British Journal of Haematology, 2003, **120**, 907–915

Correspondence

GENE POLYMORPHISMS ASSOCIATED WITH DIMINISHED ACTIVITY OF 5,10-METHYLENETETRAHYDROFOLATE REDUCTASE DO NOT EXPLAIN THE CLINICAL MANIFESTATIONS OF COBALAMIN DEFICIENCY

Why some patients with cobalamin deficiency develop neurological dysfunction and others do not is unknown. The observations that the haematological and neurological expressions of cobalamin deficiency appear to be inversely related and that the same clinical expression tends to recur with relapse (Healton *et al*, 1991) raise the possibility that a predilection for one expression over the other may have a genetic basis.

It is noteworthy that hereditary absence of 5,10-methylenetetrahydrofolate reductase (MTHFR) is characterized by severe neurological dysfunction without megaloblastic anaemia (Carmel & Rosenblatt, 2002). In the absence of MTHFR, 5,10-methylenetetrahydrofolate cannot be reduced to 5-methyltetrahydrofolate, which is required together with cobalamin in the remethylation of methionine. However, the 5,10-methylenetetrahydrofolate remains available for thymidylate synthesis, which accounts for the absence of anaemia. The possibility arises that, by altering the flow of 5,10-methylenetetrahydrofolate metabolism, genetic polymorphisms affecting MTHFR activity may influence whether individual cobalamin-deficient patients express predominantly neurological or haematological dysfunction. The 677C → T and 1298A → C polymorphisms of the *MTHFR* gene are common and are associated with mildly decreased MTHFR activity (Frosst *et al*, 1995; van der Put *et al*, 1998; Weisberg *et al*, 1998).

Therefore, we examined whether the cobalamin-deficient patient who has a mutation with reduced MTHFR activity is predisposed to neurological dysfunction and less likely to have anaemia. DNA was obtained from cobalamin-treated patients with proven pernicious anaemia (clinical and biochemical evidence of cobalamin deficiency, evidence of response to cobalamin therapy and a diagnostic Schilling test and/or intrinsic factor antibody result), for whom clinical and laboratory information was sufficient to determine the presence or absence of haematological and neurological disease. We excluded seven neurologically impaired patients who had co-existing disorders, such as diabetes or alcoholism, that can cause neurological dysfunction or who had insufficient evidence of neurological response to cobalamin therapy. All specimens were tested for the 677C → T and 1298A → C mutations by polymerase chain reaction (PCR) techniques described elsewhere (Frosst *et al*, 1995; Weisberg *et al*, 1998).

The 27 patients included 15 men and 12 women, aged 22–74 years; 15 patients were Latin American, five were white, six were black and one was Asian. None of the 13 patients in the 'central nervous system (CNS)' group, who had neurological abnormalities ranging from mild (e.g. sensory deficits limited to the feet) to severe (e.g. ataxia, involvement of hands as well as feet), were homozygous for either type of MTHFR mutation, and only four were compound heterozygotes for the two mutations (Table I). This absence of homozygotes is in disagreement with the hypothesis that we proposed. Although the frequency of heterozygosity for the mutations was slightly greater in the CNS group than in the 14 patients without neurological abnormalities (the 'non-CNS' group), even this non-significant difference was mitigated by the fact that simple heterozygosity has little effect on MTHFR activity (van der Put *et al*, 1998; Weisberg *et al*, 2001). Indeed, more patients in the non-CNS group were homozygous for the 677TT mutation than in the CNS group, which is in disagreement with the hypothesis. Exclusion of black patients, in whom the 677C → T mutation is rare (McAndrew *et al*, 1996), from analysis did not alter the results but, in any event, including blacks would not have masked the clinical expression–mutation interaction if the MTHFR polymorphism hypothesis was correct.

The lack of association between the mutations and neurological status was demonstrated further by assigning to each patient the relevant mean MTHFR activities in lymphocytes that were reported for each genotype combination by van der Put *et al* (1998). The CNS and non-CNS groups had comparable calculated 'MTHFR activities' (18·3 ± 5·0 *vs* 19·6 ± 6·8 nmol formaldehyde/mg protein/h). The same outcome was obtained by applying the values from site-directed mutagenesis studies published by Weisberg *et al* (2001), which were expressed as a percentage of wild-type enzyme activity (57·5% ± 21·7 in the CNS group *vs* 66·5% ± 25·9 in the non-CNS group).

The CNS group also had higher sex-adjusted haemoglobin levels than the non-CNS group (11·6 *vs* 7·9 g/dl, $P = 0{\cdot}02$), which confirmed past reports of an inverse relationship between anaemia and neurological symptoms (Healton *et al*, 1991). Only three out of 13 patients in the CNS group had moderate or severe anaemia, in contrast to 12 out of 14 in the non-CNS group. Analysis based on patients' haematological status, irrespective of neurological status, or based on the presence of either or both of the two clinical expressions, also showed no significant MTHFR mutation differences (Table I). Reclassification of patients into two groups based on the presence or absence of mutations with meaningful effects on MTHFR activity

Table I. 677C → T and 1298A → C mutations of the MTHFR gene in patients with pernicious anaemia stratified in several ways for the presence or absence of neurological dysfunction or anaemia.

Comparison of groups	677C → T			1298A → C			Compound heterozygote
	CC	CT	TT	AA	AC	CC	(677CT + 1298AC)
CNS	5/13	8/13	0/13	6/13	7/13	0/13	4/13
	39%	62%	0%	46%	54%	0%	31%
vs.							
Non-CNS	9/14	3/14	2/14	8/14	6/14	0/14	1/14
	64%	21%	14%	57%	43%	0%	9%
Anaemia	12/22	8/22	2/22	12/22	10/22	0/22	5/22
	55%	36%	9%	55%	45%	0%	23%
vs.							
No anaemia	4/11	6/11	1/11	5/11	6/11	0/11	2/11
	36%	55%	9%	45%	55%	0%	18%
CNS+/anaemia–	3/7	4/7	0/7	3/7	4/7	0/7	1/7
	43%	57%	0%	43%	57%	0%	14%
vs.							
CNS–/anaemia+	8/12	3/12	1/12	7/12	5/12	0/12	2/12
	67%	25%	8%	58%	42%	0%	17%
vs.							
CNS+/anaemia+	2/6	4/6	0/6	3/6	3/6	0/6	3/6
	33%	67%	0%	50%	50%	0%	50%

Comparisons were made in three subgroups: (1) patients with vs. without neurological dysfunction; (2) patients with vs. without anaemia (this analysis included the seven patients excluded from all other analyses because of uncertainty as to whether the CNS findings arose from cobalamin deficiency); (3) patients with neurological dysfunction but no anaemia vs. anaemia without neurological dysfunction vs. both neurological dysfunction and anaemia (two patients who had neither anaemia nor neurological defects were excluded from this analysis). Percentage values are shown at the bottom of each row. CNS, neurological dysfunction; +, positive/present; –, negative/absent.

(677TT homozygosity or combined 677CT/1298AC heterozygosity versus wild type or heterozygosity for only one of the mutations) also produced no significant differences in haemoglobin values between the groups, confirming that MTHFR polymorphisms did not protect against anaemia in cobalamin deficiency. Haemoglobin levels did not correlate with the MTHFR enzyme activity scores, applied as described earlier.

Our results show that the two common MTHFR polymorphisms, which help to shunt folate towards thymidylate synthesis and away from methionine synthesis, neither predispose to neurological dysfunction nor protect against anaemia in patients who develop cobalamin deficiency. Although the number of patients studied was relatively small, the trends were, if anything, opposite in direction to those that would implicate compromised MTHFR activity as the determinant of clinical expression. Other explanations must be sought for the frequent dichotomy between neurological and haematological expressions in cobalamin-deficient patients.

ACKNOWLEDGMENTS

This work was supported by grant DK32640 from the National Institutes of Health. We thank Dr Zvi Kelman for valuable advice, Drs Frank Collela and Elshami Elamin for allowing us to include their patients, and James Parker for expert technical assistance.

[1]*New York Methodist Hospital, Brooklyn, NY, and* [2]*University of Southern California School of Medicine, Los Angeles, CA, USA.*
E-mail: rac9001@nyp.org

RALPH CARMEL[1]
VINOD PULLARKAT[2]

REFERENCES

Carmel, R. & Rosenblatt, D.S. (2002) Disorders of cobalamin and folate metabolism. In: *Blood. Principles and Practice of Hematology*, 2nd edn (ed. by R.I. Handin, S.E. Lux & T.P. Stossel), chap. 45. Lippincott Williams & Wilkins, Philadelphia.

Frosst, P., Blom, H.J., Milos, R., Goyette, P., Sheppard, C.A., Matthews, R.G., Boers, G.J.H., den Heijer, M., Kluijtmans, L.A.J., van den Heuvel, L.P. & Rozen, R. (1995) A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nature Genetics*, **10**, 111–113.

Healton, E.B., Savage, D.G., Brust, J.C., Garrett, T.J. & Lindenbaum, J. (1991) Neurologic aspects of cobalamin deficiency. *Medicine*, **70**, 229–245.

McAndrew, P.E., Brandt, J.T., Pearl, D.K. & Prior, T.W. (1996) The incidence of the gene for thermolabile methylene tetrahydrofolate reductase in African Americans. *Thrombosis Research*, **83**, 195–198.

van der Put, N.M.J., Gabreëls, F., Stevens, E.M.B., Smeitink, J.A.M., Trijbels, F.J.M., Eskes, T.K.A.B., van den Heuvel, L.P. & Blom, H.J. (1998) A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? *American Journal of Human Genetics*, **62**, 1044–1051.

Weisberg, I., Tran, P., Christensen, B., Sibani, S. & Rozen, R. (1998) A second polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. *Molecular Genetics and Metabolism*, **64**, 169–172.

Weisberg, I.S., Jacques, P.F., Selhub, J., Bostom, A.G., Chen, Z., Ellison, R.C., Eckfeldt, J.H. & Rozen, R. (2001) The 1298A → C polymorphism in methylenetetrahydrofolate reductase (MTHFR): in vitro expression and association with homocysteine. *Atherosclerosis*, **156**, 409–415.

Keywords: cobalamin deficiency, folate, megaloblastic anaemia, methylenetetrahydrofolate reductase, neurological dysfunction.

RECOMBINANT FACTOR VIIa FOR REFRACTIVE HAEMORRHAGE IN AUTOIMMUNE IDIOPATHIC THROMBOCYTOPENIC PURPURA

Published guidelines on the management of patients with autoimmune idiopathic thrombocytopenic purpura (AITP) fail to make recommendations for the subset of patients with platelet counts $< 30 \times 10^9$/l and persistent bleeding despite standard treatment (George *et al*, 1996). We report here the successful treatment of a paediatric patient with recombinant Factor VIIa (rFVIIa; NovoSeven®, NovoNordisk, Copenhagen, Denmark).

An 8-year-old girl with a 3-year history of AITP presented with epistaxis. On admission, her platelet count was 3×10^9/l and haemoglobin was 7·4 g/dl. Serum iron was 7·9 μmol/l, unbound iron-binding capacity 82 μmol/l, total iron-binding capacity 90 μmol/l and ferritin < 3 μg/l. Prothrombin time, partial thromboplastin time and plasma fibrinogen concentration were all normal. Intravenous immunoglobulins were contraindicated because of a previous severe allergic reaction, so she was managed with a deep nose tampon, corticosteroids, erythrocyte transfusions and tranexamic acid.

The patient had a second brief episode of epistaxis at 24 h that stopped spontaneously, but otherwise did well initially. On d 9, epistaxis recurred, accompanied by severe headache. Her platelet count was 13×10^9/l, fibrinogen 0·8 g/l and FXIII 51 iu/dl. The other coagulation parameters were normal. Initial management with a deep nose tampon, corticosteroids, platelet transfusions (6 units) and tranexamic acid was ineffective, and her condition deteriorated until she received an intravenous bolus injection of 85 μg/kg rFVIIa. Bleeding subsequently decreased within a few minutes and stopped within 1 h. Her headache resolved with no neurological deficit.

Despite treatment with corticosteroids and tranexamic acid, the patient presented on d 30 with severe incessant epistaxis. Her haemoglobin was 9·1 g/dl, and her platelet count was 13×10^9/l. She received platelet transfusions (4 units) and a deep nose tampon with no effect. The bleeding stopped 1 h after a single bolus injection of 85 μg/kg rFVIIa. At d 34, she was stable with a platelet count of $13–42 \times 10^9$/l. There was no further bleeding. No adverse effects were observed; FXIII and fibrinogen levels were normal.

rFVIIa represents a rational management approach to bleeding complications resulting from platelet disorders (Hedner, 1998; Negrier & Lienhart, 2000), but we believe that this is the first reported use in a child with AITP. At physiological concentrations, FVIIa initiates haemostasis through its interaction with tissue factor (TF) at the site of injury, forming a catalytically active complex. The TF/FVIIa complex leads to the activation of FX and, thus, to the formation of small amounts of thrombin, leading in turn to the activation of coagulation Factors VIII, V, XI and platelets. At the higher concentrations found after therapeutic administration, FVIIa also binds directly to activated platelets to initiate thrombin formation. Platelet-bound FVIIa in higher concentrations is functionally active and initiates thrombin formation via direct activation of FX. This pathway is independent of the presence of TF (Monroe *et al*, 1997).

Infusion of rFVIIa has been shown to reduce or stop bleeding in patients with thrombocytopenia, and high doses may help to provide haemostasis in these patients by increasing the initial thrombin generation (Kristensen *et al*, 1996; Kjalke *et al*, 2001). We suggest that, in this patient, the dual action of rFVIIa played a crucial role in maximizing platelet activation and thrombin generation.

We conclude that rFVIIa provided safe and effective therapy for severe bleeding in a child with AITP who was unresponsive to other therapy. As there is currently a lack of consensus about how such patients should be managed, this approach should be studied further.

Clinical Hospital Split, SRĐANA ČULIĆ
Department of Pediatrics, Split,
SpinčIćeva 1, Croatia.
E-mail: srdjana.culic@st.tel.hr

REFERENCES

George, J.N., Woolf, S.H., Raskob, G.E., Wasser, J.S., Aledort, L.M., Ballem, P.J., Blanchette, V.S., Bussel, J.B., Cines, D.B., Kelton, J.G., Lichtin, A.E., McMillan, R., Okerbloom, J.A., Regan, D.H. & Warrier, I. (1996) Idiopathic thrombocytopenic purpura: a practice guideline developed by explicit methods for the American Society of Hematology. *Blood*, **88**, 3–40.

Hedner, U. (1998) Recombinant activated Factor VII as a universal haemostatic agent. *Blood Coagulation and Fibrinolysis*, **9**, S147–S152.

Kjalke, M., Ezban, M., Monroe, D.M., Roberts, H.R. & Hedner, U. (2001) High-dose Factor VIIa increases initial thrombin generation and mediates faster platelet activation in thrombocytopenia-like conditions in a cell-based model system. *British Journal of Haematology*, **114**, 114–120.

Kristensen, J., Killander, A., Hippe, A., Helleberg, C., Ellegard, J., Holm, M., Kutti, J., Mellqvist, U.H., Johansson, J.E., Glazer, S. & Hedner, U. (1996) Clinical experience with recombinant Factor VIIa in patients with thrombocytopenia. *Haemostasis*, **26**, 159–164.

Monroe, D.M., Hoffman, M., Oliver, J.A. & Roberts, H.R. (1997) Platelet activity of high-dose factor VIIa is independent of tissue factor. *British Journal of Haematology*, **99**, 543–547.

Negrier, C. & Lienhart, A. (2000) Overall experience with NovoSeven®. *Blood Coagulation and Fibrinolysis*, **11**, S19–S24.

Keywords: autoimmune idiopathic thrombocytopenic purpura, bleeding, case report, children, recombinant Factor VIIa.

Ca^{2+} IONOPHORE-INDUCED DENDRITIC CELL DIFFERENTIATION IN A PATIENT WITH DEFECTIVE Ca^{2+} IONOPHORE-INDUCED PLATELET AGGREGATION

Fuse *et al* (2001) reported three cases of platelet dysfunction characterized by defective Ca^{2+} ionophore-induced platelet aggregation without impaired production of thromboxane A_2 (TXA_2). Platelet stimulation with strong agonists such as thrombin and collagen generates diacylglycerol (DG) and inositol 1,4,5-triphosphate (IP_3) from membrane phospholipid by hydrolysis with phospholipase C (PLC). DG induces phosphorylation of a 47-kDa protein (pleckstrin) by activating protein kinase C (PKC). IP_3 triggers cytoplasmic Ca^{2+} mobilization, which induces various important signal transductions, such as the activation of the 20-kDa myosin light chain kinase (MLCK) via the Ca^{2+} receptor protein, calmodulin. The activation of MLCK gives rise to the interaction between actin and myosin and a release reaction, with morphological changes of platelets. Although intracellular Ca^{2+} mobilization, IP_3 generation and phosphorylation of 47-kDa pleckstrin and 20-kDa MLC were demonstrated not to be impaired after stimulation with thrombin, STA_2 (TXA_2 mimetic) and A23187 (Ca^{2+} ionophore), platelet aggregation was not induced when collagen, arachidonic acid, STA_2 or A23187 (Ca^{2+} ionophore) were used as stimulating agents in the three cases reported by Fuse *et al* (2001). In these cases, the processes at the upper stream of the phosphorylation of both pleckstrin by PKC in the DG pathway and myosin light chain (MLC) by the Ca^{2+} calmodulin complex in the IP_3 pathway were demonstrated to be normal. From these findings, it was suggested that Ca^{2+} ionophore-induced platelet aggregation is defective because of impairment (on the impaired site) in the downstream processes of phosphorylation of pleckstrin in the DG pathway, the phosphorylation of MLC in the IP_3 pathway or the Ca^{2+} mobilization-related processes (which differ from the phosphorylation of MLC).

Recently, dendritic cells were successfully generated from normal peripheral blood monocytes, blood dendritic cell precursors, marrow $CD34^+$ cells and peripheral blood cells from patients with chronic myelogenous leukaemia by culturing with A23187 or thapsigargin (Koski *et al*, 1999). A23187 mobilized Ca^{2+} into the cytoplasm through the cell membrane, and thapsigargin elevated the concentration of cytoplasmic Ca^{2+} by inhibiting the ATP-dependent Ca^{2+} pump of smooth-surfaced endoplasmic reticulum. In order to determine whether the same signalling mechanisms are activated in the generation of dendritic cells and in platelet aggregation by the stimulation of Ca^{2+} ionophore, we attempted to generate dendritic cells from the peripheral blood cells of Patient 1 (as designated by Fuse *et al*, 2001) by culturing them with A23187.

Peripheral blood (PB) was collected in a heparinized syringe from Patient 1 (Fuse *et al*, 2001) and a normal volunteer (control) after obtaining informed consent. Mononuclear cells (MNCs) were separated by Ficoll-Hypaque (Lymphoprep; Nyomed, Oslo, Norway) gradient centrifugation and cultured in a plastic dish for 2 h. The adherent cells, which were left on the dish after the removal of non-adherent cells, were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Nipro, Tokyo, Japan) with 375 ng/ml (0·7 mmol/l) A23187 (Calbiochem, San Diego, CA, USA) and 10% fetal bovine serum (FBS, CCS, Sanko Pure Chemicals, Japan) in 5% CO_2 at 37°C. The cells were cultured for 2 d and then cytospun on a slide glass and stained with May–Grunwald–Giemsa solution for morphological observation. Cultured cells were also stained for surface phenotypes by the standard direct procedure using the following phycoerythrin (PE)-conjugated monoclonal antibodies: anti-CD1a (T6; Coulter, Hialeah, FL, USA), anti-CD80 (L307.4; Becton Dickinson, San Jose, CA, USA), anti-CD86 (IT2.2; PharMingen, San Diego, CA, USA), anti-CD83 (IM2218; Immunotech) and anti-HLA-DR (Becton Dickinson). Cell fluorescence was analysed with a FACScan flow cytometer (Becton Dickinson). Ten thousand events were collected, and the data were analysed by Lysis II (Becton Dickinson). After 2 d of culture of PBMNCs from Patient 1 with A23187, cell aggregates of dendritic-like cells with multiple cytoplasmic projections were observed by phase-contrast microscopy. Similar cell aggregates were also observed in the control. Cytospin preparation of cultured cells also demonstrated the presence of some dendritic-like cells with hairy cytoplasmic projections in both Patient 1 and the control. Flow cytometric analysis of these cultured cells from the patient for 2 d revealed the generation of cells with the phenotype $CD1a^+$, $CD80^+$,

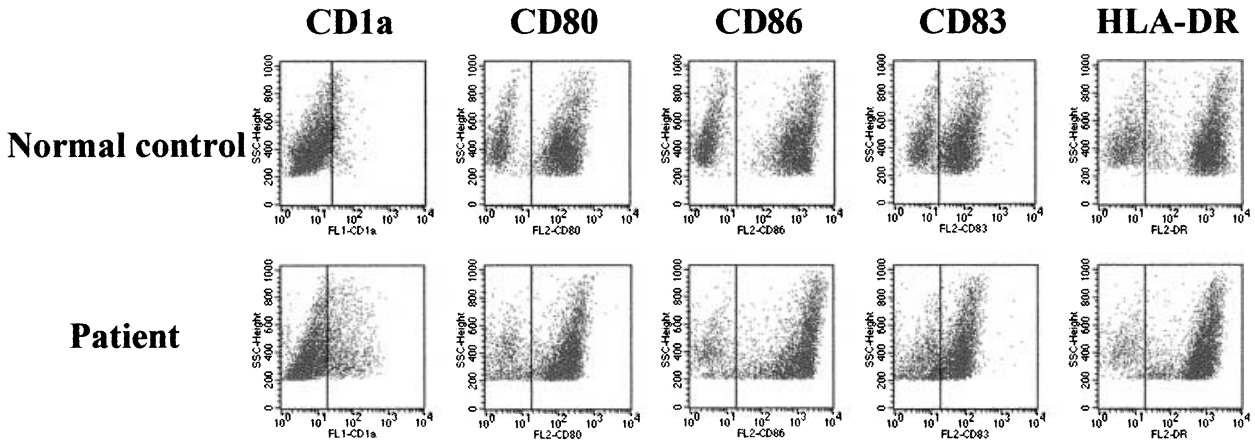

Fig 1. Surface phenotypes of dendritic cells generated from PBMNCs from a normal control and a patient with platelet dysfunction caused by impaired utilization of intracellular Ca^{2+} by culture with A23187 (375 ng/ml) for 2 d.

$DC86^+$, $CD83^+$ and HLA-DR^+ (Fig 1). The level of expression of these dendritic cell-associated molecules was similar in the cultured cells from both the patient and the control.

The present study demonstrated that, although platelets were not aggregated by the stimulation of Ca^{2+} ionophore, dendritic cells were generated from monocytes by culturing with Ca^{2+} ionophore in this particular patient, who had a bleeding disorder. The generation of dendritic cells by culturing with A23187 has been demonstrated previously to be associated with increased cytoplasmic Ca^{2+}, as 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid (acetoxy-methyl) ester (BAPTA AM, an intracellular Ca^{2+}-chelating agent) and 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8, a blocking agent of Ca^{2+} release from the endoplasmic reticulum) blocked the generation of dendritic cells in the culture with A23187 (unpublished observation). Signalling mechanisms following an increased level of cytoplasmic Ca^{2+} have been investigated in the interleukin-2 gene expression of T lymphocytes. Intracellular Ca^{2+} mobilization activates the Ca^{2+}-binding protein calmodulin, which upregulates serine/threonine protein kinases and serine/threonine protein phosphatase 2B and calcineurin (Stemmer & Klee, 1994). As to the generation of dendritic cells by the stimulation of Ca^{2+} ionophore, calcineurin antagonists such as cyclosporin A and tacrolimus (FK-506), as well as calmodulin antagonists such as trifluoroperazine dimalate and W-7, were demonstrated to be suppressive (Koski *et al*, 1999). These findings showed that, although both platelet aggregation and the generation of dendritic cells were induced by an increased level of cytoplasmic Ca^{2+}, signalling mechanisms downstream of Ca^{2+} mobilization might differ between platelet aggregation and dendritic cell differentiation. In addition, a signalling defect in Ca^{2+} ionophore-dependent platelet aggregation was demonstrated to have no effects on the differentiation of dendritic cells stimulated with Ca^{2+} ionophore in a study of the enrolled patient.

[1]*School of Health Science,* [2]*Division of Blood Transfusion,* and [3]*First Department of Internal Medicine, Faculty of Medicine, Niigata University, Asahimachi, Niigata, Japan. E-mail: naritami@clg.niigata-u.ac.jp*

Miwako Narita[1]
Masuhiro Takahashi[1]
Ichiro Fuse[2]
Yoshihusa Aizawa[3]

REFERENCES

Fuse, I., Higuch, W., Uesugi, Y. & Aizawa, Y. (2001) Pathogenetic analysis of three cases with a bleeding disorder characterized by defective platelet aggregation induced by Ca^{2+} ionophores. *British Journal of Haematology*, **112**, 603–608.

Koski, G.K., Schwartz, G.N., Weng, D.E., Czerniecki, B.J., Carter, C., Gress, R.E. & Choen, P.A. (1999) Calcium mobilization in human myeloid cells results in acquisition of individual dendritic cell-like characteristics through discrete signaling pathways. *Journal of Immunology*, **163**, 82–92.

Stemmer, P.M. & Klee, C.B. (1994) Dual calcium ion regulation of calcineurin by calmodulin and calcineurin B. *Biochemistry*, **33**, 6859–6866.

Keywords: Ca^{2+} ionophore, intracellular Ca^{2+}, platelet dysfunction.

A SPECTRUM OF SKIN REACTIONS CAUSED BY THE TYROSINE KINASE INHIBITOR IMATINIB MESYLATE (STI 571, GLIVEC®)

Imatinib mesylate (STI 571, Glivec®) has now been established as an effective treatment for chronic myeloid leukaemia (CML). However, the optimal duration of therapy is unclear, particularly in the light of recent data showing a rarity of durable molecular remissions in complete cytogenetic responders (Feng *et al*, 2002). As

chronic treatment with this agent may be required, it is important to characterize its non-haematological side-effects fully. According to the National Cancer Institute/National Institutes of Health (NCI/NIH) Common Toxicity Criteria, these are generally of grade 1 or 2 severity (i.e. mild) and include nausea, oedema, myalgias and diarrhoea (Druker *et al*, 2001). In the largest series published to date, of 532 late chronic-phase patients receiving 400 mg of imatinib daily, 32% developed a rash (Kantarjian *et al*, 2002). Although these reactions were not characterized fully, they were deemed to be serious drug-related adverse events in four patients. We followed a large cohort of imatinib-treated patients prospectively to characterize the cutaneous side-effects further. Between August 2000 and November 2001, we enrolled a total of 82 patients within the UK imatinib programme (Novartis Pharma) for chronic-phase (400 mg/d, $n = 50$), accelerated-phase (600 mg/d, $n = 28$) and blast crisis (600 mg/d, $n = 4$) CML. Follow-up data was available for 78 patients who were reviewed regularly for the development of side-effects. Skin toxicity was documented as per NCI/NIH Common Toxicity Criteria (Druker *et al*, 2001). Only cases that were attributable to imatinib are reported. Skin biopsy was performed on patients with unusual rashes, but not for typical dermatitic rashes. Treatment was empirical and based on clinical signs and symptoms. Rashes were observed that could be directly attributed to imatinib in a total of nine patients (12%, five men and four women; Table I).

Standard management of cutaneous drug reactions generally includes cessation of the offending drug and future avoidance. Unfortunately, however, there is no equally effective alternative to imatinib for CML patients (the majority in this cohort were interferon failures), and it is therefore important to establish the natural history and appropriate management of common side-effects. We have demonstrated that rashes are a relatively common side-effect of imatinib, although at a lower incidence than found in other studies. This may result from the exclusion of cases in which cutaneous reactions to other drugs were possible (most frequently in the context of concomitant allopurinol therapy), where there was a history of pre-existing skin pathology and where there was no clear temporal relation to the introduction (or increase in dose) of imatinib. As in a previous report (Brouard & Saurat, 2001), our data suggest that a rash is more likely with higher doses, supporting the hypothesis that it is due to a pharmacological effect, rather than true hypersensitivity. Although the majority of rashes are dermatitic, self-limiting and easily treated, progression to erythroderma may occur, and cessation of therapy may be required. We also report the occurrence of a graft-versus-host (GVH)-

Table I. Clinical details of patients developing cutaneous reactions to imatinib.

UPN	Type of reaction	Grade (NIH/NCI criteria)	Biopsy	Dose and duration of imatinib before rash	Imatinib interrupted?	Treatment	Outcome
1	Dermatitis	1	ND	400 mg/19 d	No	None	Resolved (3 d)
2	Dermatitis	2	ND	600 mg/47 d	Yes	Oral steroids	Resolved within 14 d (imatinib restarted)
3	Dermatitis	2	ND	600 mg/17 d	No	Oral piriton	Resolved within 14 d
4	Dermatitis	2	ND	600 mg/13 d	No	None	Resolved within 50 d
5	Dermatitis	2	ND	400 mg/70 d	No	Oral steroids and piriton	Settling at 130 d
6	GVH-like drug rash	2	Yes	600 mg/359 d	No	Oral and topical steroids	Ongoing at 170 d
7	Dermatitis	4	ND	400 mg/21 d	Yes	Oral and topical steroids	Resolved on stopping imatinib
8	Erythema nodosum	3	Yes	600 mg/73 d	Yes	Oral steroids and azathioprine	Resolved on stopping imatinib
9	Vasculitis	2	Yes	600 mg/42 d	Yes	Oral steroids	Resolved, 5 mg pred at present, on imatinib

Six cases occurred in 30 patients on 600 mg/d (20%) and three cases in 48 patients on 400 mg/d (6%). Median duration of treatment before development of skin rash was 42 d (range 13–359 d). Patients 1–5 developed itchy, dermatitic eruptions, mainly on their limbs (grade 1–2). Patients 1 and 4 required no treatment, and their rashes resolved spontaneously. Patients 2, 3 and 5 required oral prednisilone, chlorpheniramine (piriton) or both respectively. Patient 6 developed a symptomatic, indurated, red/brown reticulate eruption affecting the trunk, buttocks and limbs, 14 d after increasing the imatinib dose from 400 to 600 mg/d. Biopsy demonstrated an unusual GVH-like eruption (see text). Patient 6 has required ongoing intermittent use of topical and oral steroids. Patient 7 required interruption of treatment for 2 weeks because of a dermatitic rash on the face and limbs. On rechallenge, he became erythrodermic with an exfoliative dermatitis after two doses, necessitating permanent discontinuation of therapy (grade 4 toxicity). Patient 8 developed tender subcutaneous nodules that were clinically and histologically consistent with erythema nodosum. The rash was not controlled with oral prednisilone alone and required the addition of azathioprine and, ultimately, cessation of therapy (grade 3 toxicity). Patient 9 developed biopsy-proven small vessel vasculitis of his lower legs, forearms and abdomen (grade 2 toxicity) requiring a tapering dose of oral prednisilone. There was no evidence of systemic vasculitis, and imatinib was continued. A minor flare-up subsequent to this was controlled with 10 mg of prednisilone (since reduced to a maintenance dose of 5 mg). UPN, unique patient number; pred, prednisolone.

like drug reaction, erythema nodosum and small vessel vasculitis, skin eruptions not reported previously with this treatment. The appearance of these rashes was not typically dermatitic and prompted skin biopsy. The unusual appearance of the rash in patient 6 resulted in several skin biopsies being performed, all of which were suggestive of a GVH-like reaction. The patient had no history of bone marrow transplant or transfusion, modalities typically associated with this pathology. Drug eruptions resembling GVH disease are extremely rare, but have been recognized (Eming *et al*, 2001). Other groups have reported an acute generalized exanthematous pustulosis, a well-characterized cutaneous drug reaction (Brouard & Saurat, 2001), and Stevens–Johnson syndrome (Hsiao *et al*, 2002). We would therefore advise dermatological review in rashes that are not typically dermatitic in nature or persist despite simple treatment measures.

[1]*Dermatology Department, Monklands Hospital, Lanarkshire,* [2]*Haematology Department, Crosshouse Hospital, Ayrshire,* [3]*Haematology Department, Monklands Hospital, Lanarkshire,* [4]*Dermatology Department, Royal Alexandra Hospital, Paisley, and* [5]*ATMU, Department of Medicine, Glasgow University, Glasgow, UK.*
E-mail: mwd3z@clinmed.gla.ac.uk

A. Drummond[1]
P. Micallef-Eynaud[2]
W. S. Douglas[1]
J. A. Murphy[3]
I. Hay[4]
T. L. Holyoake[5]
M. W. Drummond[5]

REFERENCES

Brouard, M. & Saurat, J.-H. (2001) Cutaneous reactions to STI571. *New England Journal of Medicine*, **345**, 618–619.

Druker, B.J., Talpaz, M., Resta, D.J., Peng, B., Buchdunger, E., Ford, J.M., Lydon, N.B., Kantarjian, H., Capdeville, R., Ohno-Jones, S. & Sawyers, C.L. (2001) Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *New England Journal of Medicine*, **344**, 1031–1037.

Eming, S.A., Peters, T., Hartmann, K., Scharfetter-Kochanek, K. & Mahrle, G. (2001) Lichenoid chronic graft-versus-host-disease-like acrodermatitis induced by hydroxyurea. *Journal of the American Academy of Dermatology*, **45**, 321–323.

Feng, L., Drummond, M., Byrne, J., Shepherd, P., Apperley, J., Clarke, R., Craddock, C., O'Brien, S., Cervantes, F., Lennard, A., Holyoake, T., Goldman, J.M. & Kaeda, J.S. (2002) Molecular remission following treatment with STI571 for chronic myeloid leukaemia: a report from the UK STI571 Study Group. *British Journal of Haematology*, **117**, Abstract 10.

Hsiao, L., Chung, H., Lin, J., Chiou, T., Liu, J., Fan, F.S., Wang, W., Yen, C. & Chen, P. (2002) Stevens–Johnson syndrome after treatment with STI571: a case report. *British Journal of Haematology*, **117**, 620–622.

Kantarjian, H., Sawyers, C., Hochhaus, A., Guilhot, F., Schiffer, C., Gambacorti-Passerini, C., Niederwieser, D., Resta, D., Capdeville, R., Zoellner, U., Talpaz, M., Druker, B. and the International STI571 CML Study Group (2002) Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. *New England Journal of Medicine*, **346**, 645–652.

Keywords: chronic myeloid leukaemia, erythema nodosum, imatinib mesylate, skin rash, vasculitis.

PLASMA PROTEIN Z DEFICIENCY IS COMMON IN WOMEN WITH ANTIPHOSPHOLIPID ANTIBODIES

Antiphospholipid antibodies (APA) may be associated with venous thrombosis, arterial thrombosis, recurrent/late fetal loss and thrombocytopenia (Greaves, 1999). The mechanisms involved in such sequelae are complex as these antibodies may act at various stages of coagulation (Greaves, 1999). Protein Z (PZ) is a vitamin K-dependent glycoprotein that functions as a natural anticoagulant by acting as a cofactor for a plasma protease inhibitor (ZPI) to inhibit coagulation factor X (activated; FXa) (Broze, 2001). Recent data indicate that PZ may play a role in the aetiology of ischaemic stroke (Kobelt *et al*, 2001; Vasse *et al*, 2001) and early fetal loss (Gris *et al*, 2002). We have assayed PZ concentrations in women with evidence of APA to determine if inhibition of the PZ natural anticoagulant pathway may represent an additional thrombotic mechanism in subjects with antiphospholipid syndrome.

The subjects of study consisted of women who had suffered previous objectively confirmed venous thromboembolism (VTE) with no evidence of APA ($n = 40$; non-APA VTE group), women with evidence of APA ($n = 28$; APA group) and healthy female control subjects ($n = 47$). Twenty patients on warfarin with a stable International Normalized Ratio (INR) (between 2 and 4) were also recruited to allow confirmation of the effect of warfarin on PZ.

The APA group were women investigated for a variety of clinical indications [previous VTE ($n = 2$), arterial thrombosis ($n = 3$), recurrent fetal loss ($n = 3$), family history of VTE ($n = 4$), connective tissue disorder ($n = 2$), thrombocytopenia ($n = 2$), prolonged APTT ($n = 6$), neurological symptoms ($n = 2$), unclear indication ($n = 4$)] and who had laboratory evidence of APA [positive lupus anticoagulant test (LAC) and/or elevated titre of immunoglobulin class G (IgG) or IgM anticardiolipin antibodies (ACA)]. PZ was assayed using an enzyme-linked immunosorbent assay technique on citrated plasma (Asserachrom protein Z; Diagnostica Stago, France).

Age (mean ± standard deviation, SD) at blood withdrawal in the non-APA VTE and control groups were 36·9 ± 8·3 years and 45·9 ± 10 years respectively ($P < 0{\cdot}0001$). Mean age at sampling in the APA group was 46·2 ± 19·2 years ($P = 0{\cdot}93$ *vs* control subjects). APA investigations demonstrated six patients with positive LAC and elevated ACA, two with positive LAC only and 20 with elevated ACA only. The mean ± SD INR in 20 patients on stable warfarin therapy was 2·55 ± 0·35.

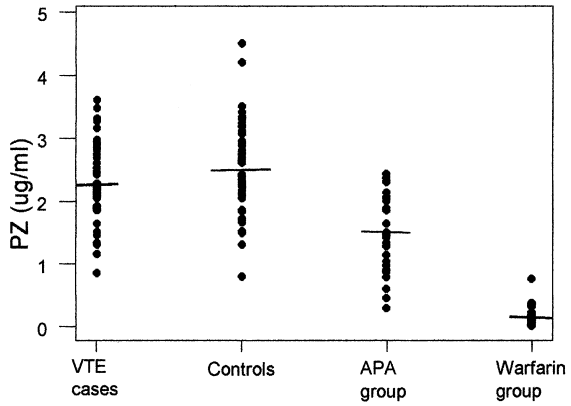

Fig 1. PZ concentrations in the four subject groups. Lines represent mean PZ values for each group. $P < 0{\cdot}001$ for APA group *vs* control subjects. VTE cases = non-APA VTE group.

Mean PZ in 40 patients with previous non-APA VTE (2·35 ± 0·66 μg/ml) was not significantly different from control subjects (2·52 ± 0·72 μg/ml; age-adjusted $P = 0{\cdot}73$) (Fig 1).

However, mean PZ in the APA group (1·49 ± 0·61 μg/ml) was significantly lower than control subjects ($P < 0{\cdot}001$). There was no significant difference in mean PZ concentrations between subjects with APA who had previously been symptomatic (1·34 ± 0·77 μg/ml, $n = 10$) or were asymptomatic (1·58 ± 0·51 μg/ml, $n = 18$) (adjusted $P = 0{\cdot}35$) and concentrations were significantly lower in both groups compared with control subjects.

One subject in each of the non-APA VTE (0·85 μg/ml) and control (0·79 μg/ml) cohorts had PZ concentrations below the normal range of our control [derived normal range (mean ± 2 SD) 1·08–3·96 μg/ml]. However, 8/28 (28·6%) subjects in the APA cohort had PZ concentrations below our normal range. Of this group, 3/8 had a positive LAC in association with elevated ACA and five had elevated ACA only.

One other study has suggested that plasma PZ concentrations may be reduced in subjects with APS (Steffano *et al*, 2001). Our findings confirm this and suggest that further research is required to determine whether the PZ deficiency observed in subjects with APA is prothrombotic or merely an epiphenomenon.

[1]*Department of Haematology, Crosshouse Hospital, Kilmarnock, Ayrshire,* [2]*University Department of Obstetrics, Royal Infirmary, and* [3]*Department of Haematology, Royal Infirmary, Glasgow, UK.*
E-mail: Mark.mccoll@aaaht.scot.nhs.uk

M. D. McColl[1,2]
A. Deans[1]
P. Maclean[3]
R. C. Tait[3]
I. A. Greer[2]
I. D. Walker[3]

REFERENCES

Broze, Jr, G.J. (2001) Protein Z-dependent regulation of coagulation. *Thrombosis and Haemostasis*, **86**, 8–13.

Greaves, M. (1999) Antiphospholipid antibodies and thrombosis. *Lancet*, **353**, 1348–1353.

Gris, J.C., Quere, I., Dechaud, H., Mercier, E., Pincon, C., Hoffet, M., Vasse, M. & Mares, P. (2002) High frequency of protein Z deficiency in patients with unexplained early fetal loss. *Blood*, **99**, 2606–2608.

Kobelt, K., Biasiutti, F.D., Mattle, H.P., Lammle, B. & Wuillemin, W.A. (2001) Protein Z in ischaemic stroke. *British Journal of Haematology*, **114**, 169–173.

Steffano, B., Forastiero, R., Martinuzzo, M. & Kordich, L. (2001) Low plasma protein Z levels in patients with antiphospholipid antibodies. *Blood Coagulation and Fibrinolysis*, **12**, 411–412.

Vasse, M., Guegan-Massardier, E., Borg, J.Y., Woimant, F. & Soria, C. (2001) Frequency of protein Z deficiency in patients with ischaemic stroke. *Lancet*, **357**, 933–934.

Keywords: protein Z deficiency, antiphospholipid antibodies.

INTENSIVE TRIATHLON TRAINING INDUCES LOW PERIPHERAL CD34$^+$ STEM CELLS

Watson & Meiklejohn (2001) recently reported that high-intensity training undertaken by professional football players could affect the physiological processes of the bone marrow, as peripheral leucopenia was observed in these athletes. However, the mechanisms by which this hypothesized process could occur remain unclear. Here, we report for the first time that the number of CD34$^+$ stem cells (CD34$^+$ SC) measured in peripheral blood samples from elite triathletes (swimming, biking, running) was found to be significantly lower than in healthy sedentary subjects.

All triathletes included in this study were competing at national level. They trained at least 45 weeks per year on a basis of between 14 h and 28 h of training per week. Controls were sedentary, healthy men who underwent less than 1 h of sports or intense physical activity per week. Thirty male triathletes (mean age 28·7 years, standard deviation ± 2·5 years) and 38 male sedentary controls (27·4 ± 2·1 years; all Caucasians) were studied on one occasion, in a resting state, during the early morning.

CD34$^+$ SC of venous peripheral blood from controls, triathletes (at least 36 h after the last training session) and CD34$^+$ SC standardized samples (Fluotrol, Bioergonomic, MN, USA) for intralaboratory quality assurance were analysed simultaneously every day. Whole-blood samples were incubated at room temperature with phycoerythrin-conjugated CD34 monoclonal antibodies (HPCA2, Becton Dickinson, CA, USA), and erythrocytes were lysed on a TQ-Prep workstation (Beckman Coulter, FL, USA). Absolute CD34$^+$ SC counts were determined

Table I. Comparison (unpaired *t*-test) of peripheral leucocyte subpopulations and $CD34^+$ SC counts between elite triathletes and controls.

	Sedentary controls ($n = 38$)	Triathletes ($n = 30$)	*P*
Leucocytes (10^9/l)	7·6 (0·8)	6·0 (1·4)	< 0·01
Neutrophils (10^9/l)	4·4 (0·6)	3·4 (1·0)	0·01
Lymphocytes (10^9/l)	2·6 (0·9)	2·1 (0·6)	0·06
$CD34^+$ cells (10^9/l)	0·006 (0·0018)	0·0033 (0·0015)	< 0·01

Results are given as the mean (standard deviation).

using a calibrated flow cytometer (MCL-XL flow cytometer, Beckman Coulter, FL, USA) using the standardized Flow-Count procedure (Flow-Count Fluorospheres, Beckman Coulter, FL, USA).

Our results showed low counts of leucocytes, neutrophils and lymphocytes (Table I), as reported previously by Lesesve *et al* (2000) and Bain *et al* (2000) in elite cyclists and marathon runners respectively. Interestingly, the highly trained triathletes showed significantly lower $CD34^+$ SC counts than their sedentary peers (Table I).

These results are clinically relevant, as neutropenia and low lymphocyte counts could contribute to the increased incidence of upper respiratory tract infections often observed in these sportsmen (Nieman, 1995). At least three hypotheses can be drawn to explain these comparatively low peripheral $CD34^+$ SC counts. Firstly, intense aerobic training of elite triathletes could induce deleterious effects on bone haematopoiesis by the inhibition of central $CD34^+$ SC growth, as suggested by Watson & Meiklejohn (2001). Thus, quantification of peripheral $CD34^+$ SC from elite athletes could be helpful for the prediction of leucopenia and subsequent infections. Secondly, intensive training could depress the mobilization of peripheral blood $CD34^+$ SC. Thirdly, low $CD34^+$ SC counts observed in elite athletes could be explained by the aetiology of the damage/repair process, as reported recently in heart or bone marrow transplantation (Orlic *et al*, 2001; Korbling *et al*, 2002). We believe that systematic $CD34^+$ SC quantification in regular biological surveys of elite sportsmen should be helpful for both basic science researchers and sport clinicians.

Departments of [1]*Histology, and* [2]*Exercise Physiology and Sports Medicine, University of Nice Sophia-Antipolis Medical School – Unit of Cellular and Tissular Functional Testings, Hôpital Pasteur, Nice Cedex 1, France. E-mail: bermon@unice.fr*

Patrick Philip[1]
Stéphane Bermon[2]

REFERENCES

Bain, B.J., Phillips, D., Thomson, K., Richardson, D. & Gabriel, I. (2000) Investigation of the effect of marathon running on leucocyte counts of subjects of different ethnic origins: relevance to the aetiology of ethnic neutropenia. *British Journal of Haematology*, **108**, 487.

Korbling, M., Katz, R.L., Khanna, A., Ruifrok, A.C., Rondon, G., Albitar, M., Champlin, R.E. & Estrov, Z. (2002) Hepatocytes and epithelial cells of donor origin in recipients of peripheral-blood stem cells. *New England Journal of Medicine*, **346**, 46.

Lesesve, J.F., Guinot, M., Andolfatto, S., Bene, M.C. & Dine, G. (2000) Effect of high-level cycling on leucocyte counts. *British Journal of Haematology*, **110**, 1009.

Nieman, D.C. (1995) Upper respiratory tract infections and exercise. *Thorax*, **50**, 1231.

Orlic, D., Kajstura, J., Chimenti, S., Limana, F., Jakoniuk, I., Quaini, F., Nadal-Ginard, B., Bodine, D.M., Leri, A. & Anversa, P. (2001) Mobilized bone marrow repair of the infracted heart improving function and survival. *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 10349.

Watson, H.G. & Meiklejohn, D.J. (2001) Leucopenia in professional football players. *British Journal of Haematology*, **112**, 824.

Keywords: $CD34^+$, leucopenia, sports, stem cells, training, triathlon.