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Unusual phenotype and function of an expanded subpopulation of T cells in patients with haemopoietic disorders

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SUMMARY

We have studied two patients, one with red cell aplasia and the other with neutropenia. Both showed lymphocytosis. In both cases, 90–100% of E rosetting cells were T cells as defined by the monoclonal antibodies UCHT1 and OKT3. The majority of these cells also carried the OKT8 suppressor/cytotoxic marker and were HLA-DR- and FcγR-positive. In spite of the similarity of this phenotype to that reported for suppressor cells, these cells failed to suppress pokeweed mitogen-induced polyclonal Ig synthesis. Cells from both patients also failed to respond significantly to Con A and PHA. They were, however, unable to suppress the Con A responses of normal donors although cells from one patient were able to suppress completely a normal PHA response. These results demonstrate the existence of a genuine subset of T cells with Fcγ receptors but suggest that not all such cells have typical suppressor function.

INTRODUCTION

Human mononuclear cells which form rosettes with sheep red cells (E^+) are usually equated with T cells and can be divided, by several different methods, into functionally distinct subpopulations. Initially, two subsets were identified by their ability to bind the Fc portion of IgM or IgG (Ferrarini *et al.*, 1975; Moretta *et al.*, 1975). These were termed respectively Tμ, associated with helper/inducer activity, and Tγ, with suppressor activity (Moretta *et al.*, 1977). Apparently similar subsets have also been identified by monoclonal antibodies; in particular a helper/inducer subset defined by the OKT4 marker (Reinherz *et al.*, 1979; Reinherz *et al.*, 1980a) and a suppressor/cytotoxic subset defined by the OKT5 and 8 markers (Reinherz *et al.*, 1980b: Reinherz & Schlossman, 1980).

More recently, a discrepancy between E rosette-forming cells and those defined by a monoclonal anti-T cell serum (UCHT1) has come to light (Beverley, Linch & Callard, 1980). A proportion of E^+ cells are not stained by UCHT1 suggesting either that UCHT1 does not react with a small subset of E^+ cells or that some non-T cells form E rosettes. There is also evidence that E^+ cells bearing Fc receptors for IgG (FcγR) belong to a subpopulation with no T cell markers defined by monoclonal antisera (Reinherz *et al.*, 1980c). These results have cast doubt on the existence of genuine T cells with Fc receptors for IgG (Tγ).

We now report that in two patients with disturbances of haemopoiesis, the majority of E^+ cells also carry FcγR and the T cell markers UCHT1 and OKT3 and 8.

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MATERIALS AND METHODS

Patient 1. This 56-year-old male had a 1-year history of neutropenia discovered on investigation for abdominal pain. There were no abnormal physical signs. Investigations showed Hb 10·8 g/dl (MCV 116 fl), WBC $12{\cdot}5 \times 10^9$/l. Lymphocytes 92%, neutrophils 5%, monocytes 3%, platelets 252×10^9/l. ESR 26 mm. Bone marrow aspirate showed 30% lymphocytes and a relatively reduced granulocytic series with an excess of early forms and normal erythropoiesis.

Patient 2. This 64-year-old male had an 11-month history of lethargy and palpitations with no abnormal physical signs except anaemia. Investigations showed Hb 4·4 g/dl, WBC $10{\cdot}5 \times 10^9$/l. Lymphocytes 76%, neutrophils 23%, monocytes 1%. ESR 125 mm. Bone marrow aspirate showed severe red cell aplasia with red cell precursors representing less than 1% of total bone marrow nucleated cells. Sixty per cent of the bone marrow cells were mature lymphocytes and granulopoiesis was normal.

Cell separation procedures. Peripheral blood mononuclear cells (PBM) were isolated from venous blood by centrifugation over Ficoll–Hypaque, density 1·077. E rosettes were formed with SRBC (sheep red blood cells) treated with AET (*S*-2-aminoethylisothiouronium bromide hydrobromide; Aldrich Chemical Co., Wisconsin, USA) (Kaplan & Clark, 1974). One volume of packed SRBC was incubated with 5 vol. of AET (40·2 mg · ml^{-1} in H_2O, pH 9·0) for 20 min at 37°C. Two volumes of 2% AET–SRBC were mixed with 1 vol. of PBM at 10^7 cells · ml^{-1} and 0·5 vol. of FCS (fetal calf serum). The mixture was centrifuged at 250 *g* for 10 min and incubated on ice for 1 hr. The pellets were resuspended by gentle rotation of the centrifuge tube and the proportion of rosettes determined following addition of acridine orange to stain viable mononuclear cells. Separation of E rosette-forming (E^+) cells was accomplished by centrifugation at 1,500 *g* over Percoll (Pharmacia) at a density of 1·080. This method resulted in purer fractions than conventional Ficoll–Hypaque separation. E rosette-negative (E^-) cells were recovered from the interface, and, in general, were contaminated with less than 2% E^+ cells. E^+ cells were obtained from the pellet by lysis of the SRBC with haemolytic Gey's solution. All cell preparations were washed and kept for use in RPMI 1640 containing 20 mM HEPES and 5% FCS.

Lymphocytes with Fc receptors for IgG or IgM were detected by rosetting with ox red cells sensitized with rabbit anti-ox IgG (Hallberg, Gurner & Coombs, 1973) or IgM (Burns *et al.*, 1977) respectively. Lymphocytes at 2×10^6 · ml^{-1} were mixed with an equal volume of 1% sensitized ox red cells in Hanks' BSS supplemented with 0·2% bovine serum albumin, centrifuged at 800 *g* for 1 min, and incubated for 1 hr on ice before reading. The percentage of cells with Fc receptors for IgM was determined on fresh cells and after culture for 24 hr.

Mitogen responses. PBM were cultured with optimal doses of PHA (phytohaemagglutinin), Con A (concanavalin A) or pA (protein A) in flat-bottomed microtitre trays. Each well contained 2×10^5 PBM in 200 μl of RPMI 1640 containing 2 g · l^{-1} of $NaHCO_3$, 25 mM HEPES and 10% FCS. Cultures were incubated in humidified atmosphere of 10% CO_2-in-air for 3 days and then pulsed for 4 hr with 0·5 μCi of ^{125}I-deoxyuridine. Cells were harvested using a MASH, and incorporated ^{125}I-UdR determined on a Wallac LKB ultragamma counter. All cultures were set up in triplicate.

Antisera. UCHT1 is an IgG1 mouse monoclonal antibody derived from an immunization of BALB/c mice with human thymocytes followed by Sézary T cells. It identifies a determinant present only on mature T lymphocytes and some thymocytes (Beverley, Linch & Callard, 1980; Beverley & Callard, submitted). DA2 is a monoclonal antibody of IgG1 class with specificity for a non-polymorphic determinant of HLA-DR (Brodsky *et al.*, 1979). It was a gift of Dr M. Crumpton. OKT1, 3, 4 and 8 monoclonal antisera were kindly supplied by Dr G. Goldstein. OKT1 and 3 are mature T cell markers (Kung *et al.*, 1979). OKT4 and 8 identify helper/inducer (Reinherz *et al.*, 1979; Reinherz *et al.*, 1980a) and suppressor/cytotoxic (Reinherz *et al.*, 1980b; Reinherz & Schlossman, 1980) T cell subsets respectively.

Indirect immunofluorescence and cell sorting. Cells were incubated for 30 min on ice using saturating amounts, previously determined by titration, of monoclonal antibody (culture supernatant or purified Ig). They were then washed twice with RPMI 1640 containing 20 mM HEPES and 5% FCS, and stained with immunoabsorbent-purified, human immunoglobulin-absorbed FITC sheep anti-mouse immunoglobulin antibody for 30 min on ice. After a further three washes, the stained

cells were examined on a Zeiss fluorescence microscope with epi-illumination or analysed on a Becton Dickinson FACS-1.

Controls included cells stained with the second layer only or with culture supernatants from hybridomas of irrelevant specificity. In most experiments, normal sheep serum (10%) was included in the second incubation to compete for Fc receptors.

Pokeweed mitogen-induced antibody production. For polyclonal antibody production, 2×10^6 PBM were cultured in 1 ml of RPMI 1640 containing 2 $g \cdot l^{-1}$ $NaHCO_3$, 25 mM HEPES, 10^{-5} M hydrocortisone and 10% horse serum (GIBCO–BIOCULT) in 12×75 mm capped Falcon tubes. Cultures were stimulated with 20 μl of PWM (pokeweed mitogen; GIBCO–Biocult) and incubated at 37°C for 6 days in an atmosphere of 5% CO_2-in-air. At the completion of the culture period, the cells were washed twice, resuspended in 0·5 ml of RPMI 1640 containing 20 mM HEPES and 5% FCS and incubated for a further 20 hr at 37°C in air. Culture supernatants (SN) were then removed and stored at −20°C before assay for total HIg (human Ig). The concentration of HIg in culture SN was determined by a solid-phase, competition EIA (enzyme immunoassay). Sixty microlitres of SN was incubated with 15 μl of alkaline phosphatase-coupled immuno-purified goat anti-human Ig (AP Ga-HIg) for 1 hr at 37°C and then added to non-sterile, flat-bottomed microtitre trays (Flow Laboratories) which had been coated by incubating with HIg at 100 $\mu g \cdot ml^{-1}$ for 1 hr at 37°C. The plates were incubated for a further 1 hr at 37°C, washed thoroughly with saline and 100 μl of NPP substrate (nitrophenylphosphate; Sigma) at a concentration of 1 $mg \cdot ml^{-1}$ in carbonate buffer, pH 8·6, added to each well. The plates were incubated overnight at room temperature for colour development and the OD (optical density at 405 nm) in each well determined using a Titertek multiskan (Flow Laboratories). The concentration of HIg present in each SN was determined from a standard curve obtained from known concentrations of HIg and expressed in $ng \cdot ml^{-1} \pm$ s.e.

RESULTS

The phenotype of mononuclear cells

In the two patients studied there was both a moderate lymphocytosis and a grossly abnormal distribution of surface markers in the mononuclear cell fraction (PBM). Both patients had slightly higher numbers than normal of E rosette-positive (E^+) cells and a striking increase in FcγR-positive

Table 1. Phenotype of peripheral blood mononuclear cells

	Per cent cells in unfractionated PBM	
	Patient 1	Patient 2
E rosettes	83	80
FcγR	90	81
	Per cent cells in E^+ fraction	
UCHT1	99	91
OKT1	70*	40*
OKT3	99	n.d.
OKT4	4	13
OKT8	95	77
Anti-HLA-DR	45	62
FcγR	97	70
FcμR 0 hr	0	n.d.
FcμR 24 hr	13	n.d.

* Very dim staining, see Fig. 1.

cells (Table 1). This indicated the existence of a genuine subset of T cells bearing Fc receptors for IgG. This point was investigated more thoroughly by first isolating E^+ cells and then examining their phenotype in detail using monoclonal antibodies. Table 1 shows that 90–100% of the E^+ cells from both patients are T cells as judged by staining with UCHT1. This was confirmed for one patient by staining with OKT3.

In both cases the vast majority of T cells also stained with OKT8 antiserum but few cells were stained by OKT4. This is a reversal of the normal distribution (65% $OKT4^+$, 30% $OKT8^+$ in E^+ cells) (Reinherz & Schlossman, 1980). In addition, the proportion of cells with Fc receptors for IgG was very similar to that staining with OKT8. In fact, from the figures given in Table 1, it can be calculated that at least 92% of the E^+ $UCHT1^+$ cells in patient 1 have both OKT8 and FcγR markers. Similarly, patient 2 had significant numbers of T cells with this phenotype. In patient 1 the enhancement of FcγR^+ cells was accompanied by a diminution of T cells bearing Fc receptors for IgM. FcμR-bearing cells were not determined for patient 2.

In both patients there was also a high proportion of HLA-DR-positive cells (Table 1) and it can be calculated that some of these cells at least must also be $OKT8^+$ and FcγR^+.

Weak expression of OKT1 antigen

The OKT1 antigen has been claimed to be a marker for all mature peripheral T cells. In our initial examination of these patients, however, we noted that the monoclonal OKT1 antibody stained fewer E^+ cells than UCHT1 and that the staining was weak. This finding was confirmed by FACS

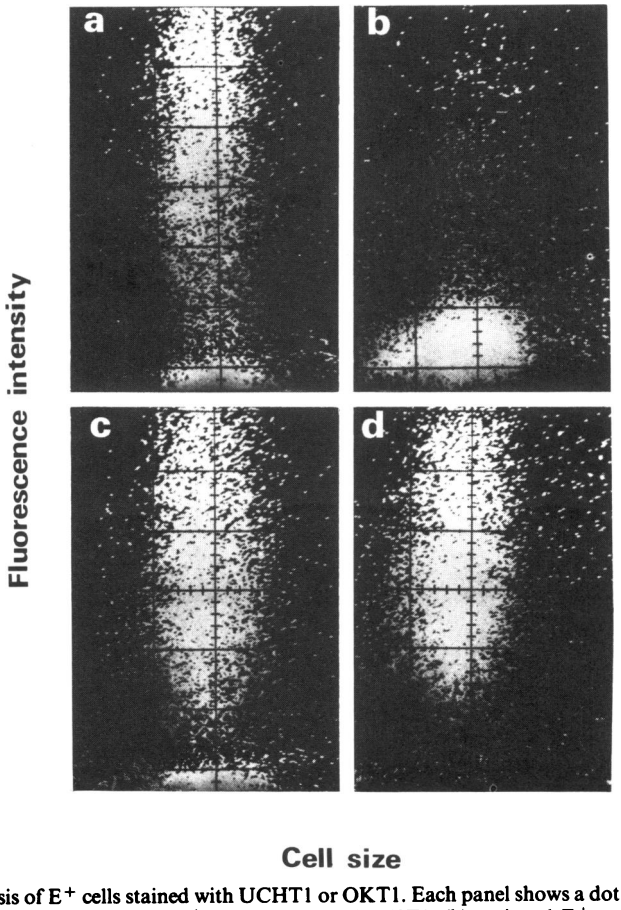


Fig. 1. FACS analysis of E^+ cells stained with UCHT1 or OKT1. Each panel shows a dot plot of 10^4 cells. Red cells have been gated out. (a) Normal E^+ cells stained with OKT1, (b) patient 1 E^+ stained with OKT1, (c) normal E^+ stained with UCHT1, (d) patient 1 E^+ stained with UCHT1.

analysis of E^+ cells from a normal donor and patient 1 stained under identical conditions on the same day. The results showed that while OKT1 stained normal E^+ cells as intensely as UCHT1 (Fig. 1a, c), the staining of the patient's E^+ cells was very much weaker (Fig. 1b). UCHT1, on the other hand, stained both control and patient E^+ cells equally well although the E^+ UCHT1$^-$ subpopulation found in normal individuals was absent from the patient (compare Fig. 1c and d).

Are OKT8$^+$ FcγR$^+$ cells T suppressors?

Suppressor T cells have been reported to bear the OKT5 and OKT8 markers (Reinherz *et al.*, 1980b; Reinherz & Schlossman, 1980). Others have demonstrated suppressor activity of E^+ cells carrying the Fc receptor for IgG (Moretta *et al.*, 1977) although recent work has suggested that these cells may not be T lymphocytes (Reinherz *et al.*, 1980c). It was, therefore, clearly of interest to determine whether OKT8$^+$ FcγR$^+$ cells from these patients exhibited suppressor activity.

PBM from both patients had profoundly reduced responses to PHA and Con A and to the T and B cell mitogen protein A (Sakane & Green, 1978) (Table 2). To test for suppression, E^+ cells from the patients were added to normal allogeneic PBM at a ratio of 1:2 and cultured with mitogens. Both patients significantly reduced the PHA response of normal cells, in one case to background (Table 2). By contrast, both patients only marginally inhibited the Con A and protein A responses. Control E^+ allogeneic cells had no effect on any mitogen responses.

An incidental finding was that neither patient mounted an MLC against the normal PBM. This can be seen from the medium control of cultures containing both normal PBM and E^+ cells from patients. By contrast, the normal E^+ cells in Expt 1 (Table 2) did give a small but significant MLR at day 3. The absence of a normal MLR in experiment 2 was due to the known HLA-DR compatibility between the donors of the normal PBM and added, normal, allogeneic E^+ cells.

The E^+ cells from both patients were also tested for their ability to suppress B cell immunoglobulin (Ig) synthesis in response to pokeweed mitogen. The E^+ cells from patient 1 had no effect on Ig synthesis by PBM from two normal donors when added at a ratio of 1:2 (Table 3, Expt 1). Normal allogeneic E^+ cells similarly failed to modify the response of PBM. E^+ cells from patient 1 were, however, unable to provide optimal helper function when combined with normal E^- cells. E^+ cells from patient 2 likewise failed to suppress Ig synthesis of one normal donor but did have some inhibitory effect on PBM from a second donor (Table 3, Expt 2).

PBM from patient 2 failed totally to respond on their own to PWM, which may also indicate a loss of helper function as was demonstrated for patient 1.

Table 2. Effect of OKT8$^+$ FcγR$^+$ T cells on mitogen responses

	PHA	Con A	pA	Medium
Experiment 1				
Control	20,900 (300)*	16,300 (800)	14,000 (600)	1,000 (100)
Patient 1	1,000 (80)	2,900 (60)	6,700 (200)	300 (4)
Control + allogeneic E^+ from normal donor	22,300 (1,200)	15,200 (400)	14,900 (800)	3,700 (300)
Control + E^+ from patient 1	2,800 (50)	12,700 (300)	13,500 (500)	1,200 (100)
Experiment 2				
Control	20,900 (700)	14,600 (700)	9,000 (600)	700 (200)
Patient 2	1,900 (200)	1,700 (300)	1,700 (300)	500 (50)
Control + allogeneic E^+ from normal donor	17,100 (2,000)	13,600 (1,100)	9,600 (500)	400 (50)
Control + E^+ from patient 2	10,600 (308)	10,100 (800)	6,100 (500)	500 (50)

* Data expressed as c.p.m. The figures in parentheses represent s.e.m.

Table 3. Effect of OKT8$^+$ FcγR$^+$ T cells on immunoglobulin synthesis

PBM donor	PWM	Donor of added E$^+$	Response (ng · ml^{-1})
Experiment 1			
Normal D.M.	−	—	200 ± 20
Normal D.M.	+	—	1,900 ± 210
Normal D.M.	+	Normal, allogeneic	1,400 ± 380
Normal D.M.	+	Patient 1	1,000 ± 120*
Normal R.C.	−	—	130 ± 20
Normal R.C.	+	—	2,150 ± 680
Normal R.C.	+	Normal, allogeneic	2,400 ± 360
Normal R.C.	+	Patient 1	1,840 ± 530*
Normal E$^-$	−	—	90 ± 10
Normal E$^-$	+	Normal, autologous	1,610 ± 680
Normal E$^-$	+	Patient 1	440 ± 40
Experiment 2			
Normal R.C.	−	—	20 ± 20
Normal R.C.	+	—	330 ± 40
Normal R.C.	+	Patient 2	380 ± 80*
Normal E.Z.	−	—	70 ± 10
Normal E.Z.	+	—	490 ± 20
Normal E.Z.	+	Patient 2	180 ± 50†
Patient 2	−	None	20 ± 9
Patient 2	+	None	20 ± 9

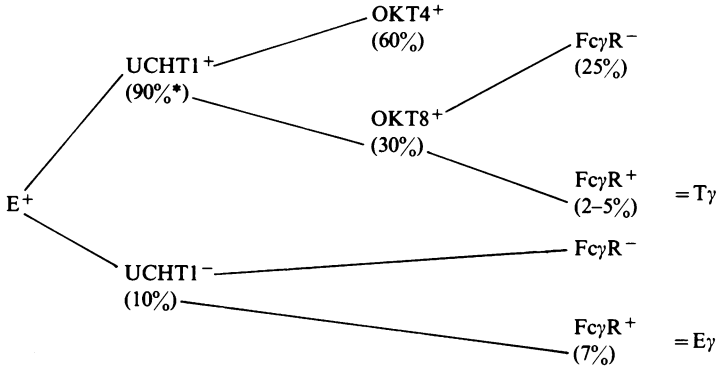
* No significant suppression.
† Significant suppression ($P < 0.05$).

DISCUSSION

Several authors have separated cells capable of forming E rosettes into subsets on the basis of their ability to bind the Fc portion of IgM or IgG (Ferrarini *et al.*, 1975; Moretta *et al.*, 1975). Of these, the FcγR$^+$ subset has been shown to suppress pokeweed mitogen-induced immunoglobulin synthesis (Moretta *et al.*, 1977) and is commonly thought of as a T suppressor cell. However, it has now been shown that non-specific suppressor cells induced with Con A can be derived from the FcμR$^+$ (Tμ) subset of E$^+$ cells (Hayward *et al.*, 1978). Moreover, there is evidence that the Fc receptor for IgG can be passively acquired by previously negative cells (Samarut & Revillard, 1980). There is thus some doubt whether the IgG Fc receptor is a stable marker for human suppressor T cells.

T cells identified with the monoclonal antiserum OKT3 can be subdivided into an OKT4$^+$ helper/inducer subset (Reinherz *et al.*, 1979; Reinherz *et al.*, 1980a) and an OKT5$^+$/8$^+$ suppressor/cytotoxic subset (Reinherz *et al.*, 1980b; Reinherz & Schlossman, 1980). The relationship of OKT5$^+$/8$^+$ cells to the suppressor subset previously identified by FcγR was not clear. At first sight these might have been expected to be the same but several lines of evidence suggest that they are not. Using the anti-T cell monoclonal UCHT1, we have shown that E$^+$ cells can be divided into a majority population (*ca* 90%) which carries UCHT1 antigen and a minority (*ca* 10%) which does not (Beverley *et al.*, 1980). Up to 70% of this latter population have FcγR (unpublished data). Similarly, it has been reported that the majority of cells isolated by E rosetting followed by FcγR rosetting do not carry T cell antigens defined by monoclonal antibodies but do carry the OKM1 antigen which is also present on monocytes and granulocytes (Reinherz *et al.*, 1980c; Breard *et al.*, 1980). These results have cast doubt on the existence of a T cell subset carrying FcγR, i.e. Tγ.

By contrast, there is some data to suggest that FcγR T cells do exist. Kaszubowski, Goodwin & Williams (1980) have examined the phenotype of E^+ FcγR cells isolated from normal peripheral blood and, on the basis of both their response to mitogens and the presence of antigens defined by conventional antisera, have concluded that T cells with Fcγ receptors are present in small but significant numbers. Such cells are clearly demonstrated in our own studies, reported here, of two patients with haemopoietic disturbances. We combined conventional methods of E and Fc rosetting with analysis by indirect immunofluorescence using monoclonal anti-T cell sera. Both patients had a high proportion of E^+ lymphocytes with Fcγ receptors, which also carry the T cell antigens UCHT1, OKT3 and OKT8 identified with monoclonal antisera. We conclude that these are true T lymphocytes with Fc receptors for IgG. Moreover, we now have preliminary data that such cells also exist in very small numbers in normal individuals. These results have led us to agree with Cooper (1980) that there are two types of E^+ $FcγR^+$ cells – those which are truly T cells (Tγ) defined with monoclonal anti-T cell sera UCHT1 and OKT3, and those which belong to the $UCHT1^-$, non-T cell population (Eγ). This scheme is summarized below:



* Approximate percentages of total E^+ cells.

Many of the cells detected in our patients also carry HLA-DR antigens which have been shown to appear on activated T lymphocytes (Evans *et al.*, 1978; Kaszubowski *et al.*, 1980). It could be argued, therefore, that there is an expansion of the $OKT8^+$ subset with the acquisition of HLA-DR on activation. Alternatively, our results could be explained by expansion of a pre-existing $HLA\text{-}DR^+$, $FcγR^+$ T cell subset. The evidence for such a subset is, however, inconclusive. Reinherz *et al.* (1980c) found no HLA-DR on their E^+, $FcγR^+$, $OKM1^+$ subset in contrast to Greaves *et al.* (1979) who have described an E^+, $HLA\text{-}DR^+$, $FcγR^+$ subset separated from fresh peripheral blood. Our own data (unpublished) suggest that 2–3% of E^+ cells are $HLA\text{-}DR^+$ and that these cells are in the $UCHT1^+$ (T) cell fraction from which the $HLA\text{-}DR^+$ cells in our patients could have been derived.

An additional curiosity of the phenotype of the cells in our patients is the weak expression of the OKT1 antigen previously thought to be expressed on all mature T cells (Kung *et al.*, 1979). This low expression of OKT1 is reminiscent of mouse data showing low levels or absence of Lyt-1 on the suppressor/cytotoxic $Lyt\text{-}2^+3^+$ subset (Feldmann *et al.*, 1975; Beverley *et al.*, 1976). It is possible either that OKT1 is lost from some T cells on activation or that the T cells in these patients are an expansion of a subset with low expression of OKT1. In either case, our data suggest that OKT1 may not be a reliable marker for all T lymphocytes.

Suppressor activity has been ascribed to both $FcγR^+$ and $OKT5^+$, 8^+ cells (Moretta *et al.*, 1977; Reinherz *et al.*, 1980b; Reinherz & Schlossman, 1980). In the case of $FcγR^+$ cells, suppression of both PWM-induced Ig synthesis and Con A responses has been reported but it is not known whether this was due to Eγ or Tγ cells (see above). Similarly, $OKT5^+$, 8^+ cells have been shown to suppress an MLC following Con A activation but, since only a small minority of $OKT5^+$ cells carry FcγR (Reinherz *et al.*, 1980c), it is unclear whether the suppression was mediated by $FcγR^+$ cells.

In our patients we failed to detect suppression of PWM or Con A responses which is consistent

with the results obtained by Bom-van Noorloos *et al.* (1980) on two other neutropenic patients. In contrast, $OKT5^+$ $HLA\text{-}DR^+$ T cells from patients with infectious mononucleosis are able to suppress PWM-induced Ig synthesis (Reinherz *et al.*, 1980d). There are at least two explanations for this discrepancy. It is possible that in our patients and those of Bom-van Noorloos, the T cells are an expansion of an antigen-specific subset and therefore fail to suppress a polyclonal B cell response to PWM. Against this explanation is the previously unreported finding that the patients' cells can suppress the PHA response of normal lymphocytes (Table 2). The alternative explanation is that the $OKT8^+$ cells in our patients are a different subpopulation to those which suppress MLC or PWM responses. This is supported by reports that the expanded T cell population in infectious mononucleosis, although $OKT5^+$ (Reinherz *et al.*, 1980d), does not bear Fc receptors for either IgG or IgM (Haynes *et al.*, 1979).

Both patients studied have a heavy lymphoid infiltrate in their bone marrow, the majority of which are $OKT8^+$ (Linch *et al.*, 1981), implying a role for these cells in the pathogenesis of the marrow suppression. It is not clear, however, how suppression of a response to PHA but not PWM or Con A relates to this phenomenon and further clarification must await development of new assays for suppression more relevant to the *in vivo* function of lymphocytes.

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