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Serum thrombopoietin levels in patients with aplastic anaemia

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Summary. Endogenous serum thrombopoietin (TPO) levels were measured in 31 patients with aplastic anaemia (AA) using an enzyme immunoassay with a sensitivity of 20 pg/ml. The median platelet count for all AA patients was $30 \pm 29 \times 10^9$/l (range 5–102) compared with a median of $284 \pm 59 \times 10^9$/l (range 148–538) for normal controls. Serum TPO levels were significantly elevated in all patients compared with normals (1706 ± 1114·2, range 375–5000 *v* 78 ± 54, range 16·5–312·9, $P < 0{\cdot}0001$). There was no correlation between serum TPO levels and the degree of thrombocytopenia in AA patients, but TPO levels were significantly higher in patients who were platelet transfusion dependent than in patients who were transfusion independent ($P<0{\cdot}01$). There was a trend for higher TPO levels in patients with severe AA compared with non-severe AA patients. Clinical trials of TPO and a related truncated, pegylated molecule, megakaryocyte growth and development factor (PEG-rHuMGDF), are awaited to determine whether treatment with these drugs will result in increased platelet counts in patients with AA.

Keywords: thrombopoietin, TPO, aplastic anaemia.

Thrombopoietin (TPO) is the critical cytokine regulator of platelet production. Human TPO is encoded by a single gene located on chromosome 3q27-28 (Gurney *et al*, 1995). TPO is the ligand for the c-Mpl receptor, a member of the haemopoietin cytokine receptor family (Bartley *et al*, 1994; De Sauvage *et al*, 1994; Lok *et al*, 1994). It stimulates the formation and proliferation of megakaryocyte colonies from human $CD34^+$ cells (Nichol *et al*, 1995) and purified murine haemopoietic stem cells (Zeigler *et al*, 1994), as well as maturation of megakaryocytes, resulting in increased megakaryocyte size and ploidy with profound increases in platelet count (Kaushansky, 1995).

When administered to normal mice and non-human primates, TPO induces a marked increase in bone marrow and spleen megakaryocyte colonies, megakaryocytes and platelet count (Kaushansky *et al*, 1994; Farese *et al*, 1995). Mice which were myelosuppressed following irradiation and carboplatin were protected against severe thrombocytopenia by TPO and showed, as expected, rapid platelet recovery, but also earlier reticulocyte recovery and higher haemoglobin levels when compared with controls not receiving TPO (Grossman *et al*, 1995; Hokom *et al*, 1995). Erythroid progenitors *in vitro* are known to respond to TPO (Kaushansky *et al*, 1995; Kobayashi *et al*, 1995), and thus TPO appears to stimulate erythropoiesis as well as thrombopoiesis in states of bone marrow failure. Therefore, in aplastic anaemia (AA), although circulating levels of other haemopoietic growth factors such as GM-CSF (Schrezenmeier *et al*, 1993), G-CSF (Kojima *et al*, 1996) and erythropoietin (Gaines *et al*, 1992; Schrezenmeier *et al*, 1994) are elevated in most cases, the therapeutic use of TPO may be greater than initially anticipated.

Evaluation of serum TPO levels in various disorders accompanied by thrombocytopenia reveals that the levels are variable and may correlate with megakaryocyte mass rather than platelet count. Emmons *et al* (1996) reported that serum TPO levels were elevated in patients with AA where megakaryocytes were reduced or absent, but decreased or undetectable in immune thrombocytopenia in association with normal or increased megakaryocytes.

We have measured serum TPO levels in 31 patients with AA and determined whether there is any correlation with platelet count, disease severity, and response to treatment with immunosuppression.

PATIENTS AND METHODS

Patients. Serum TPO levels were measured in 31 patients with AA from February 1995 to August 1995. Patient details are summarized in Table I. All patients had acquired AA, except for three with Fanconi anaemia. There were 17

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Table I. Patient details including serum TPO levels and platelet counts.

Patient	Age (yr)/ sex	Disease duration (months)	Aetiology	Severity	Status	Current treatment	Previous treatment	TPO (pg/ml)	Platelets (10^9/l)
1	60/M	0	Allopurinol	NS	New, TD	Nil	Nil	1410	21
2	26M	37	PNH	NS	R	Pred	Pred, CSA	666	50
3	60/F	24	Gold, salazopyrine, NSAID	NS	R	Nil	CSA, ATG	1520	99
4	40/M	49	Idiopathic	NS	R	Nil	ATG × 2, CSA, Oxy	2659	35
5	55/F	53	Idiopathic	NS	R	CSA	ATG	375	91
6	26/F	10	Pregnancy	S	NR	CSA	Nil	2764	7
7	28/M	59	Idiopathic	NS	R	Nil	ATG	2525	67
8	49/M	25	Idiopathic	VS	NR	Oxy	ATG × 2, CSA	1293	54
9	65/F	5	Idiopathic	NS	NR	Nil	ATG	3899	8
10	29/F	85	Idiopathic	NS	TI	Nil	Nil	2472	22
11	26/M	5	FA	VS	NR	Danazol	Oxy, Pred	1504	43
12	44/M	3	Idiopathic	NS	NR	CSA	Nil	435	35
13	58/F	162	Idiopathic	NS	R	Nil	ATG, Oxy	1589	28
14	22/M	1	MDMA	S	New, TD	Nil	Nil	3354	32
15	19/M	129	Idiopathic	NS	R	Nil	ATG × 2, Oxy	1157	44
16	79/F	10	Idiopathic	VS	NR	Nil	CSA	3133	29
17	17/F	12	Constitutive	S	NR	CSA	ATG × 2	2325	18
18	45/F	1	Idiopathic	NS	New, TD	Nil	Nil	1299	11
19	26/F	10	Idiopathic	NS	NR	CSA	ATG, IL-6	2325	5
20	63/F	11	Salazopyrine	S	NR	CSA	Oxy	4250	7
21	25/F	79	Idiopathic	NS	R	Nil	ATG	2015	102
22	16/F	132	FA	NS	NR	Oxy	Nil	5000	19
23	22/M	31	Idiopathic	NS	R	Oxy	ATG × 2, CSA	1656	92
24	31/M	19	Idiopathic	NS	R	CSA	ATG × 2, Oxy	1270	28
25	69/M	31	Idiopathic, PNH + MDS	NS	NR	G-CSF	ATG × 2, CSA	3219	7
26	38/M	27	Cimetidine	NS	R	Oxy	ATG × 2, CSA	1407	62
27	46/F	26	Remoxipride	NS	R	CSA	ATG × 2	1594	30
28	31/M	37	Idiopathic	NS	R	Oxy	ATG, BMT	824	77
29	56/M	4	Idiopathic	NS	NR	Nil	ATG	1962	8
30	48/M	2	Benzene	NS	New, TI	Nil	Nil (spont. recov.)	1706	26
31	62/M	5	Idiopathic	VS	NR	Nil	CSA	3133	31

Severity of AA was classified according to the criteria of Camitta *et al* (1976) and Bacigalupo *et al* (1988); VS = very severe; S = severe; NS = nonsevere. PNH = paroxysmal nocturnal haemoglobinuria; NSAID = non-steriod anti-inflammatory drug; FA = Fanconi anaemia; MDMA = methylenedioxymethamphetamine ('Ecstasy'); MDS = myelodysplastic syndrome. TD = transfusion dependent; R = response; NR = no response; TI = transfusion independent. Pred = prednisolone; CSA = cyclosporin A; Oxy = oxymetholone; G-CSF = granulocyte colony-stimulating factor; ATG = antithymocyte globulin; IL-6 = interleukin-6; BMT = bone marrow transplantation; spont. recov. = spontaneous recovery.

males and 14 females, and the median age was 40 years (range 16–79). 23 patients had nonsevere AA, four severe AA and a further four had very severe AA, as defined by Camitta *et al* (1976) and Bacigalupo *et al* (1988), respectively. At the time of testing, four patients were newly diagnosed and had received no treatment, although three had received platelet and/or red cell transfusions. A further patient had been transfusion independent since diagnosis, 2 months earlier. Specific treatment for AA was given to 26 patients, of whom 13 had responded to therapy with antithymocyte globulin (ATG), cyclosporin, oxymetholone or bone marrow transplantation (BMT), and seven of these remained on treatment at the time of testing. There were 13 non-responders (NR) to treatment (see Table I), requiring continued red cell and platelet transfusional support. Response to specific treatment for AA was defined as independence from platelet and red cell transfusions.

Collection of serum. 50 ml of peripheral blood was collected from AA patients into glass tubes containing no additives or anticoagulant. The clotted blood was centrifuged at 1500 rpm for 10 min and the separated serum stored at −20°C. Batches of frozen samples were despatched for assay on dry ice.

TPO ELISA: antibody preparation. Rabbits were immunized with either full-length recombinant human Mpl-ligand (rHuTPO) purified from CHO cells or the receptor binding domain of TPO purified from *E. coli* (Amgen, Thousand Oaks, Calif.). Antibodies from the sera were purified using affinity chromatography and protein A chromatography. A polyclonal antibody directed against the receptor binding portion of TPO was selected as the capture antibody, based on the low signal to noise ratio. A separate polyclonal antibody to full-length rHuTPO was selected for use as the signal antibody. The signal antibody was coupled to horseradish peroxidase (HRP) using iminothane HCl and N-succinimidyl 6-maleimidocaproate (Fluka Chemical Corp., Ronkonkoma, N.Y.), isolated by size exclusion (200 kD range), and concentrated in a Centricon 30 concentrator (WR Grace & Co, Beverly, Mass.) prior to use as the signal antibody (Rabbit anti-rHuTPO-HRP).

TPO ELISA method. The capture antibody (2 μg/ml in 0·1 M $NaHCO_3$) was incubated in wells of EIA plates (Costar, Cambridge, Mass.) for 18–24 h. The following day the antibody was diluted with buffer containing, 1% Bovine Serum Albumin (BSA) (Sigma Chemical Co., St Louis, Mo.), 5% sucrose, 5 mM Tris base, 15 mM NaCl, 1 mM ethylenediaminetetrasodium (EDTA) and 0·001% thimerosol (pH 7·35, Sigma Chemical Co.), and incubated for an additional 18–24 h. Excess solution was removed and the antibody coated wells treated with Super Block (Pierce, Rockford, Ill.) for 5 min and dried overnight at room temperature without washing. Dried plates were stored for up to 90 d at 5°C in sealed chambers prior to use.

Serum samples, warmed to 37°C, were diluted with buffer containing 50 mM Tris-HCl, 150 mM NaCl and 0·1% Tween 20 (pH 7·5, Sigma Chemical Co.). The dilutions for screening were at final concentrations of 10% and 50% by volume to ensure measurements in the linear range of the standard curve. Occasionally additional dilutions were necessary in order to measure samples containing excessive levels of TPO. A total serum concentration of 50% of the well volume was maintained in all conditions tested by diluting the standards and samples in a background of FCS which was pre-screened for undetectable endogenous TPO and negligible background optical density.

TPO concentrations were assigned based on the standard curve generated from serial dilutions of a single batch of full-length rHuTPO into 50 mM Tris-HCl, 150 mM NaCl and 0·1% Tween 20 (pH 7·5) plus pre-screened FCS (50% final concentration). Stability of this batch of TPO is confirmed by the reproducibility of the standard curves shown in Fig 1A.

The diluted samples and standards were added directly to re-hydrated, antibody-coated EIA plates for 18–24 h at room temperature, then washed well with 0·05% Tween 20, 5 mM

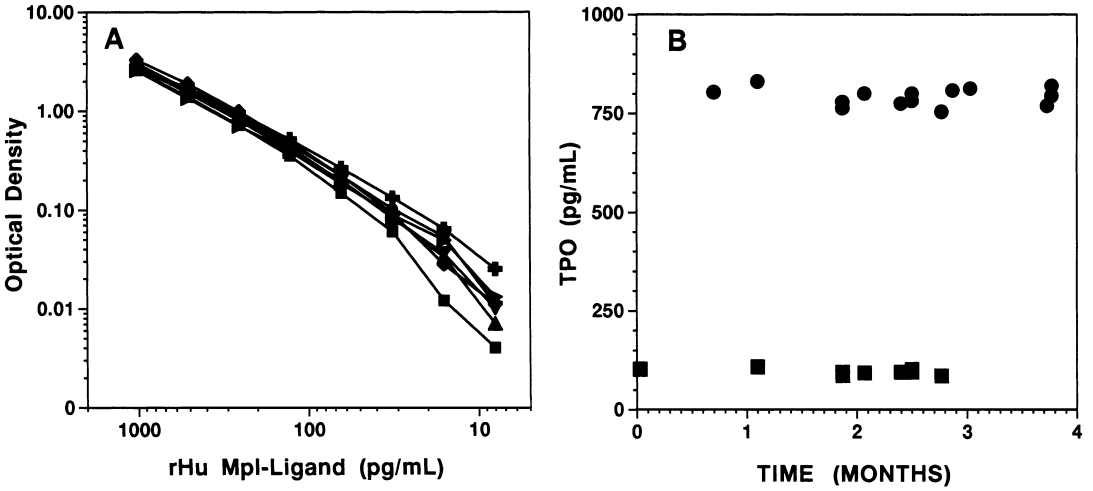

Fig 1. (A) The standard curves from seven experiments (SEM 0·005, inter-assay of coefficient of variation <20% for the linear range of the standard curve), and (B) TPO values of selected plasma samples over a 4-month period (mean concentrations of 798 ± 8 for high values and 96 ± 2 for the low values, in all assays coefficient of variation 7·7% and 2·8%, respectively), are shown.

Tris Base, 150 mM NaCl, 1 mM EDTA and 0·001% thimerosol (pH 7·5). Rabbit anti-rHuTPO-HRP signal antibody, diluted to 200 ng/ml in PBS with 2% FCS was incubated for 4 h at room temperature followed by washing (0·05% Tween 20, 5 mM Tris base, 150 mM NaCl, 1 mM EDTA and 0·001% thimerosol (pH 7·5)). HRP was demonstrated with the TMB Microwell Peroxidase Substrate System (Kirkegard & Perry, Gathersburg, Md.) as per the manufacturer's instructions. The reaction was allowed to proceed for 45 min at room temperature and stopped with 0·5 N H_2SO_4 solution. Optical density was read on a VMax microplate reader (Molecular Devices, Sunnyvale, Md.) at 450 nM minus 650 nM. Only samples within the linear range of the standard curve were assigned a TPO value.

In order to monitor assay reproducibility, pre-aliquoted plasma samples of previously determined TPO concentration were included in all assays.

Assay validation. The inter-assay reproducibility of the TPO ELISA is demonstrated in Fig 1A, in which the standard curves from seven experiments (SEM 0·005, inter-assay coefficient of variation <20% for the linear range of the standard curve) are shown TPO values of selected plasma samples over a 4-month period (mean concentrations of 798 ± 8 for the high values and 96 ± 2 for the low values, in all assays coefficient of variation 7·7% and 2·8% respectively) are shown in Fig 1B. In order to control for interference from other serum components on the ELISA, serial dilutions of a serum sample containing approximately 800 pg/ml of TPO (the high sample) were run in the same assay with dilutions of pre-screened FCS spiked with full-length rHuTPO at an equivalent starting concentration. Consistency was confirmed by the parallel dose–response curves of these samples (data not shown).

In addition, studies were performed on 13 normal donors from which samples were collected daily for 30 consecutive days at the same time of day. The mean percent change from baseline platelet count was 5·6 ± 0·3% (median 4·8%, range 0–20·3%) and the mean percent change from baseline TPO level was 7 ± 0·4% (median 5·7%, range 0–32%). There was no correlation between platelet number and TPO levels.

Statistical analysis. Statistical analysis was performed using the Student's *t* test.

RESULTS

Serum TPO levels

The median serum TPO level in 31 AA patients was 1706 pg/ml (range 375–5000) (see Table I). This was significantly higher than the TPO levels in serum from 117 normal controls, median 78 pg/ml (range 16·5–312·9), $P < 0·0001$.

Platelet counts

Platelet counts performed at the time of estimation of serum TPO levels are shown in Table I. The median platelet count for all AA patients was 30×10^9/l (range 5–102). This was significantly lower than in 99 of the normal controls tested (median 284×10^9/l, range 148–538), $P < 0·0001$.

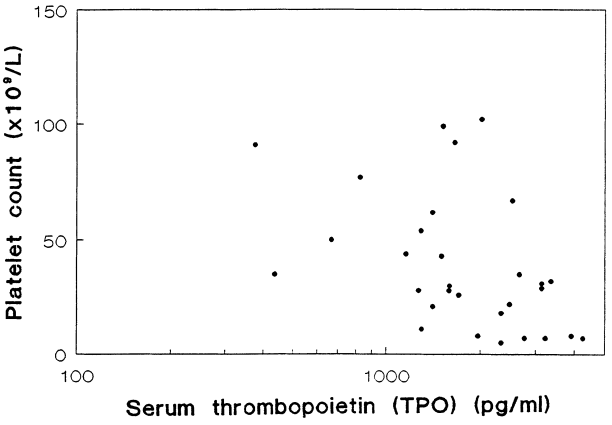

Fig 2. Serum thrombopoietin levels and platelet counts in aplastic anaemia patients. Serum thrombopoietin levels as measured by ELISA (see Methods) are plotted against peripheral blood platelet counts for all patients with aplastic anaemia. There was no correlation between the two parameters (r=0·47).

Correlation of factors with serum TPO levels

As shown in Fig 2, there was no correlation between serum TPO levels and platelet counts in AA patients (r=0·47). If AA patients who were platelet transfusion dependent were excluded from the analysis there was also no correlation for patients who were not receiving platelet transfusions. TPO levels were significantly higher in patients who were either transfusion dependent or had not responded to treatment than those who were either transfusion independent or had responded to therapy ($P < 0·01$). There was a trend for higher TPO levels in patients with severe AA compared with patients with non-severe AA, although this was not statistically significant ($P = 0·06$). The following factors showed no correlation with serum TPO levels in AA: disease duration, age and sex.

DISCUSSION

We have shown that serum TPO levels were markedly elevated in all patients with AA. The median TPO level was 22 times higher than the median level among normal controls. There was no correlation between TPO levels and platelet count, but TPO levels were higher in those patients who required platelet transfusions compared with those patients who had higher platelet counts so that platelet transfusions were not required. There was also a trend for higher TPO levels in patients with more severe AA. However, the definition of disease severity takes into account not only the platelet count but also the neutrophil count, reticulocyte count and marrow cellularity, with the neutrophil count differentiating severe from very severe AA (Camitta *et al*, 1976; Bacigalupo *et al*, 1988).

The high circulating levels may be explained by up-regulation of TPO production due to the feedback mechanism stimulated by a reduced number of platelets. However, a preliminary report indicates that serum TPO levels may be more closely related to megakaryocyte mass rather than the

peripheral blood platelet count (Emmons *et al*, 1996), as discussed earlier. Since all our patients had either reduced or absent bone marrow megakaryocytes one may postulate that a minimum number of megakaryocytes is required to negatively feedback on TPO production, and that such a number of megakaryocytes were not present in our patients' bone marrows. Alternatively, in AA there may be abnormal regulation of platelet production due, for example, to a reduced number of Mpl receptors or reduced responsiveness of the receptors to TPO.

Using bioassays, serum levels of colony stimulating activity (detecting G-CSF, GM-CSF and burst promoting activity) in AA patients are increased (Nissen *et al*, 1983, 1985). Enzyme immunoassays or chemoluminescent enzyme immunoassays have enabled a more accurate assessment of levels of specific haemopoietic growth factors, and have confirmed increased serum levels in many patients (Schrezenmeier *et al*, 1993; Kojima *et al*, 1996). Bone marrow stromal cells release and express increased amounts of these growth factors and others such as stem cell factor, interleukin 6 (IL-6) and IL-1β (Kojima *et al*, 1992; Stark *et al*, 1993; Hirayama *et al*, 1994). Nevertheless, when used clinically, some growth factors such as G-CSF do increase the neutrophil count in some patients with AA (Kojima & Matsuyama, 1994). Thus pharmacological doses of G-CSF can induce a response in patients despite their having detectable or elevated endogenous serum G-CSF levels. Clinical trials of recombinant Mpl ligands such as TPO and PEG-rHuMGDF in AA would now seem timely and relevant.

Many patients with AA require long-term platelet transfusional support. This is complicated by HLA alloimmunization in a large proportion of patients, resulting in platelet refractoriness and the consequent requirement for HLA-matched platelet transfusions (Grumet & Yankee, 1970; Klingemann *et al*, 1987). AA patients are also at increased risk of graft rejection following allogeneic BMT because of sensitization from multiple blood products to minor histocompatibility antigens (McCann *et al*, 1994). As has been shown with G-CSF, the greatest benefit in terms of neutrophil response occurs in those patients with residual granulopoiesis, one might anticipate an analogous response to TPO. If administration of TPO increases the platelet count in AA patients, even if by just an amount sufficient to abolish the requirement for prophylactic platelet transfusions in patients with severe thrombocytopenia, this may reduce the risk of HLA alloimmunization and graft rejection post BMT, and could have major cost implications in view of the expense involved in the long-term use of platelet transfusions in patients with AA.

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