Anti-Cancer Drugs 1997, **8**, pp. 204–209

Effect of granulocyte colony stimulating factor on pig blood platelets treated with cisplatin *in vitro*

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The effects of granulocyte colony stimulating factor (G-CSF) and cisplatin *in vitro* on the peroxidation of pig platelet endogenous arachidonate were investigated. Estimation of the level of malonyldialdehyde (MDA) after stimulation of platelets with thrombin served as an indicator of this process. MDA concentration was determined by means of a modified method with thiobarbituric acid. The effects of G-CSF on thrombin-induced formation of MDA in platelets was dependent on the concentration and the time of platelet preincubation with this cytokine. In the platelets obtained from the whole blood preincubated with the highest concentration of G-CSF (8 μg/ml), a significant increase of MDA formation was observed ($p < 0.01$). Cisplatin (20 μM) had a strong inhibitory effect on arachidonic acid metabolism in platelets ($p < 0.001$). In the presence of G-CSF (0.8 μg/ml) in incubation medium the inhibitory effect of cisplatin on platelet arachidonate pathway was reduced. The results showed that G-CSF had a protective effect against cisplatin action on platelets.

Key words: Blood platelets, cisplatin, G-CSF.

Introduction

Platelets are anuclear cytoplasmic fragments which originate from megakaryocytes in the bone marrow. Blood platelets play an important role in hemostasis. Activated blood platelets undergo a rapid series of morphological and biochemical changes including arachidonate liberation and peroxidation.[1,2] Clinical studies indicate that platelets may be activated *in vivo* in a number disorders such as atherosclerosis, venous thrombosis and cancer.[3,4] Activation of these cells can be stimulated by some tumor cells and it appears to be a precursor of the metastatic process. Platelets from patients with cancer exhibit a variety of functional abnormalities. These include increased or reduced activation of cells and hypersensitivity to various platelet agonists. On the other hand, some chemotherapeutic drugs can affect platelet function. The mechanism by which these drugs interfere with platelets is not entirely understood.[3]

Cisplatin is a widely used chemotherapeutic agent against a broad spectrum of human malignancies.[5,6] However, the clinical use of cisplatin is restricted by severe hematological toxicity including thrombocytopenia.[3] Our preliminary results have shown that *in vitro* cisplatin changes the function of blood platelets.[7,8] and has an inhibitory effect on the activation of these cells.[9] The exact mechanism of action of cisplatin on blood platelets remains unclear; however, it is known that cisplatin may affect the enzymatic peroxidation of platelet endogenous arachidonate.[9]

The studies *in vitro* and *in vivo* have indicated that combination with cytokines before and during cytostatic drug treatment can improve the toxic effect of cytostatic drugs.[10–12] A tendency towards shorter duration of leukocytopenia and thrombocytopenia for patients receiving the hematopoietic growth factors during chemotherapy was observed.[13–15] Hematopoietic growth factors have made a significant influence on the prevention of infections associated with chemotherapy-induced neutropenia, shorting of neutropenia following high-dose chemotherapy and progenitor cell transplantation, and chemotherapy-associated anemia.[10,16–18] Chemotherapy plus granulocyte colony stimulating factor (G-CSF) induced mobilization of peripheral blood progenitor cells, and accelerated both neutrophil and platelet recovery after high-dose chemotherapy.[18]

In addition to the clinical benefit of hematopoietic growth factors, their relative toxicity should be taken into account.

In this studies the effect of G-CSF on pig blood platelets was investigated. Whereas the antitumor properties of cisplatin have been well characterized and its influence on blood platelets *in vitro* has been described,[7–9] the direct effect of G-CSF on

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blood platelets and on cisplatin platelet interaction have not been clarified.

In consideration of the our previous observation that cisplatin *in vitro* has an inhibitory effect on thromboxane A_2 production in blood platelets stimulated by thrombin,[9] we studied the effects of G-CSF alone and in combination with cisplatin on the arachidonate pathway in blood platelets by the thiobarbituric acid assay using malonyldialdehyde (MDA) as a marker of this process.

Materials and methods

Blood platelet preparation

Platelets were obtained from fresh pig blood by differential centrifugation of blood collected into ACD solution (citric acid/citrate/dextrose) 5:1 v/v. Blood was centrifuged for 20 min at 750*g*. The platelet pellet was gently suspended and washed twice by centrifugation in a buffer containing 154 mM NaCl, 10 mM Tris–HCl and 5 mM glucose at pH 7.4.

Preincubations at 37°C (30 min to 2 h) with growth factor alone or with cisplatin were carried out in two systems: one with G-CSF, cisplatin or G-CSF plus cisplatin added to a suspension of platelets in buffered saline obtained as described above; the other with G-CSF, cisplatin or G-CSF plus cisplatin added to whole blood.

G-CSF was purchased from Roche as Neupogen. Stock solution (3000 ng/ml) was stored at 4°C. The final concentrations used were 0.08, 0.8 and 8 μg/ml of platelet suspension and 20 μg/ml of blood.

Cisplatin (Sigma) dissolved in buffered saline (pH 7.4) was added to platelet suspensions alone or with G-CSF (final concentration of cisplatin 20 μM) and to the whole blood (final concentration 500 μM) alone or with G-CSF. After preincubation of blood with tested drugs the platelets were isolated as described above. Platelets after preincubation with drugs were stimulated by thrombin (1.5 U/10^9 platelets) (Polfa).

Thrombin-induced activation of washed pig platelets

Suspensions of pig platelets in buffer (control platelets and platelets preincubated for 0.5, 1 and 2 h at the different concentrations of G-CSF or cisplatin) were treated for 5 min at 37°C with bovine thrombin (10 U/ml). Incubation was stopped by cooling the samples in an ice-bath. Samples of thrombin-activated platelets were transferred to an equal volume of 20% (v/v) cold trichloroacetic acid in 0.6 M HCl and centrifuged at 12 000 *g* for 15 min. One volume of clear supernatant was mixed with 0.2 volume of 0.12 M thiobarbituric acid in 0.26 M Tris at pH 7.0 and immersed in a boiling water bath for 15 min. Absorbance at 532 nm was measured and results were expressed as nmol of MDA formed due to thrombin action.[19]

Platelet protein was determined by the modified Lowry method.[20] Statistical analysis was performed by Student's *t*-test for paired data.

Results and discussion

Pre-treatment of pig platelets (suspended in buffered saline or present in blood) with G-CSF alone led to significant changes in the enzymatic, thrombin-stimulated peroxidation of platelet endogenous arachidonate as assessed by measuring MDA formed during this process. The action of G-CSF on platelet arachidonate transformation was time and dose dependent (Figures 1 and 2). We observed a significant increase of MDA formation in thrombin-treated platelets ($p < 0.01$) when whole blood was preincubated with the highest concentration of G-CSF (8 μg/ml). The increase was time dependent (Figure 2) ($p < 0.01$).

We showed that after a short pre-exposure (0.5 h) of platelets to G-CSF at concentrations of 0.08 and 0.8 μg/ml, thrombin appeared to cause a decreased production of MDA in comparison with the control platelets ($p < 0.05$), while long lasting (2 h) preincubation of blood platelets with Neupogen (0.08–8 μg/ml) had a stimulatory effect on thrombin activation of platelets and enhanced the production of MDA ($p < 0.05$) (Figures 1 and 2).

Our studies demonstrate that cisplatin alone (20 μM; 0.5 h) had a strong inhibitory effect (45% of inhibition) on the production of MDA in platelets ($p < 0.001$) (Figure 3). After exposure of platelets to cisplatin (20 μM; 0.5 h) and G-CSF (0.8 μg/ml; 0.5 h), the inhibitory action of cisplatin was decreased ($p < 0.05$). The presence of G-CSF in the incubation medium reduced the inhibitory effect of cisplatin on the platelet arachidonate pathway (only 10% of inhibition) (Figure 3). A protective effect of G-CSF against the cisplatin action on the production of thromboxane A_2 (TXA_2) MDA in platelets was also observed when G-CSF to was added whole blood (Figure 4).

Platelets are thought to be the major source of TXA_2 biosynthesis under physiological and some

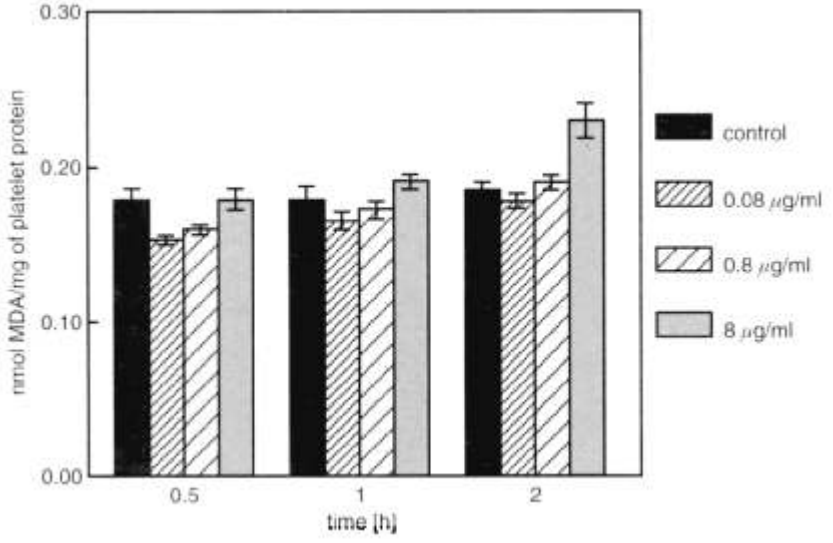

Figure 1. Thrombin-induced (10 U/ml; 5 min; 37°C) production of MDA in control platelets and platelets preincubated with G-CSF (0.08, 0.8 and 8 μg/ml; 0.5 h; 37°C).

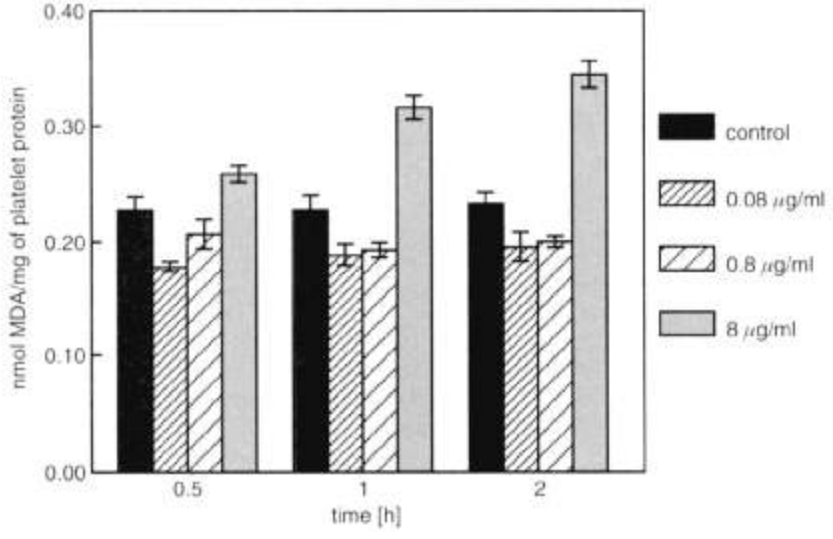

Figure 2. Thrombin-induced (10 U/ml; 5 min; 37°C) production of MDA in control platelets and platelets treated with G-CSF (0.08; 0.8 and 8 μg/ml; 0.5 h; 37°C) in whole blood.

pathophysiological conditions. TXA_2 is a labile product formed from arachidonic acid in activated platelets during cyclo-oxygenase and thromboxane synthase catalyzed reactions, and it appears to contribute to normal hemostasis. It is well known that in the arachidonate cascade MDA and TXA_2 are formed in approximately equimolar amounts.[21,22] A commonly used test with thiobarbituric acid was applied for the detection of MDA.[19] Our earlier studies indicate that cisplatin affects blood platelet function[7,8] and there is evidence for a mechanism involving arachidonic acid metabolism.[9] Cisplatin alone reduced the TXA_2/MDA formation in blood platelets (45% of inhibition after 30 min action of cisplatin on platelets). It seems that cisplatin affects the enzyme(s) participating in the first step of the arachidonate pathway, i.e. the release of free arachidonate acid from membrane phospholipids via phospholipases.[9] The liberation of arachidonate from the *sn-2* position of cellular phospholipids is an important step in the formation of TXA_2, and this enzyme may be a key contributor to cellular arachidonic acid mobilization and eicozanoid formation.[22]

Our preliminary results indicate that G-CSF alone at a concentration of 8 μg/ml enhanced TXA_2/MDA formation in thrombin-stimulated platelets (Figures 1 and 2). In combination with cisplatin it reduced the inhibitory action of cisplatin on platelets (Figure 3). G-CSF seems to have a protective effects against the toxic influence of cisplatin on platelets. The inhibitory effects of G-CSF on platelets were confirmed in whole blood (Figure 4).

G-CSF is a glycoprotein that influences the proliferation and differentiation of a neutrophilic granulocytic precursor. It not only increases granulocyte production in bone marrow but can stimulate activation of mature granulocytes.[23] The action of this cytokine on blood platelet is now unknown. Our preliminary results presented in this paper indicate that *in vitro* G-CSF affected the arachidonate cascade in platelets stimulated by thrombin, although the exact biochemical mechanism underlying this phenomenon remains to be determined. It seems that metabolism of arachidonate plays an important role in the interaction of G-CSF with platelets. Whether the observed increased of MDA/TXA_2 formation in thrombin-induced platelets, pretreated with the highest dose of G-CSF, is consequent to direct effects of this cytokine on platelets remains to be elucidated. The response of blood platelets to G-CSF action in whole blood (8 μg/ml;

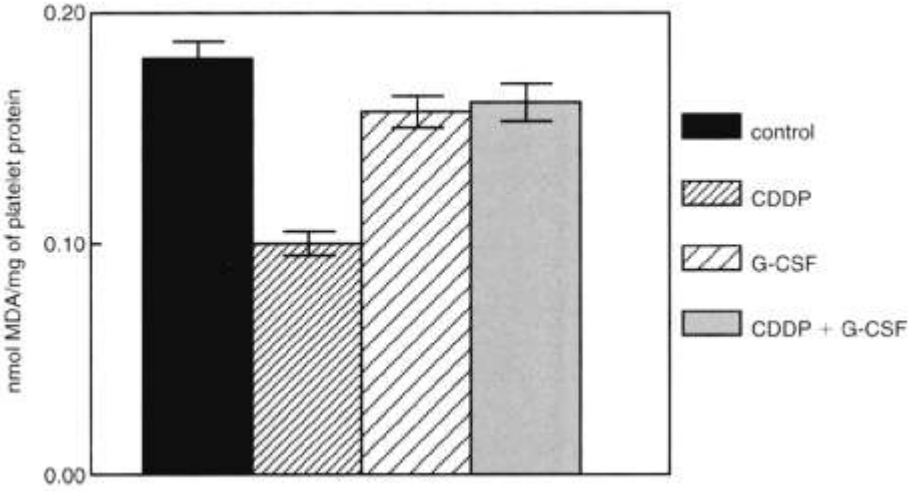

Figure 3. Thrombin-induced (10 U/ml; 5 min; 37°C) production of MDA in control platelets and in platelets preincubated with G-CSF (0.8 μg/ml; 0.5 h; 37°C), cisplatin (20 μM; 0.5 h; 37°C) and G-CSF together with cisplatin.

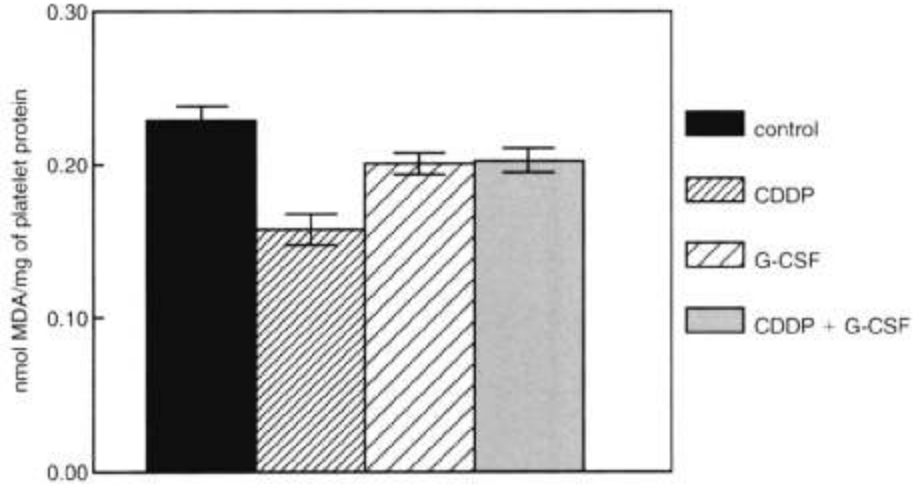

Figure 4. Thrombin-induced (10 U/ml; 5 min; 37°C) production of MDA in control platelets isolated from pig blood and from blood preincubated with G-CSF (0.8 μg/ml; 0.5 h; 37°C), with cisplatin (20 μM; 0.5 h; 37°C) and with cisplatin together with G-CSF.

2 h) (Figure 2) indicates that this process is more complex. There are important differences between platelet behavior in whole blood and in a buffer system. G-CSF is one of the cytokines involved in a complex network of interactions.[24,25] It can activate mature granulocytes; degranulation of PMNs (polymorphonuclear leucocytes, neutrophils) in turn results in the activation of surrounding platelets associated with an increase of arachidonic acid metabolism that is largely mediated by the released compounds and generates superoxide anions.[24–26] The response of platelets to G-CSF in whole blood is due to PMN-derived substances, mainly to the combinated effects of catepsin G and elastase, two serine proteinases released from activated PMN, the essential mediators of platelet activation.[27,28] In stimulated PMN the production of reactive oxygen species also takes place.[24,29] Superoxide anions ($O_2^{\cdot-}$) generated by activated PMN are platelet proaggregatory agents and may directly activate blood platelets. It has been shown that in whole blood PMN-derived oxygen-reactive species stimulate platelets[30] and a low level of hydrogen peroxide (dismutation product of $O_2^{\cdot-}$) enhances platelet activation by cyclo-oxygenase stimulation.[31] A synergy between the action of proteinases and oxygen metabolites on platelets may also exist. Thrombin-activated platelets express P-selectin on their surface and bind neutrophils via this receptor.[2] Platelet and PMN interactions have been extensively studied.[24,25] The responses of PMN to various stimuli, including granulocyte macrophage colony stimulating factor are enhanced in the presence of platelets or products released from platelets (PF4).[25] It seems highly probable that G-CSF added to blood modulates the reactivity of platelets mainly via interaction with neutrophils.

In view of current interest in G-CSF as a therapeutic agent by chemotherapy, the side-effects of this cytokine, including its influence on platelets, deserve attention and the exact biochemical mechanism underlying this phenomenon remains to be elucidated.

References

1. Blockmans D, Deckmyn H, Vermylen J. Platelet activation. *Blood Rev* 1995; **9**: 143–56.
2. Kroll MH, Schafer AJ. The analysis of ligand–receptor interactions in platelet activation. *Immunopharmacol Platelets* 1995; 31–65.
3. Goad KE, Gralnick HR. Coagulation disorders in cancer. *Haematol Complicat Cancer* 1996; **10**: 457–64.
4. Poggi A, Rossi C, Beviglia L, *et al.* Platelet-tumor cell interactions *Immunopharmacol Platelets* 1995; 151–65.
5. Treskes M, van der Vijgh WJF. WR2721 as a modulator of cisplatin and carboplatin-induced side effects in comparison with other chemoprotective agents: a molecular approach. *Cancer Chemother Pharmacol* 1993; **33**: 93–106.
6. Dabholkar M, Reed E. Cisplatin. *Cancer Chemother Biol Res Mod Ann* 1996; **7**: 88–100.
7. Wachowicz B, Olas B. Changes of platelet cytoskeleton induced by cisplatin in the relation to platelet activation. *Biomed Lett* 1995; **52**: 181–90.
8. Olas B, Wachowicz B. Cisplatin-induced changes of biological activity of blood platelets; thiol-related mechanisms. *Anti-Cancer Drugs* 1996; **7**: 476–82.
9. Wachowicz B. Inhibitory effect of cisplatin on the enzymatic peroxidation of platelet endogenous arachidonate. *Cytobios* 1993; **75**: 157–62.
10. Vose JM, Armitage JO. Clinical applications of hematopoietic growth factors. *J Clin Oncol* 1995; **13**: 1023–35.
11. Sundman-Engberg B, Tidefelt U, Paul C. Effect of cytokine on the toxicity of cytostatic drugs on leukemic cells *in vitro* and *in vivo*. *Eur J Haematol* 1996; **56**: 1–6.
12. Kreuser ED, Wadler S, Thiel E. Interactions between cytokines and cytotoxic drugs: putative molecular mechanisms in experimental hematology and oncology. *Semin Oncol* 1992; **19**: 1–7.
13. Tepler I, Elias L, Smith JW, *et al.* A randomized placebo-controlled trial of recombinant human interleukin-11 in cancer patients with severe thrombocytopenia due to chemotherapy. *Blood* 1996; **87**: 3607–54.
14. Brugger W, Birken R, Bertz H, *et al.* Peripheral blood progenitor cells mobilized by chemotherapy plus granulocyte-colony stimulating factor accelerate both neutrophil and platelet recovery after high-dose VP16, ifosfamide and cisplatin. *Br J Haematol* 1993; **84**: 402–7.
15. Shepherd FA, Goss PE, Rusthoven J, *et al.* Phase I trial of granulocyte-macrophage colony-stimulating factor with high-dose cisplatin and etoposide for treatment of small-cell lung cancer. *J Natl Cancer Inst* 1992; **84**: 59–70.
16. Farese AM, Hunt P, Grab LB, *et al.* Combined administration of recombinant human megakaryocyte growth and development factor and granulocyte colony-stimulating factor enhances multilineage hematopoietic reconstitution in nonhuman primates after radiation-induced marrow aplasia. *J Clin Invest* 1996; **97**: 2145–51.
17. Kohno A, Takeyama K, Narabayashi M, *et al.* Low-dose granulocyte colony-stimulating factor enables the efficient collection of peripheral blood stem cells after disease-oriented, conventional-dose chemotherapy for breast cancer, malignant lymphoma and germ cell tumor. *Bone Marrow Transplant* 1995; **15**: 49–4.
18. Tanaka R, Matsudaira T, Aizawa J, *et al.* Characterization of peripheral blood progenitor cells (PBPC) mobilized by filgrastin (rHuG-CSF) in normal volunteers: dose–effect relationship for filgrastim with the character of mobilized PBPC. *Br J Haematol* 1996; **92**: 795–803.

19. Panse M, Block HU, Forster T, *et al.* An improved malonyl dialdehyde assay for estimation of thromboxane synthase activity in washed human blood platelets. *Prostaglondins* 1985; **30**: 1031–8.
20. Vatassary GT, Smith WE. Determination of α-tocopherolquinone (vitamin E quinone) in human serum, platelets, and red cell membrane samples. *Anal Biochem* 1987; **167**: 411–7.
21. Hamberg M, Samuelsson B. Prostaglandin endoperoxides. Novel transformations of arachidonic acid in human platelets. *Proc Natl Acad Sci USA* 1974; **71**: 3400–4.
22. Practico D, FitzGerald GA. Testosterone and thromboxane of muscles, mice, and men. *Circulation* 1995; **91**: 2694–8.
23. Rapaport AP, Abboud CN, Di Persio JF. Granulocyte macrophage colony-stimulating factor (G-CSF), receptor biology, signal transduction and neutrophil activation. *Blood Rev* 1992; **6**: 43–57.
24. Renesto P, Tahar MS, Chignard M. Modulation by superoxide anions of neutrophil-mediated platelet activation. *Biochem Pharmacol* 1994; **47**: 1401–14.
25. Aziz K, Cawley AJC, Zuzel M. Platelets prime PMN via released PF4: mechanism of priming and synergy with GM-CSF. *Br J Haematol* 1995; **91**: 846–53.
26. Qudinet JP, Srear M, Bens M, *et al.* Influence of polymorphonuclear leukocytes on the metabolism of arachidonate in human platelets. *Thromb Haemost* 1988; **60**: 59–62.
27. LaRosa C, Rohrer MJ, Benoit SE, *et al.* Human neutrophil cathepsin G is a potent platelet activator. *J Vasc Surg* 1994; **19**: 309–19.
28. Ruf A, Patscheke H. Platelet-induced neutrophil activation: platelet-expressed fibrinogen induces the oxidative burst in neutrophils by an interaction with CD11C/CD18. *Br J Haematol* 1995; **90**: 791–6.
29. Selak MA. Cathepsin G activates platelets in presence of plasma and stimulates phosphatidic acid formation and lysosomal enzyme release. *Platelets* 1984; **4**: 85–99.
30. Practico D, Iuliano L, Ghiselli A, *et al.* Polymorphonuclear leukocyte-derived O_2-reactive species activate primed platelets in human whole blood. *Am J Physiol* 1993; **264**: 1582–97.
31. Hecker G, Utz J, Kupferschmidt RJ, *et al.* Low levels of hydrogen peroxide enhance platelet aggregation by cyclooxygenase activation. *Eicosanoids* 1991; **4**: 107–13.

(Received 31 October 1996; accepted 12 November 1996)