THE EGYPTIAN JOURNAL OF IMMUNOLOGY Vol. 17 (2), 2010
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T - Cell Function, T - Cell Phenotype and Its Role in Responsiveness to Recombinant Human Erythropoietin in Hemodialysis Patients

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Resistance to recombinant human erythropoietin (Epo) occurs in a small proportion of hemodialysis (HD) patients. In this study we investigated the relationship between T-cell phenotype (using flow cytometry), T-cell function (by measuring *in vitro* cytokine production) and responsiveness to Epo in HD patients and to compare the results with those from healthy controls. T-cell phenotypes were assessed and T-cell function was studied. The study included 24 chronic renal failure (CRF) patients on HD treated with rHuEPO as well as 14 normal control subjects. Dual-colour immunofluorescence and flow cytometry were used to compare the surface antigen expression on freshly isolated CD4+ and CD8+ T-cells from PBMC of the studied groups. Levels of a panel of selected cytokines (IL-4, IFN-γ, sIL-2R and IL-10) were determined in PBMC culture supernatants and in plasma samples (TNF-α, IFN-γ, IL-6, sIL-2R) using (ELISA) kits. Patients were followed-up for 24 months and a survival study was carried out. T-cells from poor responders showed increased proportions of CD4+/CD28- cells and CD8+/CD28- cells compared with both good responders and controls. Compared with their CD28+ counterparts, CD4+/CD28- T-cells produced significantly more IFN- γ, enabling them to function as pro-inflammatory cells. There was no difference in secretion of IFN- γ, sIL-2R or IL-4 in PBMC cultures obtained from HD patients and controls. However, Unstimulated PBMC from poor responders generated increased levels of IL-10 poor compared with both good responders and controls. Plasma sIL-2R and IL-6 were significantly elevated in both good and poor responders compared with controls. Plasma levels of IFN- γ and TNF- α were undetectable in both HD patients and controls. In the follow up period, more deaths were occurring among the poor responders than the good responders. Based on the finding of the this study we may suggest that, in the absence of any obvious cause, poor response to Epo may be mediated by generation of cytokines from a subpopulation of activated T-cells, which might promote apoptosis in erythroid progenitor cells in the bone marrow.

The invention of recombinant human erythropoietin (rHuEpo) for the treatment of renal anaemia was a hallmark in the care of patients with renal insufficiency (Aguilera *et al.,* 2002). The latest published guidelines (Macdougall *et al.,* 2007) have set the target haemoglobin to be reached by treatment with rHuEPO to >11 g/dl. Normalizing haemoglobin levels may reduce morbidity and mortality and improve quality of life in (HD) patients (Stenvinkel & Barany, 2002). However, the dose requirements of erythropoietin (Epo) to achieve correction of anaemia vary enormously, with some patients requiring as much as 10 times the dose of other patients (Amacdougall, 1995). Up to 10% of patients with renal anemia receiving rHuEPO therapy show poor responsiveness to the drug (Macdougall *et al.,* 2005). Even in patients who do respond to rHuEPO, there is a marked variability in drug sensitivity. Several factors have been recognized as causing resistance to rHuEPO, notably iron deficiency, infection/inflammation, and under dialysis. However, when these factors are excluded, the wide variation in responsiveness to rHuEPO persists (Macdougall & Cooper, 2002). The mechanism of this effect needs to be fully elucidated. Uraemia is known to be a chronic inflammatory state, with some patients showing considerably increased laboratory

markers of inflammation and immune activation. Chronic inflammation can modify the process of erythropoiesis, probably mediated via pro-inflammatory cytokines such as interleukin-1 (IL-1), tumour necrosis factor-alpha (TNF-alpha) and interferon-gamma (IFN-gamma) (Sitter *et al.,* 2000; Copper *et al.,* 2003).

Cytokines are critical effectors in immune-cell function and it is therefore likely that the uraemic state leads to complex alterations in the cytokine network. The haemodialysis (HD) procedure itself may cause further changes in cytokine generation (Sitter *et al.,* 2000; Coponat *et al.,* 2008). Haemodialysis patients have increased circulating levels of cytokines compared with healthy controls and monocytes from HD patients have been shown to produce high levels of interleukin-6 (IL-6), IL-10 and tumour necrosis factor- α (TNF- α) *in vitro* (Girndt *et al.,* 1995; Kimmel *et al.,* 1998).

One of the clinical consequences of aberrant cytokine production may be impaired erythropoiesis. In this regard, TNF-α and interferon- γ (IFN- γ) have been shown to suppress erythroid colony formation *in vitro* (Allen *et al.,* 1999), by promoting apoptosis of these cells (Dai & Krantz 1999). These findings may explain, in part, the observation that HD patients with inflammation or infection are resistant to Epo therapy. Production of IL-6 and TNF- α from cultured peripheral blood mononuclear cells (PBMC) has been shown to correlate with Epo dose (Goicoechea *et al.,* 1998).

It is hypothesized, therefore, that some patients showing resistance to Epo may have enhanced levels of immune activation, causing increased release of pro-inflammatory cytokines in the bone marrow (Macdougall *et al.,* 2005). In the present study this hypothesis has been investigated by studying T-cell phenotypes by flow cytometry, along with cytokine release from T cells and monocytes in 'good' and 'poor' responders to Epo.

Therefore the aims of this study were to investigate the relationship between T-cell phenotype, T-cell function and responsiveness to Epo in HD patients and to compare the results with those from healthy controls. The patients selected as poor responders for the study had all the usual causes of Epo resistance excluded, such as iron deficiency, under dialysis and severe hyperpara-thyroidism. T-cell phenotypes were assessed using flow cytometry and T-cell function was studied by measuring *in vitro* cytokine production.

Subjects and Methods

Patients

Twenty four patients of chronic renal failure (CRF) treated with (HD) and 14 normal controls were included in this study. Two distinct groups of HD patients were evaluated: (I) HD patients not responding to Epo with Hb <10 g/dl, Epo/Hb ratio >15 (defined as the dose of Epo (International Units/kg body weight) divided by the Hb concentration); and (II) HD patients responding well to Epo with Hb >10 g/dl and Epo/Hb ratio <10. The mean time on HD did not vary between the two groups (30±18 months for good responders *vs.* 41±36 months for poor responders; P=0.41).

The causes of renal failure in the patient population were as follows: hypertensive nephropathy (n=10), ischaemic nephropathy (n=1), nephrocalcinosis (n=1), obstructive nephropathy (n=1), polycystic kidney disease (n=1), renal amyloid (n=1), Reno vascular disease (n=1), cortical necrosis (n=1) and unknown (n=7).Of the 12 good responders, 4 and 2 subjects were on Ramipril and Enalapril, respectively of the 12 poor responders 4,3 and one subject were taking Ramipril, enalapril and perindopril respectively.

The study did not include patients with autoimmune disease, malignancy, haematological disorders, diabetes mellitus, systemic vasculitis, iron deficiency, liver cirrhosis, overt acute and chronic infection or those taking immunosuppressive therapy as all these conditions could secondarily increase proinflammtory cytokines. No patient had occult blood in stool.

There were no patients with serum aluminum levels >20μg/l and the dialysate aluminum levels were kept

less than 5µg/l. Patients with symptoms or signs of aluminum intoxication, with history of steroid or on aluminum containing phosphate binder or with history of parathyroidectomy all were excluded.

The patients were dialysed three times per week for 3–5 h (mean dialysis time: 3.6±0.5 h), using either a cellulose diacetate (good responders, n=8; poor responders, n=8), haemophan (good responders, n=3; poor responders, n=3) or polyacrylonitrile (good responders, n=1; poor responders, n=1) dialyser. A total of 19 patients had an arteriovenous fistula as vascular access, three patients had a polytetrafluoroethylene graft and two patients had a tunnelled dialysis catheter as vascular access. Healthy volunteers were used as normal controls and, as far as possible; the three groups were age- and sex-matched.

Methods

Blood samples (25 ml) were drawn into plastic tubes containing 1000 U sodium heparin (Leo Laboratories Ltd, Princes Risborough, UK). Blood from dialysis patients was taken immediately prior to a dialysis session. Since laboratory data were stable during the 3 months of the study, we conducted analytical determinants using a single sample. Serum urea nitrogen, creatinine, total protein, albumin, total cholesterol, Hb, and bicarbonate were measured by standard laboratory techniques using an automatic analyzer XL-300 (Fully Automated Biochemistry Analyser). The efficiency of dialysis was assessed from urea reduction rate (URR), calculated from the monthly blood tests and based on the formula (1–post-BUN/pre-BUN)x100, and from the delivered dose of dialysis (Kt/Vurea) using a single-pool urea kinetic model (Mercadal *et al.,* 2005). Normalized protein catabolic rate (PCRn), an indirect indicator of protein intake, was calculated from dialysis urea removal and serum urea levels. The creatinine generation rate was calculated from changes in body weight and blood creatinine levels during a single HD session. Serum ferritin and ß2-microglobulin were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (BioSource International, Camarillo, CA, USA). Transferrin was measured by Nitroso-PSAP method (Soichi *et al.,* 2005) and the thrombin time were determined using commercially available kits (Gerotziafas *et al.,* 2009).

The patients were all maintained on the same dose of subcutaneous Epo for at least 3 months prior to investigation.

T-cells from all HD patients and controls were subjected to flow cytometry, for the cytokine studies, only 10 subjects from each of the three groups were studied.

- Cell Preparation and Culture Conditions

Peripheral blood mononuclear cells were isolated from whole blood on a Lymphoprep® (Robbins Scientific Europe Ltd, Solihull, UK) density gradient at 400x*g* for 30 min. The upper plasma layer was taken for cytokine measurement. The mononuclear cell layer was harvested and washed three times using phosphate-buffered saline (PBS) the pH 7.19-7.59 Osmolarity: 282-288 mOsm, Total Molarity 0.150 (Gibco BRL, Life Technologies Ltd, Paisley, UK) at 300x*g* for 20 min. Cell viability was assessed using trypan blue and samples that were <99% viable were discarded.

The PBMC were cultured at 2x106/ml in Iscoves-modified buffer containing Glutamax (Gibco BRL, Life Technologies Ltd, Paisley, UK), supplemented with 10% foetal calf serum (FCS) (Gibco BRL, Life Technologies Ltd, Paisley, UK), 100 U/ml penicillin (Gibco BRL, Life Technologies Ltd, Paisley, UK) and 50 µg/ml streptomycin (Gibco BRL, Life Technologies Ltd, Paisley, UK).

The cultures were set up either in the absence of stimulus, in the presence of 10 µg/ml lipopolysaccharide (LPS) (Sigma Chemical Co., Poole, UK) or in the presence of monoclonal antibodies (mAbs) to CD3 (5 µg/ml) (Clone HIT3a, Becton Dickinson (UK) Ltd, Oxford, UK) *and CD28* (2 µg/ml) (Clone M1456, Eurogenetics UK Ltd, Hampton, UK). Lipopolysaccharide activates monocytes while the combination of mAbs to CD3 and to CD28 activates T-lymphocytes. Each experimental condition was performed in duplicate. The PBMC were incubated at 37°C, 5%, CO2 for 48 h and the cells were then precipitated by centrifugation at 15000x*g* for 30 min. The supernatants were then collected and stored at -80°C until analysed.

- Cytokine Measurement in Culture Supernatants and Plasma Samples

Levels of a panel of selected inflammatory and proinflammtory cytokine levels were determined in PBMC culture supernatants (IL-4, IFN-γ, sIL-2R and IL-10) and in plasma samples (TNF-α, IFN-γ, IL-6, sIL-2R) using commercially available enzyme-linked immunosorbent assay (ELISA) kits (BioSource International, Camarillo, CA, USA) according to the manufacturer's instructions. The assay ranges for detection of TNF-α, IFN-γ, IL-10 and sIL-2R were 0–10 000, 0–1000, 0–500 and 0–800 pg/ml, respectively, and the lower detection limits were 10, 4, 5, and 16 pg/ml, respectively. Plasma IL-6 and sIL-2R levels were determined using commercially available ELISA kits (BioSource International, Camarillo, CA, USA). The IL-6 assay detection range was 0–500 pg/ml with a lower detection limit of 2 pg/ml.

- C-Reactive Protein

C-reactive protein (CRP) levels were measured on a Behring Nephelometer Analyser II, using a mAb to CRP and the latex enhanced nephelometric reaction. The detection range was 0–10 mg/ml.

- Estimation of Blood Loss in HD Patients

Estimation of blood losses by each patient was determined by tracing every blood sample obtained throughout the study period and the volume of blood usually collected per test. Volume of blood per HD treatments (include blood loss from access plus blood lost in dialyzer and tubing) was assumed to be constant 14 (8 ml access+3 ml Blood tubing+3 ml in dialyzer). Since the number of HD setting performed by each patient was known total blood loss in each studied patient could be calculated.

- Flow Cytometry

Dual-colour immunofluorescence was performed by labelling cells with fluorescein isothiocyanate-conjugated and phycoerythrin-conjugated mAbs (Becton Dickinson (UK) Ltd, Oxford, UK). Peripheral blood mononuclear cells were aliquoted, and then incubated with the optimal concentration of appropriate mAb (together with the appropriate isotype matched controls) for 30 min at 4°C in the dark. The samples were washed with PBS containing 1% FCS and centrifuged at 200x*g* for 10 min and resuspended in PBS containing 1% paraformaldehyde (Sigma Chemical Co., Poole, UK). The labelled PBMC were stored at 4°C in the dark until analysed within 1 week. Measurements were performed on a Becton Dickinson FACScan flow cytometer and the Cellquest software 3.1. Initial gating was performed using forward and side scatter to identify T-lymphocytes. A total of (20 000 cells) was collected. The percentage of cells with fluorescence intensity ≥95% of control events (per cent positive) was determined.

Statistical Analysis

Statistical analysis was carried out using Prism V3.0 statistical software (Graphpad, San Diego, USA). Results are expressed as means±SD. Differences between the means were analysed using the Mann–Whitney test for unpaired data and were considered significant at $P<0.05$. Differences between survival curves were analysed using the Mantel–Haenszel test and were considered significant at $P<0.05$.

Results

Demographic and Haematological Characteristics

Table 1 and table 2 summarize the demographic and haematological characteristics of the three studied groups. The good responders and the poor responders were age-matched (Table 1). Although age matching was attempted, the mean age of the normal control group was found to be slightly lower than both the HD patient groups.

As regards the total volume of blood loss (determined by adding the size of the sample used for laboratory investigations and blood loss from HD therapy) for all patient groups during the study period was similar for all groups and there was no statistical significance difference between poor and good responder groups.

The iron status of the good responders was equivalent to that of the poor responders and all the HD patients in both groups received intravenous iron as required for maintenance of serum ferritin >100 μg/l. The serum albumin levels were comparable in the two groups. The urea reduction ratio was not significantly different between the good and poor responder groups. Likewise, there was no significant difference in parathyroid hormone levels between the two groups.

The mean Epo dose in the poor responders was significantly higher than that of the good responders. The poor responders were significantly more anaemic than the good responders. Thus, the Epo/Hb ratio was significantly different between the two HD groups. C-reactive protein levels were higher in the poor responders (17.5±21.2 mg/l) compared with the good responders (9.6±8.0 mg/l; $P<0.05$), which in turn were higher than in the normal controls (3.2±1.0 mg/l; $P<0.002$).

The white-cell count in the three tested groups did not differ significantly (Table 2).

However, lymphocyte counts were significantly lower in both the poor responders and the good responders compared with the normal controls. There was no significant difference in the lymphocyte count between good and poor responders. Monocyte counts were significantly elevated in the good responders compared with the normal controls, but there was no such significant increase in the poor responders. Similarly, both the neutrophil and basophil counts were significantly increased in the good responders compared with the normal controls. Eosinophil counts did not differ among the three study groups.

Table 1. Clinical Parameters in HD Patients According to rHuEpo Dose.

Variable	Normal controls n=14	Good responders n=12	Poor responders n=12
Age	43±12	52±15	53± 14
Male/female	9/5	8/4	9/3
rHuEpo-related factors			
rHuEpo (U/kg/week)	n/a	71±30*	296±147*#
Hb (g/dl)	13.5±1.1	11.5±1.2*	8.8±1.9*
Reticulocyte (%)	1.5±0.8	11±2*	16±3*
Ferritin (µg/ml)	229±62	622±287*	636±224*
Fe (µg/dl)	95±5	90±4	84±5
TIBC (µg/dl)	3.4±1.0	2.7±0.9	2.6±13
Transferrin saturation (%)	32.4±2.2	34.6±1.7	30.9±2.2
Nutrition-related factors			
Albumin (ng/l)	38±5	37±6	37±7
Transferrin (µg/dl)	310±10	201±8*	172±7
BMI (kg/m2)	22.7±0.6	19.4±0.3*	17.7±0.5*
Total cholesterol (mg/dl)	154±18	151±5	136±5*
Crt generation rate (g/kg/day)	n/a	18.2±0.5	15.2±0.7#
PCRn (g/kg/day)	n/a	1.04±0.03	0.95±0.05
Dialysis-related factors			
BUN (mg/dl)	10.1±2.8	72.2±2.1	67.9±3.2
Crt (mg/dl)	1.0±0.4	10.7±0.3*	9.2±0.5*#
URR (%)	n/a	67.2±60%	65.0±88%
ß2-MG (µg/l)	17.7±1.7	32.1±1.4	28.9±1.9
Intact PTH (ng/l)	70±8	391±438	357±371

rHuEpo= recombinant human erythropoietin; HB= haemoglobin; Fe= iron; TIBC= total iron-binding capacity; BMI= body mass index; n/a= not applicable, Crt= creatinine; PCRn= normalized protein catabolic rate; BUN=blood urea nitrogen; URR= urea reduction rate; PTH= parathyroid hormone.*P<0.01 compared with control group; #P<0.05 compared with Good responder group. Data are means ± SD

Table 2. Demographic and Haematological Characteristics of Subjects (means±SD)

	Normal controls (*n*=14)	Good responders (*n*=12)	Poor responders (*n*=12)
Age (years)	43±12	52±15a	53±14b
Hb (g/dl)	13.5±1.1	11.5±1.2c	8.8±1.9d,e
CRP (mg/l)	3.2±1.0	9.6±8.1a	17.5±20.7d,e
Epo dose (U/kg/week)	n/a	71±30	296±147e
Epo dose/Hb ratio	n/a	6.3±2.6	33.4±17.8e
White cell count (x109/l)	7.12±1.47	8.10±1.90	7.15±2.80
Lymphocytes (x109/l)	2.10±0.88	1.51±0.37a	1.18±0.38d
Monocytes (x109/l)	0.50±0.14	0.75±0.21a	0.60±2.7
Neutrophils (x109/l)	3.81±1.25	5.88±1.97a	5.71±3.12
Eosinophils (x109/l)	0.11±0.10	0.32±0.35	0.29±0.03
Basophils (x109/l)	0.03±0.04	0.07±0.10a	0.03±0.03f

Samples were taken for clinical and haematological analysis at the same time as cytokine and immunofluorescence analysis. n/a= not applicable. a= $P<0.05$ and c$P<0.001$ for good responders *vs* normal controls. b= $P<0.05$ and d= $P<0.001$ for poor responders *vs* normal controls. f= $P<0.05$ and e= $P<0.001$ for poor responders *vs* good responders.

Flow Cytometric Immunofluorescence Analysis

Immunofluorescence and flow cytometry were used to compare the surface antigen expression on freshly isolated CD4+ and CD8+ T-cells in the three study groups. The results are summarized in Table 3. Low levels of CD25 antigen expression (which comprises the alpha chain of the IL-2 receptor), was found on CD4+ T helper cells isolated from the three groups.

Figure 1A shows the percentage of CD4+ cells that expressed the T-cell co-stimulatory molecule, CD28. Both T-cell proliferation and cytokine production require two signals: one involving the T-cell receptor–CD3 complex and a second co-stimulatory signal through the B7-1 or B7-2 molecule on antigen-presenting cells and the CD28 antigen on T-cells. Interestingly, CD28 expression in the poor responders was significantly lower at 86±10% compared with both the normal controls (98±1%; $P<0.001$) and the good responders (96.5±4%; $P<0.005$).

For CD8+ T cytotoxic cells, CD25 antigen expression was found to be negligible in all three groups (Table 3). As with the CD4+ T-cells, CD28 expression in the CD8+ cells was reduced in the poor responder group compared with both the normal controls and good responders (Figure 1B) and (Table 3).

Table 3. Accessory Molecule Expression on CD4+ T-Cells (T helper cells) and CD8+ (T cytotoxic cells) (means ± SD)

	Normal controls (*n*=14)	Good responders (*n*=12)	Poor responders (*n*=12)
CD4+/CD25+	3±2	4±3	4±3
CD4+/CD28+	98±1	96±5	86±10a,b
CD8+/CD25+	<1	<1	<1
CD8+/CD28+	54±13	48±19	30±14c,b

Receptor expression is presented as percent positive determined by fluorescence intensity, gating on CD3+ T-lymphocytes. Values equate to percentage of CD4+ or CD8+ cells that express antigen. c$P<0.05$ and a$P<0.005$ for poor responders *vs* good responders. b$P<0.005$ for poor responders *vs* normal controls.

PBMC Generation of Cytokines

In vitro PBMC generation of cytokines was studied in order to compare immune reactivity among the three study groups. Table 4 summarizes the results obtained for cytokine detection in PBMC culture supernatants. Significant levels of IL-4 were detected only in PBMC cultures stimulated with mAbs to CD3/CD28 with similar levels were in the three groups (Table 4).

A high level of IFN-γ was detected in the unstimulated supernatant of the poor responders. Little IFN- γ was detected in supernatants of either the normal controls or the good responders (Table 4). Similarly, LPS-stimulation caused high IFN-γ generation in the poor responders and negligible generation in the other two groups. CD3/CD28 mAbs challenge of PBMC generated similar amounts of IFN-γ in all three groups (Table 4).

The membrane-bound CD25 antigen (alpha chain of IL-2 receptor) can be proteolytically cleaved to generate a soluble form that is present in human plasma (abbreviated to sIL-2R). In PBMC cultures, low levels of sIL-2R were detected in unstimulated and LPS-stimulated cultures. Higher levels were detected when PBMC were activated with mAbs to CD3/CD28 (Table 4). For all three culture conditions, there was no significant difference in sIL-2R levels among any of the subject groups.

Table 4. Cytokine production (pg/ml) in culture supernatants of PBMC isolated from study groups[a].

Cytokine/stimulus	Normal controls (*n*=10)	Good responders (*n*=10)	Poor responders (*n*=10)
IL-4/no stimulus	0.47±0.55	0.28±0.04	0.63±1.10
IL-4/LPS	<0.24	0.30±0.12	0.81±1.40
IL-4/CD3&CD28	16.99±15.30	16.40±13.01	15.70±8.40
IFN- γ /no stimulus	<4	6±2	163±456
IFN- γ /LPS	<4	4±1.2	65±165
IFN- γ /CD3&CD28	566±318	643±387	596±323
sIL-2R/no stimulus	15±3	23±10	29±30
sIL-2R/LPS	175±104	285±323	418±520
sIL-2R/CD3&CD28	2210±1872	3260±1873	2675±1310
IL-10/no stimulus	22±10	53±101	159±272b,c
IL-10/LPS	1405±656	2704±1064d	2986±1835b
IL-10/CD3&CD28	1477±888	2349±1205	3262±1798b

Data are presented as means ± SD a: PBMC were drawn prior to a dialysis session and cultured for 48 h before cytokine determination by ELISA. CD3&CD28, monoclonal antibodies to CD3 and CD28. b: *P*<0.05 for poor responders *vs* normal controls. c*: P*<0.05 for poor responders *vs* good responders. d:*P*<0.05 for good responders *vs* normal controls.

Figure 2A shows IL-10 generation in unstimulated PBMC. Poor responders had significantly higher levels of IL-10 compared with both good responders and normal controls. Interleukin-10 production in unstimulated PBMC from good responders did not differ statistically from normal controls. Lipopolysaccharide challenge resulted in elevated IL-10 levels in both poor responders and good responders compared with normal controls (Figure 2B & Table 4).

In contrast, mAbs to CD3/CD28-stimulation resulted in elevated IL-10 levels in the poor responders alone, compared with normal controls (Figure 2C and Table 4), while IL-10 generation in the good responder group was not statistically different from the other two groups.

Plasma IFN- γ, TNF-α, IL-6 and sIL-2R

Plasma sIL-2R and IL-6 were found to be significantly elevated in both good and poor responders compared with controls, but there was no statistical difference between the HD patient groups. Plasma levels of IFN- γ and TNF- γ were undetectable in both HD patients and normal controls (Table 5).

Mortality

Figure 3 shows the Kaplan–Meier survival curves for the good responders to Epo (n=12) and the poor responders to Epo (n=12). During the 24 month study period, seven poor responders died due to the following reasons: sepsis (n=4), cardiovascular death (n=2) and access failure (n=1).

In the good responder group, two patients died due to cardiovascular death. Using the Mantel-Haenszel log-rank test, the survival curve for the poor responder group was significantly different from that of the good responder group (P<0.05).

Table 5. Plasma cytokine levels from subjects[a] (means ± SD)

	Normal controls (n=14)	Good responders (n=12)	Poor responders (n=12)
IFN- γ (pg/ml)	<1	<1	<1
TNF- α (ng/ml)	<10	<10	<10
IL-6 (pg/ml)	3±1	29±21[b]	28±25[c]
sIL-2R (pg/ml)	225±165	1276±532[b]	1367±945[c]

a: Plasma cytokine determination by ELISA. b:P<0.05 for good responders *vs* normal controls. c:P<0.05 for poor responders *vs* normal controls.

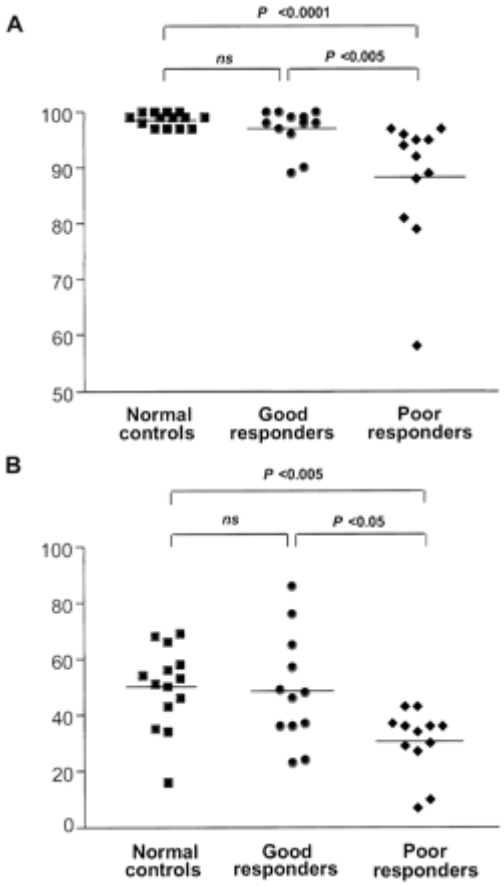

Figure 1. (A) Percentage of CD28+ CD 4 cells in all studied groups. (B) Percentage of CD28+ CD 8 cells in all studied groups. Horizontal bars indicate the mean percentage value.

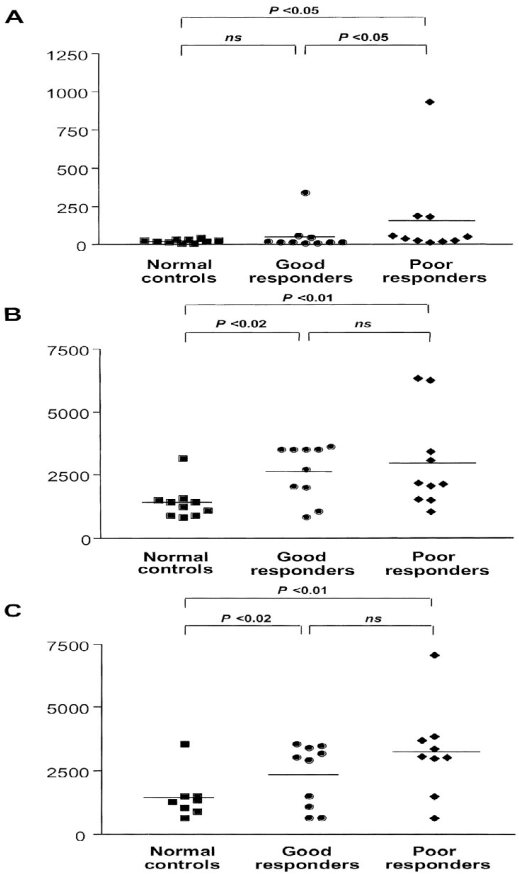

Figure 2. IL-10 levels in culture supernatants of PBMCs in studied groups. IL-10 was measured in pg/ml per2X106 cells after 48h culture. (A) Unstimualted cells (B) cells stimulated with LPS (C) cells with mAbs to CD3 and CD28. Horizontal bars indicate the mean percentage value.

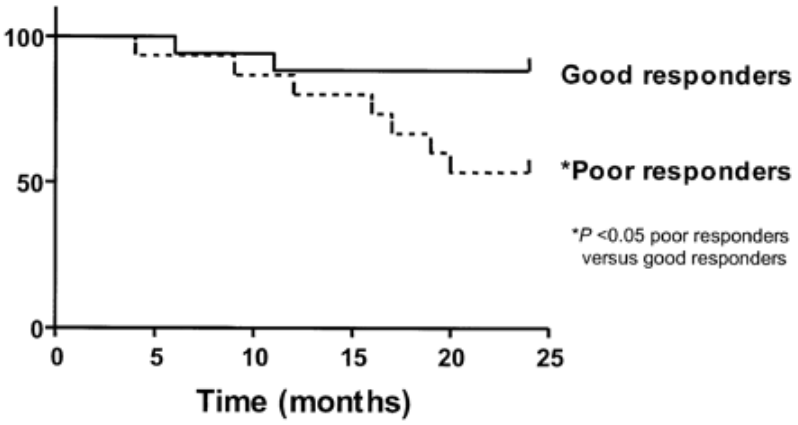

Figure 3. Kaplan-Meier survival curve for good and poor responder to Epo over the 24 months period of follow-up.

Discussion

The present study found that poor responders to Epo also had increased proportions of CD8+/CD28- compared with good responders to Epo and normal controls. The pathological relevance of CD8+/CD28- and CD4+/CD28- to the poor response to Epo is unclear. The accumulation of these unusual cell types may be an epiphenomenon, without actually contributing to the anaemia seen in poor responders to Epo.

The advantage of cytokine determination by flow cytometry is that the method is independent of variations in the proportions of Tcells to other mononuclear cells in mixed leukocyte cultures. Hence cytokine expression is determined on a single cell basis and expression can be assigned to either the CD4 or the CD8 T cell subset (Macdougall and Cooper 2005).

C-reactive protein levels were higher in the poor responders compared with the good responders which in turn were higher than in the normal controls which support the role of inflammation in EPO resistance. These results were in agreement of Barany *et al.,* 1997 who concluded that CRP is a strong predictor of resistance to erythropoietin in HD patients (Barany *et al.,* 1997).

In the present study, rHuEPO resistance is due to enhanced levels of immune activity. It has been investigated by studying T-cell phenotypes using flow cytometry, as well as cytokine release from T cells and monocytes in 'good' and 'poor' responders to rHuEPO.

Hyporesponsiveness to rHuEPO was defined as patients requiring >500 IU/kg/week or failing to achieve hematocrit (Hct) levels of <30% (Eschbach *et al.,* 2002). Consideration should be given to modifying the definition of rHuEPO hyporesponsiveness. The US Hct target of 33-36% for haemodialysis patients is narrow and the European target of Hct >33% may be significantly more practical and physiologically relevant(Eckardt, 2002).

This study demonstrates that HD patients responding poorly to Epo with no obvious underlying cause have different circulating T-cell immunophenotypes compared with good responders and normal controls. Peripheral blood T-cells from poor responders have increased proportions of CD4+/CD28- cells and CD8+/CD28- cells compared with both good responders to Epo and normal controls.

The resistance to recombinant human erythropoietin occurs in a small but important proportion of hemodialysis patients. This may be due to increased immune activation because pro-inflammatory cytokines inhibit erythropoiesis *in vitro* (Cooper *et al.,* 2003.)

It has been reported that CD28+ compared with their counterparts, CD4+/CD28- T-cells produce significantly more IFN- γ, enabling them to function as pro-inflammatory cells. CD4+/CD28- cells persist in the circulation for years and this has recently been attributed to a defect in their apoptotic pathway, Hence, CD4+/CD28- T-cells are resistant to apoptosis, accounting for their clonal outgrowth and maintenance *in vivo* (Azuma *et al.,* 1998).

In healthy individuals CD4+/CD28- T-cells are rarely found (the proportion is usually<1%) (Fagnoni *et al.,* 1996; Azuma *et al.,* 1998), but they have been described in a number of other chronic inflammatory states. Thus, increased numbers of CD4+/CD28- cells have been found in patients with rheumatoid arthritis (Martens *et al.,* 1997), unstable angina (Liuzzo *et al.,* 1999; Barany *et al.,* 2002), HIV infection (Choremi-Papadopoulou, 1994) and Wagener's granulomatosis (Moosig *et al.,* 1998).

Healthy adults usually have between 25 and 50% of CD8+ T-cells that are CD28- (Fagnoni *et al.,* 1996). Viral infections such as HIV-1 infection and Epstein–Bar virus-induced mononucleosis are associated with higher populations of CD8+/CD28- T-lymphocytes (Choremi, 1994). A higher

incidence of these cells has also been found in Wagener's granulomatosis (Moosig *et al.,* 1998).

It has been reported that CD8+/CD28- and CD4+/CD28- T-cells secrete high levels of IFN- γ since this pro-inflammatory cytokine inhibits erythroid colony growth *in vitro* and is implicated as one of the major factors causing impaired erythropoiesis and anaemia in chronic inflammatory states (Allen *et al.,* 1999; Dai &Krantz 1999; Liuzzo *et al.,* 1999).

Aguilera *et al.,* (2002) reported that treatment with recombinant human erythropoietin (rHuEPO) in dialysis patients has been associated with improvement of nutritional and immune status through an increase of cytokine production [such as tumor necrosis factor alpha (TNF alpha)]. The high cytokine production can be a double-edged sword owing to the relationship of cytokines with the systemic inflammatory process, which has been associated with many complications of uraemic status (Kato *et al.,* 2001).

This study did not find any difference in secretion of IFN- γ or IL-4 in PBMC cultures obtained from HD patients and normal controls. Furthermore, there was no difference in secretion of these cytokines between good and poor responders. A previous study (Goicoechea *et al.,* 1998) has suggested that PBMC isolated from poor responders secreted lower levels of IFN- γ compared with good responders. This disagreement with our results could be explained on the basis that our patients have reduced circulating levels of lymphocytes in peripheral blood. Therefore, the PBMC culture data do not reflect cytokine production per absolute cell number.

However, we found that unstimulated PBMC generation of IL-10 was significantly increased in poor responders compared with both normal controls and good responders. Monoclonal antibodies to CD3/CD28-stimulation also caused increased IL-10 production in poor responders compared with normal controls. Interleukin-10 is generated by monocytes, natural killer cells and T-lymphocytes. The source of IL-10 in our unstimulated cultures could be from any or all of these cell types. The CD3/CD28 mAbs exclusively stimulate T-cells and it is therefore likely that the IL-10 in these cultures is derived from T-cells alone, although it is possible that a T-cell-derived factor may have activated monocytes to produce IL-10 in our mixed leukocyte cultures.

Interleukin-10 has been shown to down-regulate inflammatory reactions, predominantly by limiting monocyte function (Moore *et al.,* 2001).

This cytokine potently inhibits both cytokine and chemokine secretion from monocytes. IL-10 also interferes with the antigen presenting capacity of monocytes by inhibiting their surface expression of MHC class II, B7-1 and B7-2, causing the inhibition of CD4+ T-cell proliferation and cytokine production (Buer *et al.,* 1998). In contrast, IL-10 has a stimulatory action on CD8+ T-cells and induces their recruitment, cytotoxicity and proliferation. Activation of T-cells in the presence of IL-10 can cause irreversible non-responsiveness known as T-cell anergy and such anergy can be induced *in vivo* by continuous antigenic challenge (Moore *et al.,* 2001).

The observation that poor responders to Epo have increased levels of IL-10 *in vitro* compared with both good responders and normal controls may indicate that leukocytes from these HD patients are primed to secrete higher levels of this anti-inflammatory cytokine. This effect may be 'adaptive', occurring as a result of increased underlying inflammation. In this context, the increased production of IL-10 would down-regulate the inflammatory process that may be associated with the poor response to Epo.

A previous study has shown that spontaneous PBMC secretion of IL-12 is increased in HD patients dialysed with cuprophan membrane (Memoli *et al.,* 1999); IL-10 is known to inhibit the production of IL-12 from activated macrophages and monocytes. T-cells from poor responders to Epo may therefore secrete more IL-10 to compensate for the elevated IL-12 production.

An earlier study of Kato *et al.,* 2001 showed that inflammation is one of the major causes of resistance to erythropoietin (rHuEpo) treatment. Tumour necrosis factor-alpha (TNF-alpha), one of the most potent proinflammatory cytokines, is known to inhibit human erythropoiesis directly in vitro. Although blood levels of soluble receptors for TNF-alpha (sTNFRs) are elevated in (HD) patients, and high blood sTNFR p80 may contribute to the development of rHuEpo resistance in female patients undergoing long-term HD (Kato *et al.,* 2001).

The present study showed that in the follow up period, more deaths were occurring among the poor responders than the good responders. This was therefore assessed using the Mantel–Haenszel log-rank test, and the survival curves were indeed different, with seven of the 12 poor responder patients dying compared with only two of the 12 good responders. This is consistent with the observation that chronic inflammation and high levels of inflammatory markers are predictive of mortality in a dialysis population.

The reported biological effects of rHuEpo include the induction of several genes in endothelial cells as well as a role for erythropoietin in the outcome of plasmodium infection. A new erythropoietin-like molecule is novel erythropoiesis stimulating protein (NESP), which is as effective and safe as rHuEpo, with the potential advantage of less frequent dosing(Sunders – Plassman& Hort 2001; Macdougall & Cooper 2002; Stenvinkel & Barany 2002).

In summary, this study shows that poor response to Epo is associated with an increase in IL-10 generation *in vitro* and this may be indicative of enhanced immune activity. The observation that poor responders have raised CRP levels compared with both good responders and normal controls, despite the absence of any overt infection or inflammation, supports this theory.

From the present study we can conclude that poor responders had increased amount of CD4+/CD28- and CD8+/CD28- because these T-cell phenotypes occur in chronic inflammatory diseases, again implying that inflammation is linked to poor response to Epo. CD4+/CD28- and CD8+/CD28- T-cells produce large amounts of the pro-inflammatory cytokine IFN- γ, which has been shown to inhibit erythropoiesis.

From the results of the this study we might suggest that, in the absence of any obvious cause, poor response to Epo may be mediated by generation of pro-inflammatory cytokines from a subpopulation of activated T-cells, which then promote apoptosis in erythroid progenitor cells in the bone marrow. Anti-cytokine antibodies may be useful for studying inflammatory cytokine secretion from T cells in patients with renal failure. Strategies utilizing anti-cytokine therapy may prove to be a useful adjuvant in optimizing the response to rHuEPO therapy.

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