and tonsil, in the blood of patients after BMT

	T4/T8 ratio	% of T8+ cells within the T population	% of T10	% of HLA-DR+	% of T1+ cells within the T8+ population	% of HNK-1+
Normal controls						
blood (normal volunteers)	2·2 ±0·21 (12)*	29±2·0 (12)†	6·2 (4)	3±0·8 (6)	96±0·9 (6)	26±3 (6)
blood (pre-transplant)‡	1·61±0·37 (6)	41·5±4·5 (6)	n.t.	18±5 (5)	n.t.	19±3 (4)
BM (normal donors)	0·51±0·11 (10)	71±4·4 (10)	40 (4)	3±0·4 (4)	98±0·3 (6)	27±6 (3)
tonsil§	8·1 (6)	21±3·8 (6)	33 (6)	4±0·9	90±0·3	<5
cord blood	4·5 ±1·0 (3)	18·6±3·1 (3)	65±8 (5)	30±10 (3)	n.t.	0 (5)
Blood values after BMT¶, patients with no complications:						
days 15–30	0·61±0·11 (13)	63±5 (13)	44±5 (4)	40±3 (10)	94±1 (7)	35,43
days 31–45	0·62±0·12 (13)	66±4 (13)	32,36	43±7 (8)	95±1 (6)	27,31
days 46–60	0·71±0·20 (8)	64±6 (8)	29,31	29±6 (5)	95±1 (5)	23,25
days 61–90	0·97±0·37 (7)	53±8 (4)	38±2 (3)	26±7 (3)	n.t.	22±3 (3)
days 91–180	0·77±0·13 (30)	62±3 (30)	30±5 (9)	26±9 (11)	n.t.	31±7 (7)
Blood values after BMT patients with GvHD and/or CMV infections:						
days 15–30	0·80±0·31 (11)	65±7 (11)	76±2 (4)	41±8 (13)	93,94	28±9 (7)
days 31–45	0·96±0·23 (13)	60±6 (15)	45	45±8 (14)	97,98	44±12 (6)
days 46–60	0·88±0·21 (11)	59±6 (11)	48,52	35±11 (7)	94±1 (5)	25±7 (10)
days 61–90	1·22±0·41 (9)	52±8 (9)	30	30,36	n.t.	23±7 (3)
days 91–180	0·49±0·08 (10)	69±4 (10)	24±8 (3)	19±12 (4)	n.t.	41,45

* ±s.e. (mean). The number of samples are shown in parentheses.

† These values were obtained by reagent combinations 1, 3, 4, and 5 respectively (Table 1). The number of T cells was determined as (T4+,T8− + T8+,T4−). This was similar to the numbers of T3+ cells.

‡ These patients received chemotherapy for leukaemia treatment.

§ Elective tonsillectomy after antibiotic therapy. Similar results were observed by histological analysis (Fig. 4).
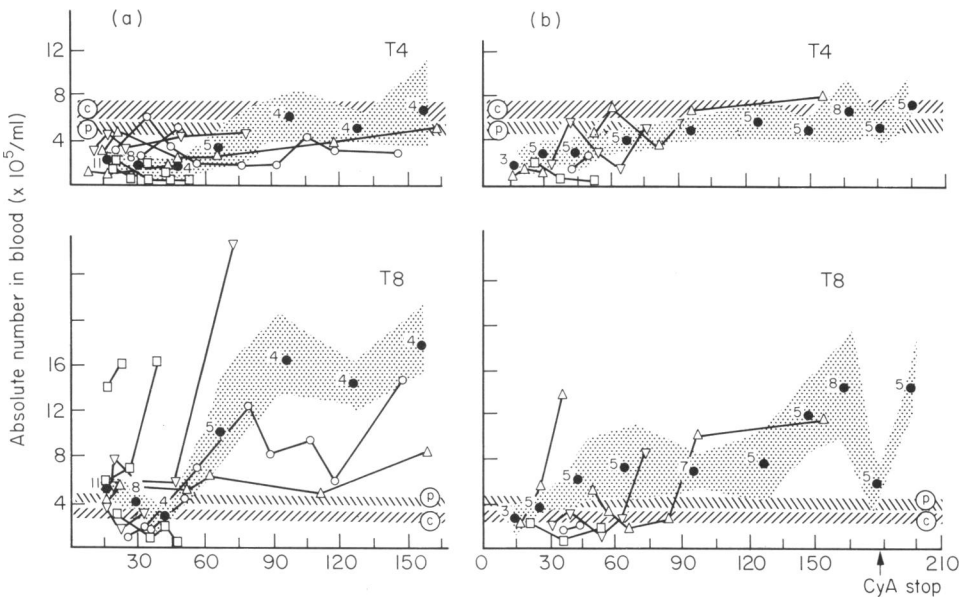
¶ Similar results were observed in BM (Fig. 3).

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Fig. 1. Regeneration of T4$^+$ and T8$^+$ T lymphocyte subsets after allogeneic BMT. In group A the BM was incubated with 1 mg antibody OKT3. Patients in group B were treated with Cy A. The mean values (●) of patients with uncomplicated course of regeneration and the range (s.e.:▦) are shown and the numbers refer to the number of observations (n) in each group. (△, ▽) = individuals with GvHD only; (○) = patients with CMV infection only; (□) = patients with severe GvHD plus verified CMV infection. The control values are: (c) normal controls; (p) patients waiting for BMT in full remission after therapy for leukaemia.

groups. From the results shown in Fig. 1 the mean values for the T4/T8 ratios have been calculated between the period of 90–180 days post-transplant. In the OKT3 group the T4/T8 ratio was more depressed (0.35 ± 0.13) than in the 'Cy A' group (1.15 ± 0.30). The P value is < 0.02.

Atkinson *et al.* (1982) reported a short wave of suboptimal T4$^+$ cell reconstitution around 15–25 days after BMT. This transient T4 dominance has also been noticed in five of 10 patients studied in group A. Patients in group B were not investigated during this early period.

Patients with complicated courses (i.e. with GvHD and/or CMV infection) showed great individual variations. Neither the mean level of T8$^+$ nor T4$^+$ cells was significantly different from the values of transplanted patients with no complications. In the patients with GvHD involvement, especially if this was complicated by CMV infections, serious T4$^+$ lymphopenia appeared to develop frequently (see below and Fig. 2).

Phenotypic features of T8$^+$ lymphocytes in the blood after BMT and in the normal thymus
In the lymphoid populations analysed 10–30 days after BMT 30–70% (mean 40.6%) of T lymphocytes expressed T10 antigen. When further studied in four selected cases the majority ($> 60\%$) of T10$^+$ cells were T8$^+$ (Table 2). This phenomenon was seen both in the 'OKT3' and 'Cy-A' group. This observation raises the possibility that immature thymocyte like cells circulate. Nevertheless in the 25 samples analysed at various intervals after BMT, cells with double T4 + T8 expression could not be identified (Table 2). Similarly, circulating cells did not express the T6 marker or nuclear terminal transferase (TdT) in the 13 patients analysed (13 for T6 and four for TdT). The same reagents simultaneously stained 60–70% of normal infant thymocytes (T8$^+$,T4$^+$,T6$^+$,TdT$^+$). In addition, the percentage of circulating T10$^+$ T lymphocytes remained elevated throughout the study (> 6 months after BMT).

In the second part of the study the T8$^+$ cells were tested for expression of HLA-DR and HNK-1 (Table 2). High levels of HLA-DR$^+$, T8$^+$ cells were seen in both the 'OKT3' and 'Cy A' groups,
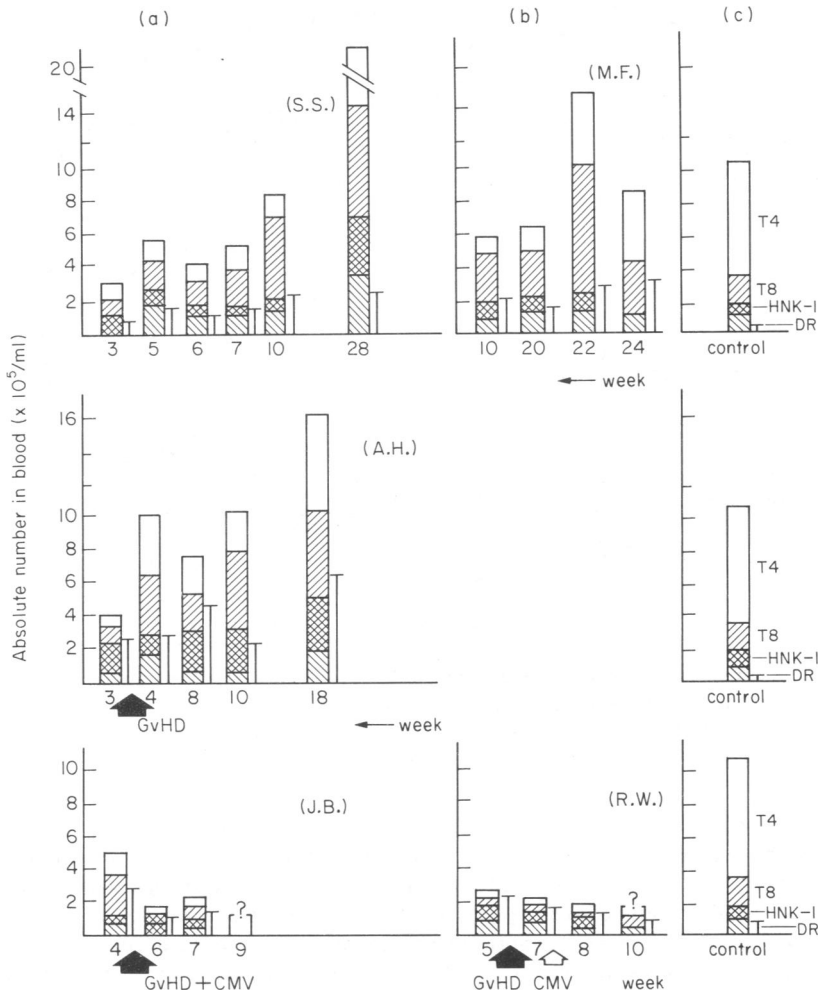
Fig. 2. The absolute numbers of T4+ (□), T8+ (■) and HNK-1+ cells (▧) in the blood of representative patients with no complications (S.S. and M.F.) with GVHD only (A.H.) and with CMV infection + GvHD (J.B. and R.W.). The overlap of T8+ and HNK-1+ cells have been directly visualized by double labelling (Table 2). The HLA-DR+ cells were also investigated in both the T8+ and HNK-1+ populations and approximate values are shown by the bars at the side of the columns. In the samples from patient A.H. (at weeks 4, 8, 10 and 18), from J.B (at week 4), from R.W. (at weeks 5, 7 and 8) most of the T8+ and HNK-1+ cells are HLA-DR positive (but see the phenotype of the infiltrating T8+ cells in the skin biopsies from patient A.H. in Fig. 4).

irrespective of whether these patients had a normal clinical status or developed overt CMV infection and/or GvHD. During the first 45 days after the graft HLA-DR was expressed on approximately 40% of the T8+ population (Table 3). After 2 months the percentage of HLA-DR+ cells among the T8+ lymphocytes remained as high as 30% in complicated cases but slightly decreased (below 30%) in patients with a normal course. Nevertheless the latter values were still higher than those in the normal control blood samples (< 5% of HLA-DR+ cells within the T8+ marker subset; Fig. 2).

The HNK1+ cells in the normal blood were larger granular lymphocytes (10–20% of all lymphoid cells). Of the T8+ cells 25–30% were HNK-1+ (Fig. 2, Table 2). In the blood of patients following BMT the percentage of HNK-1+ was high particularly in samples studied during the first month. The HNK-1+ population included both T8+ and T8− lymphocytes. The proportions of overlapping T8+ HNK-1+ cells in blood of BMT patients was similar to normal blood values but

Table 3. Heterogeneity of T8$^+$ cells in man*

	Co-expression of T4	T10	HLA-DR	HNK-1	T1
Normal samples					
thymus	+(>70)	+(>70)	−(<5)	−(<1)	+(weak)
cord blood†	−(<2)	+(>50)	−(<10)	−(<5)	+(>80)
blood†	−(<2)	−(<10)	−(<10)	25–40	+(>80)
tonsil†	−(<2)	−(<5)	−(<10)	−(<10)	+(>80)
bone marrow‡	−(<2)	−(<5)	−(<10)	−(<20)	+(>80)
intraepithelial lymphocytes in gut‡	−(<2)	−(<5)	−(<10)	−(<5)	−(<20)
BM transplants and pathological conditions					
blood during viral infections‡	−(<2)	n.t.	+(>50)	n.t.	+(>80)
regenerating BM and blood after BMT‡	−(<5)	+(>50)	20–40	20–40	+(>80)
−"−+ viral complication and GvHD‡	−(<5)	+(>70)	20–80	20–60	+(>80)
skin infiltrations in GvHD‡	−(<5)	−(<5)	−(<10)	−(<10)	−(<20)

* Percentages are shown in parentheses. It is obscure how these markers relate to function (precursor, suppressor and cytotoxic) or stage of activation.

† In these samples the T4$^+$ cells predominate over the T8$^+$ population.

‡ In these samples T8$^+$ cells dominate.

from the 5th week onward the absolute number of HNK-1$^+$,T8$^+$ circulating cells were frequently higher than the normal controls (Fig. 2).

The percentage of circulating DR$^+$ T8$^+$ cells or HNK-1$^+$, T8$^+$ cells did not show an exact correlation with the appearance of complications and these parameters could not be applied for an early diagnosis of GvHD or CMV infections. Nevertheless, differences could be seen in the lymphocyte subset markers of various patients once the disease developed. When the absolute numbers of DR$^+$, T8$^+$ cells and HNK-1$^+$ T8$^+$cells have also been counted (as shown in Fig. 2) in six patients (two with GvHD and four with GvHD and CMV infection) particularly high levels (> 70–80%) of T8$^+$ cells expressed DR antigens (see A.H. in Fig. 2). The same populations also had
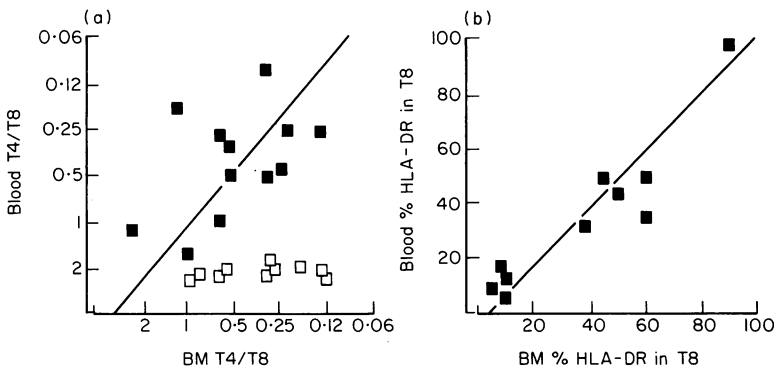

Fig. 3. Comparative study of two T cell parameters in the blood and bone marrow within the same patients after BMT (a): T4/T8 ratio; (b) percentage HLA-DR$^+$ cells within the T8$^+$ population. Empty squares refer to normal healthy volunteers as controls.

>45% HNK-1$^+$ cells. In these cases it was possible to directly demonstrate that a sizeable proportion of T cells have the T8$^+$,DR$^+$,T10$^+$,HNK-1$^{+/-}$,T4$^-$,T6$^-$,TdT$^-$ phenotype. In contrast in some patients with CMV infections and severe GvHD, severe lymphopenia developed (Fig. 2, patients J.B and R.W). Anti-T antibodies as well as anti-HLA-DR and HNK-1 antibodies reacted only weakly with the residual circulating putative T cells. These cells were, again, T6$^-$,TdT$^-$.

The final point of this phenotypic analysis is relevant to the histological investigations described below. T8$^+$ cells in the blood after BMT in all groups were mostly T1$^+$ although the staining was of variable intensity (Table 2).

Analysis of T cell subsets in the BM
Thymocyte features (T4+T8$^+$,T6$^+$ and TdT$^+$) were looked for in BM samples 10–30 days after BMT. In two, 3–3·5% of T cells were double labelled for T4+T8 but in five other samples no doubles were seen. None of the 13 samples studied contained T6$^+$,TdT$^+$ thymocytes. Next the T4/T8 ratio and the percentage of HLA-DR$^+$ cells within the T8$^+$ subset were compared in the BM and blood samples of the same patients at different intervals after BMT (Fig. 3). Large variations were seen between the different patients but the values seen in the blood and the BM of the same individual correlated closely with each other. This finding is in contrast with the observations seen in the BM and blood of normal healthy BM donors, where the subset distribution in the BM (T4/T8 = 0·5) differs from those in the blood (T4/T8 = 2·1; Fig. 3).

Finally, additional phenotypic analysis of the T8$^+$ population was carried out in the BM 45–60 days after BMT in three patients in the 'OKT3' group and three from the 'Cy A' group with uneventful BMT recovery. The BM T8$^+$ cells in the BM expressed DR (45 ± 12% s.e.), T10 (85 ± 21%) and T1 antigens (80 ± 22%). The proportion of HNK-1$^+$ cells in this population was also high (32 ± 20%). These features corresponded to the phenotype of the blood borne T8$^+$ cells.

Immunohistological analysis of T8$^+$ cells
The double marker technique was suitable to detect positive cells in tissue sections. The anti-HLA-DR reagent strongly and clearly labelled many T8$^+$ lymphocytes in the skin of a patient with lichen planus. In the thymus cortical thymocytes were T10$^+$,T8$^+$. The HNK-1 antibody strongly stained a few T8$^+$ cells infiltrating the breast cancer tissue. Finally, in tonsil 70–80% of T8$^+$ cells were positive with the T1 antibody (Table 2).

The same reagent combinations were applied for the analysis of lymphoid infiltrates in six samples of skin taken at the peak of acute GvHD. Moderate to heavy lymphoid infiltrates were seen. The majority (70–95%) of cells that had reached the epidermal regions were T8$^+$,T4$^-$ cells with typical uropods. This selective staining for T8 is attributable to a special migratory pattern since a few T4$^+$,T8$^-$ cells could be observed in the same samples within deeper regions of the dermis where they constituted 30–40% of all cells within the perivascular cuff.

The next analysis was then concentrated on the T8$^+$ cells in the GvHD skin. These were almost exclusively HLA-DR$^-$, although the reagent strongly labelled the tissue macrophages and the vascular endothelium (Fig. 4a). Both T10 and HNK-1 antibodies stained only a minority (5–10%) of the T8$^+$ cells (Figs. 4b & c). The MoAbs to T1 antigen strongly labelled a minute population of T cells but these cells were the rare T8$^-$ (T4$^+$) cells. The T8$^+$ lymphocytes had no or only weak staining for T1 (Fig. 4d). Finally it was shown that the T8$^+$ cells expressed other T cell markers such as T3 (detected by UCHT1 antibody; Beverley & Callard, 1981) and T11 antigen (identified by OKT11 antibody; Reinherz *et al.*, 1980a). The phenotype of the T8$^+$ population in the skin during GvHD is therefore T8$^+$,T3$^+$,T11$^+$ (T lymphocytes) with weak or no detectable expression of DR, T10, HNK-1 and T1 antigens. This phenotype is different from the features of the circulating T8$^+$ populations in the same patients (see legend to Figs 2 & 4).

DISCUSSION

The results of this study can be discussed in five topics. First, the pattern of T cell regeneration in the 'OKT3' and 'Cy A' groups is similar. In patients with an uncomplicated course only minimal or no
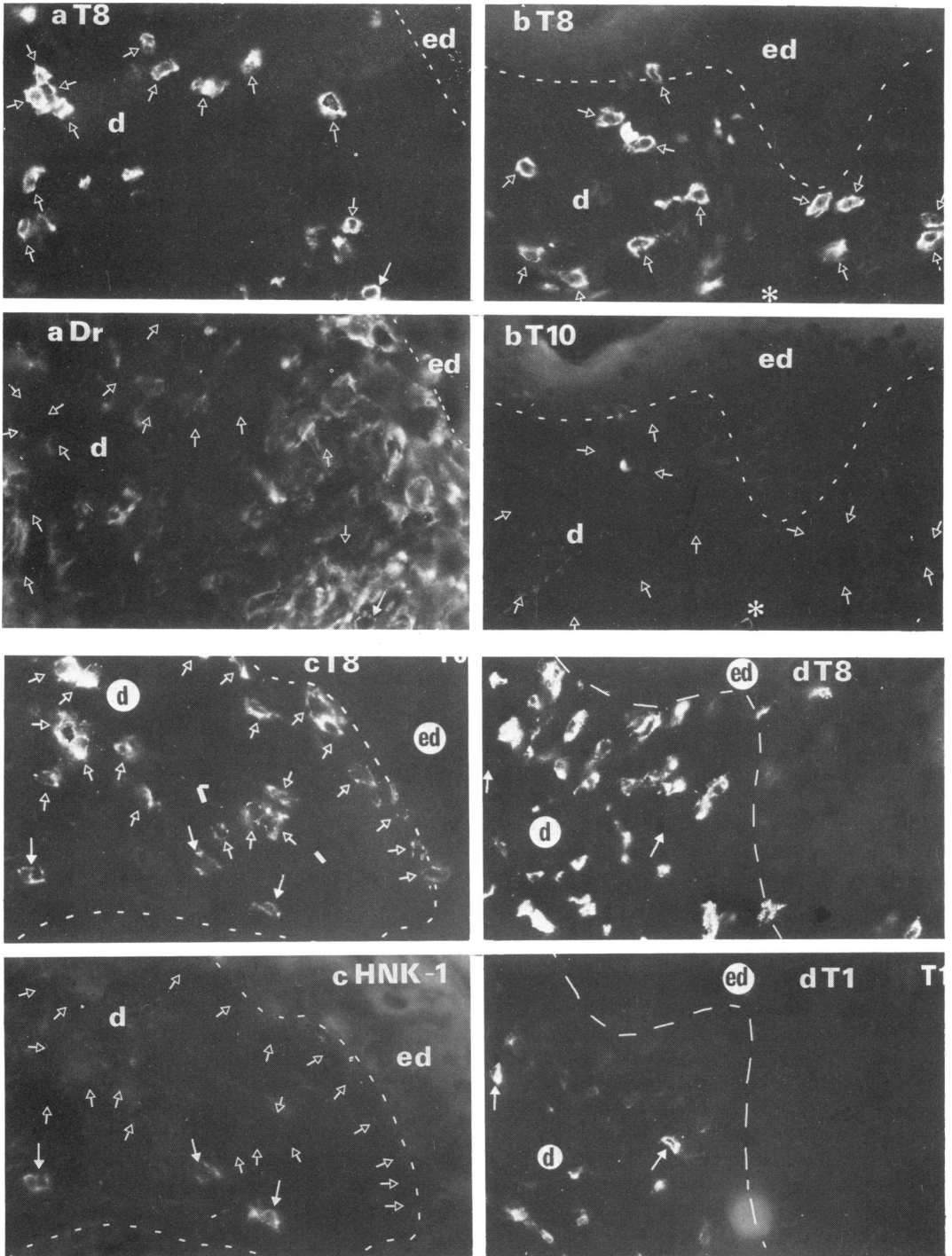
Fig. 4. The membrane phenotype of most T8$^+$ cells in the skin during GvHD is HLA-DR$^-$, T10$^-$, HNK-1$^-$ and T1$^-$. Double immunofluorescence staining for T8 (TRITC) and another antibody (FITC) was used in sections of frozen biopsies taken from patient A.H. (see Fig. 2, week 8). The same areas were photographed with selective filters for each fluorochrome. (a) T8 and HLA-DR double staining. Cells with T8 staining only are shown by empty arrows. Double stained cells are depicted with full arrow. (b) T8 and T10 double staining as in (a) except that the only T10$^+$ is T8$^-$ *). (c) T8 and HNK-1 double staining as in (a). (d) T8 and T1 double staining. The strongly T1$^+$ cells are T8$^-$ (full arrow). d = dermis; ed = epidermis.

immunosuppression was given (Prentice *et al.*, 1983; Powles *et al.*, 1980) and the number of T8$^+$ cells has reached normal values within 25 days and then continued to rise (Fig. 1). Normal levels of T4$^+$ cells have also been obtained but only within 90–120 days. The only difference between the 'OKT3' and 'Cy A' groups has been that in the 'OKT3' group the 'build-up' of T8$^+$ cells was significantly greater (Fig. 1). These values as well as the T4/T8 ratios in the 'OKT3' group are similar to those reported by Friedrich *et al.* (1982) suggesting that the lower T8 values in the 'Cy A' group may be specifically attributable to this drug's action. Nevertheless, Atkinson *et al.* (1982) compared patients receiving methotrexate with those receiving Cy A and found no difference. An additional point is that in Atkinson's study the recorded absolute values of T4$^+$ and T8$^+$ cells are lower than in our studies, while the values of 'null' cells are higher. This minor discrepancy could be due to technical differences (microscopy *vs* flow cytometer) or due to the possibility that in our study patients with severe viral infections and lymphopenia are discussed separately but are included in the study by Atkinson *et al.* (1982).

We have been unable to find thymocyte like cells (T6$^+$,TdT$^+$) after BMT in the BM and blood of adult patients. This is not surprising because thymocytes do not normally circulate and the few TdT$^+$ cells in normal and regenerating BM represent a different HLA-DR$^+$ non-T cell population (Janossy *et al.*, 1979). Nevertheless, very early in life (e.g. in normal cord blood) T6$^+$ cells circulate in small numbers (1–2%, exceptionally 10%) and it is therefore notable that no similar subpopulation was observed after BMT in this group of mostly adult patients. These observations indicate that previous findings about peanut lectin binding (Van der Griend *et al.*, 1981) and the expression of T10 markers (De Bruin *et al.*, 1981; Atkinson *et al.*, 1982) cannot be taken as evidence for thymocyte like cells circulating during the regeneration period.

The marked differences between the regenerating T8$^+$ subsets and typical thymocytes or immature (cord blood) T cells are surprising. Many newly formed T8$^+$ cells expressed HLA-DR and HNK-1 antigens and these markers are not observed on fetal or infant thymocytes or on T8$^+$ cells in the cord blood. The second point of the discussion is therefore that the more rapid regeneration of T8$^+$ (as opposed to T4$^+$) cells, together with the peculiarities of the accumulating T8$^+$ populations, indicate that T cell regeneration after BMT in adults is not an exact 're-run' of normal thymocyte/T cell ontogeny.

The third point is that after BMT close similarities exist between the phenotypic features of T cells in the regenerating BM and in the circulating blood (Fig. 3). In normal individuals within the blood T4$^+$ cells predominate and in the BM T8$^+$ cells are more numerous (Table 2; see also Janossy *et al.*, 1980). It would appear therefore that following BMT a relatively free traffic of T cells takes place between the BM and blood. This is not true for all organs because the local lesions in skin GvHD contain a T8$^+$ subset which is different from the T8$^+$ cells observed in the BM and blood (see below).

Some patients studied developed serious debilitating viral infections and/or GvHD disease. In a proportion of these patients the T8$^+$,HLA-DR$^+$,T10$^+$,HNK-1$^{+/-}$,T1$^+$ populations became even more dominant and constituted >60–80% of all T lymphocytes. This phenomena is consistent with previous studies which document the reversal of T8/T4 ratio and the presence of large T8$^+$,HLA-DR$^+$,T1$^+$ circulating cells in cases of infectious mononucleosis (Reinherz *et al.*, 1980; Crawford *et al.*, 1981) and CMV infections (Carney *et al.*, 1981). Thus one possible explanation for the differences between T lymphopoiesis in the fetus and that in regeneration after BMT is that T cell education may proceed virus free only in the fetal thymus. Admittedly only a proportion of BMT patients develop overt CMV infection but lymphopoiesis may also be influenced by other viruses (e.g. herpes simplex; Saral *et al.*, 1982). In other cases, with GvHD and severe viral exposure, however, profound lymphopenia develops with fragile, distorted T lymphocytes which expressed T cell associated antigens only at a low density. It is therefore possible that 'over-stimulation' of T8$^+$ cells or serious inhibition of T lymphopoiesis can both occur as a result of viral infection and GvHD.

The final point is the differential migration of lymphocyte subsets to special sites. During GvHD many circulating and BM borne T8$^+$,T3$^+$ cells exhibit the DR$^+$,T10$^+$, HNK-1$^+$, T1$^+$ phenotype (see above and Table 3) but the infiltrates of GvHD skin are T8$^+$,T3$^+$ but negative with all other

four markers; the same kind of $T8^+,T3^+$ cells are also seen in the normal gut epithelium (intraepithelial lymphocytes; Janossy *et al.*, 1980; Selby *et al.*, 1981; Table 3).

One can only speculate about the significance of these observations in respect of the relative contribution of thymus and BM to T lymphopoiesis after BMT in adult patients. A small number of cytolytic precursor cells are present in the athymic nu/nu mice (Wagner *et al.*, 1982) where intraepithelial 'T like' cells can also be observed in the gut (Mayrhofer, 1981). In addition, Triebel *et al.* (1981) have recently shown that in BM cultures T cell colonies develop from T cell antigen negative, HLA-DR$^+$ precursors in the absence of thymic micro-environment. This extra-thymic education may be relatively inefficient (and perhaps 'misguided' when viral antigens are present) but may contribute to the relative dominance of $T8^+$ cells over the $T4^+$ population. Thymic deficiency (or educational 'imbalance') may contribute to the development of GvHD as the disease occurs far more frequently in adults than in children (Ramsey *et al.*, 1982).

Clearly, the five points discussed all require further studies. The developmental links between the different $T8^+$ cell types are unknown and the functional correlates of these phenotypic differences need analysis. The $T8^+,DR^-,T1^-$ cells seen in the GvHD skin may perhaps simply represent a cohort of cells at their later stages of differentiation, irrespective of whether these are suppressor or cytotoxic. It is also possible, that this is a functionally distinct subset but this question cannot be effectively studied in blood samples. Further functional studies should pay attention to histology: certain cell types do not recirculate and others may show cytotoxic activity in a test tube but will be unable to exert their function if they cannot reach their target at the relevant site of the body.

Finally, one should not regard the $T8^+$ cells as the only important population in GvHD. In our study only blood, BM and pathological skin samples were studied for ethical reasons, but no information is available about the lymphoid system. Animal experiments show that the inducer T cells are indeed important in the development of GvHD. The elimination of murine Lyt-1$^+$ inducer type cells (together with the Lyt-1$^+$,2$^+$ suppressor/cytotoxic precursors) with anti-Lyt-1 plus complement from the BM inoculum prevents GvHD (Gleichman *et al.*, 1982). In contrast, the elimination of Lyt-2$^+$ (together with Lyt-1$^+$,2$^+$ precursors) with anti-Lyt-2 plus complement is effective only if performed twice consecutively (Sprent & Korngold, 1981). Our results in man suggest that the effects of inducer type cells must be easily demonstrated in this experimental system because the population with inducer phenotype regenerates slowly while the reconstitution of suppressor/cytotoxic cells is quick. These findings are not in conflict with the facts demonstrated both in experimental skin allograft rejection in rats (Delman & Mason, 1983) and in GvHD in man (Fig. 4; Lampert *et al.*, 1982) that the infiltrating cells within the lesions are dominantly of suppressor/cytotoxic type, but merely emphasize that when the probable T–T collaborations during GvHD are investigated or discussed these cells, different tissue distribution and migratory capacity should be borne in mind.

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