Platelet releasing activity in sera of patients with rheumatoid vasculitis

T J CUNNINGHAM, R L MEDCALF, J D MATHEWS, AND K D MUIRDEN

University of Melbourne, Department of Medicine, Rheumatology Unit, The Royal Melbourne Hospital, Victoria 3050, Australia

SUMMARY Platelet releasing activity was found in sera fractionated by 30% ammonium sulphate in seven of 11 patients with systemic rheumatoid vasculitis, two of 16 patients with cutaneous rheumatoid vasculitis, and three of 18 patients with clinically uncomplicated rheumatoid arthritis. Serial studies in three patients suggest a key role of platelet activation by serum factors in the pathogenesis of systemic rheumatoid vasculitis. IgG rheumatoid factor or IgG containing immune complexes, or both, may be responsible for this platelet releasing activity.

Key words: rheumatoid arthritis, platelets, platelet activation.

Platelet activation has been observed in many vascular disorders probably secondary to pre-existing vessel damage. In the vascular damage such as occurs in rheumatoid vasculitis (RV) it is possible that platelet activation may also occur as the initiating or primary event. The liberation of permeability factors could then result in enhanced vascular permeability favouring the deposition of circulating immune complexes which may provoke subsequent inflammatory events. The classic experiments of Kniker and Cochrane¹ showed the key role of increased vascular permeability in experimental immune complex mediated vascular damage. Platelets are the major intravascular source of the vasoactive amine serotonin and also contain a cationic protein which induces increased vascular permeability.² There is evidence of intravascular platelet release in rheumatoid disease.³ Furthermore, studies in vitro have shown the ability of IgG rheumatoid factor (IgG RF), IgG aggregates or complexes to activate platelets.⁴ Serum IgG RF levels correlate temporarily with the evolution of RV.⁵ The present study therefore investigated the hypothesis that levels of such serum factors capable of activating platelets correlate with the manifestation and evolution of RV.

Materials and methods

PATIENTS AND CONTROL GROUPS

Patients were selected from the outpatient clinics or

wards of The Royal Melbourne, Prince Henry's, Alfred, Repatriation General, and St Vincent's hospitals, Melbourne. All had classical or definite rheumatoid arthritis (RA) by American Rheumatism Association criteria. They were divided into two groups – rheumatoid arthritis (RA) and rheumatoid vasculitis (RV) – on the criterion of past or present clinical manifestations of vasculitis (except nodules). The RV category was further subdivided into: (a) mild RV (cutaneous vasculitis only) – MRV, (b) severe RV (visceral, neural, or extensive tissue involvement) – SRV, and (c) RV in remission (past documented vasculitis but none present at the time of examination) – RVR.

The control group consisted of healthy volunteers with no clinical evidence of disease.

SERA

10–20 ml of venous blood was drawn from each subject, placed in 10 ml sterile plain tubes, and allowed to clot at 37°C for two hours. After centrifugation at 1500 g for 10 minutes serum was isolated, recentrifuged at 1500 g for 10 minutes to remove trace cellular elements, and then stored in 300 µl aliquots at –70°C until used.

BUFFERS

Buffer A. Composition in mmol/l: Na⁺ 137.4, K⁺ 5.4, Mg²⁺ 1.2, SO₄²⁻ 1.2, HCO₃⁻ 15.0, H₂PO₄⁻ 1.2, Cl⁻ 126.6, glucose 11.5.

Bovine serum albumin (BSA) was dialysed against this salt solution and then added to give a

final concentration of 0.35%. The pH was adjusted to approximately 6.0 with 1 M citric acid.

Buffer B. Same as buffer A with the addition of 2.5 mmol/l CaCl_2 . The pH was adjusted to 7.4 by bubbling with 95% O_2 and 5% CO_2 (carbogen). Human fibrinogen (CSL) and dialysed BSA were added to give final concentrations of 0.05% and 0.35% respectively.

Buffer C. Same as buffer B without fibrinogen.

FRACTIONATION OF SERA

335 μl of saturated ammonium sulphate $[(\text{NH}_4)_2\text{SO}_4]$ was added to 500 μl of serum dropwise and with stirring to give a final concentration of 30% $(\text{NH}_4)_2\text{SO}_4$. The procedure was performed at 4°C , and the solution was stirred for a further 20 minutes, then centrifuged at 10 000 g for 20 minutes at 4°C . The supernatant was aspirated and discarded and the protein precipitate redissolved in 500 μl buffer C. This solution was then reprecipitated in 30% $(\text{NH}_4)_2\text{SO}_4$, centrifuged, and the supernatant discarded. The protein was finally resuspended in 250 μl of buffer C, effectively concentrating the fractionated sera by a factor of two. Each sample was then dialysed against BSA-free buffer C at 4°C for 24 hours to remove remaining $(\text{NH}_4)_2\text{SO}_4$. Dialysed serum fractions were then split into 60 μl volumes and stored at -70°C until use. Monomeric and heat aggregated IgG (see below (10 g/l)) were subjected to the same process and used as negative and positive controls respectively.

PREPARATION OF MONOMERIC IgG AND HEAT AGGREGATED IgG

Human Cohn fraction II was dissolved in buffer C (initially without BSA) to a concentration of 60 g/l and centrifuged at 37 000 g for 20 minutes at 4°C to remove unwanted aggregates of IgG. The supernatant was removed and assayed for protein concentration (Lowry).⁶ Heat aggregated IgG (HAIgG) was prepared by heating approximately half of this in the stock solution of monomeric IgG at 63°C for 10 minutes, then cooling it on ice. Equal volumes of buffer C containing 0.4% BSA were added to both monomeric and HAIgG to give final stock solutions in 0.2% BSA. HAIgG and monomeric IgG were stored in aliquots at -70°C and used within six weeks of preparation.

PLATELET DONORS

A panel of eight volunteers who had normal clinical examinations and full blood examinations were bled at approximately three to four weekly intervals. None had taken drugs known to affect platelet function in the preceding three weeks. Fractionated serum samples were tested on at least three different

donors' platelets on at least three different occasions.

PREPARATION OF WASHED HUMAN PLATELETS

Suspensions of washed human platelets were prepared as described by Medcalf *et al.*⁷ 50 ml of blood was drawn from the antecubital vein through a 19 gauge needle into acid citrate dextrose (ACD) in a polypropylene syringe. The final ACD/blood ratio was 1/6. After a sample had been taken for platelet counting the mixture was transferred to polypropylene tubes and centrifuged at 125 g for 20 minutes at room temperature. The platelet rich plasma (PRP) was removed by a polypropylene syringe and the pH adjusted to 6.0 with 1 M citric acid. PRP was then centrifuged at 1100 g at room temperature for 20 minutes, the supernatant discarded, and the platelet pellet resuspended in 7–10 ml of buffer A. The resuspended platelets were recentrifuged at 1000 g for 7.5 minutes at room temperature, the supernatant aspirated, and the platelet pellet resuspended and centrifuged as before. After aspiration of the supernatant the platelets were finally resuspended in buffer B to a concentration of 350 000/ mm^3 ($350 \times 10^9/\text{l}$). Apyrase (potato grade III, Sigma Chemical Company, Missouri) initially dissolved in saline to a concentration of 3.4 g/l was added to the platelet suspension to give a final concentration of 34 mg/l. During the experiments the platelet suspension was kept in 10 ml polypropylene syringes (allowing no air exposure) at 37°C .

[^3H]SEROTONIN RELEASE ASSAY

Platelet suspensions at concentrations of 350 000/ mm^3 ($350 \times 10^9/\text{l}$) were incubated with [^3H]serotonin (5-hydroxy[G- ^3H]tryptamine creatine sulphate aqueous solution containing 500 mCi/mmol (Amersham, UK) for at least one hour, to give a final concentration of 5 $\mu\text{Ci/ml}$ (5 mCi/l). 450 μl of platelet suspension was initially reacted with 20 μl of heparin (David Bull Laboratories) to give a final concentration of 20 U/ml. The inclusion of heparin was necessary as it had been previously shown in initial studies that trace amounts of thrombin coprecipitated with the IgG fraction. Added heparin had negligible effect on the platelet activating ability of both monomeric IgG and HAIgG (data not shown). The platelet suspensions were then allowed to react with 50 μl of reagent (HAIgG, monomeric IgG, or fractionated serum) in small polypropylene tubes. Each tube was placed horizontally in a 37°C reciprocating water bath (Paton Industries, Australia) and shaken at 220 oscillations/minute for 10 minutes. The reactions were stopped by the addition

of 50 μ l of paraformaldehyde (final concentration 1%). The complete volume of each reaction was layered on top of 1 ml of percoll solution (adjusted to 1.0426 g/ml (1042.6 g/l) at 20°C with buffer C) in plastic P4 tubes. The tubes were centrifuged at 1500 g for 15 minutes at 4°C to remove platelets, aggregates, and debris. 200 μ l of the supernatant was added to 6 ml of scintillant, mixed, and counted in a beta counter (Packard liquid scintillation spectrometer model 3380). 200 μ l of unactivated and uncentrifuged platelets diluted with equivalent volumes of reagents to match test solutions was similarly treated to determine the total radioactivity present in the platelet preparation. The percentage serotonin release was calculated as:

$$\frac{(\text{activity (cpm) in test sample} - \text{background activity}) \times 100}{\text{total activity} - \text{background activity}}$$

Platelet releasing activity (PRA) was considered to be significant if test samples induced a release of greater than 13% of [3 H]serotonin on the basis of studies on control sera and the lack of associated platelet aggregation below this level. In a study by Medcalf *et al.*,⁷ the release of approximately 13% of endogenous serotonin from washed human platelets was necessary to contract a human smooth muscle preparation in vitro. This level of serotonin release could be achieved by 20 mg/l of heat aggregated IgG.

IMMUNE COMPLEXES AND IgG RF

The C1q binding assay and IgG RF were assayed as previously described.^{8,9}

Results

Variations in sensitivity of platelet preparations to aggregating agents were noted between donors and in the same donor bled on different occasions. In general, female donors showed greater sensitivity than male, in agreement with the findings of Moore *et al.*¹⁰ Fractionated test sera and control solutions were therefore allowed to react with the same panel of donors, and the mean release of [3 H]serotonin from at least three reactions was calculated (Fig. 1). To determine whether the fractionation process itself was creating non-specific platelet releasing activity (i.e. IgG clumping), 1 g/l monomeric IgG and various control sera were similarly fractionated but failed to cause significant platelet serotonin release.

In seven of 11 fractionated sera from patients with active severe RV (SRV) and in two of 16 patients with cutaneous rheumatoid vasculitis (MRV) significant platelet releasing activity (PRA) was shown

($p < 0.05$, Mann-Whitney U test). All of six patients with past severe RV currently in remission (RVR) and nine of 10 control subjects (C) showed negligible serotonin releasing activity. Three of 18 patients with RA lacking clinical signs of RV had significant PRA in their fractionated sera (Fig. 2). In three cases of RV tested serially fractionated serum from the active disease phase caused significant serotonin release, in contrast with the effect of fractionated serum taken after treatment and clinical remission (Fig. 3). In the RA and RV categories PRA did not correlate significantly with platelet count, IgG RF (Fig. 4), Rose-Waaler titre, erythrocyte sedimentation rate, or immune complex levels by the C1q binding assay (r values = -0.09, 0.18, 0.01, -0.18, 0.09 respectively).

Fig. 1 Heat aggregated IgG concentration dependent platelet serotonin release.

Fig. 2 Percentage platelet serotonin release v diagnosis.

Fig. 3 Serial studies of releasing activity and C1q binding in SRV. (ng/ml= μ g/l).

Fig. 4 Percentage platelet serotonin release v IgG RF ($r=0.18$ NS). (μ g/ml= μ g/l).

Discussion

This study indicates that fractionated sera of some RA and some RV patients have the ability to cause the release of significant concentrations of serotonin from normal platelets and permits speculation of similar events in vivo. The fractionation of serum with 30% ammonium sulphate does not remove IgM RF, which Wager *et al.*¹¹ found to be an inhibitor of immune complex induced platelet aggregation in vitro. IgM RF was still present in fractionated serum as shown by the persistence of positive latex and Rose-Waaler agglutination tests. Thus it could be suggested that the inhibitory effect of IgM RF may be overcome in vivo if sufficient concentrations of platelet releasing substance(s) are present. Although some fractionated sera induced platelet activation, this was not seen in the corresponding

unfractionated serum samples, indicating the improbability of antiplatelet antibodies having any significant role in RV. The fractionation process is mainly operative in reducing the concentration of monomeric IgG, the major inhibitory component of normal human serum to platelet activation by immune complexes.¹² The work of Clark *et al.*¹³ indicated that platelet activation by immune complexes may still occur in the presence of plasma. Platelet aggregation and release occurred exclusively during the formation of precipitating immune complexes at antigen-antibody equivalence. The inhibition offered by C1q to platelet activation by IgG containing complexes,¹⁴ if significant at all, may be reduced during complement consumption, which also occurs in RV.

It is suggested that platelet release by serum factors is a localised event in view of the local

clinical manifestations of vasculitis and the lack of hypertension which would result from the vasoconstrictive effect of generalised platelet serotonin release. The inhibition of platelet release may alter with changes in microcirculatory blood flow and the varying molecular environment of platelets.

This study found no correlation between PRA of fractionated RA or RV sera and platelet count. Platelet release without aggregation may occur,¹⁵ and platelets that have undergone the release reaction may circulate with a near normal life span.¹⁶ It thus appears that activation and release need not necessarily be coupled to sequestration of platelets. Furthermore, a mild degree of platelet loss may be compensated for by increased production. An increase in platelet turnover has been recorded in rheumatoid disease.¹⁷

The temporal relationship of PRA to active clinical vasculitis in three patients tested serially and the lack of PRA in RV in remission suggest a possible pathogenic role. The presence of PRA in a few patients without clinical vasculitis may relate to a higher prevalence of subclinical vasculitis as shown by the histological studies of Kulka,¹⁸ who found an incidence of vasculitis in skeletal muscle tissue in 20% of RA autopsies. It also appears that the subsequent pathogenic processes leading to clinical vasculitis (probably starting with complement activation) need not necessarily follow platelet release and immune complex deposition. Skin biopsy studies have in fact shown a relatively high incidence of positive immunofluorescence in histologically and clinically normal skin.¹⁹

The nature of PRA in RV sera remains unknown. However, it seems likely that it is due to IgG RF- or IgG-containing immune complexes. The lack of correlation between IgG RF, C1q, and PRA does not negate this. The IgG RF assay used in this study, like most other techniques, probably does not give a true indication of levels of aggregated or complexed IgG RF. The C1q assay was performed on serum, whereas the PRA was performed on serum fractions. It is also possible that the PRA is related to non-complement binding immune complexes. Valone *et al.*¹⁵ identified the PRA in synovial fluid as IgG complexes. In RA the majority of material measured as immune complexes is composed of IgG and rheumatoid factors of several immunoglobulin classes.²⁰ Furthermore, clinical correlative studies such as those by Scott *et al.*,⁵ Theofilopoulos,²¹ and Allen *et al.*²² show that high levels of IgG RF are strongly associated with clinical RV, serum anticomplementary activity, and depressed C4 levels. As immune complexes containing only IgG RF are reported to be incapable of activating complement,²³ it may be that their putative action on

platelets enables the deposition of other complement fixing complexes. Alternatively, mixed IgG RF and IgM RF complexes, which activate complement, may also cause platelet release. Such complexes have been isolated from RA sera,²⁴ but their ability to activate platelets has not been tested.

The PRA detected in a proportion of systemic lupus erythematosus (SLE) sera is probably due to antiplatelet antibodies.²⁵ A recent study suggests that specific antiplatelet antibodies exist in RA – 24% of decomplexed RA sera causing significant platelet serotonin release.²⁶ The present study found no significant serotonin release by unfractionated RA sera: the disparity does not appear totally explicable by methodological differences between the studies. Norberg²⁷ found indirect evidence of platelet antibodies in only two of 36 RA sera. Further work is required to characterise PRA in RA.

Comparative platelet sensitivity to activating agents between RA patients (treated and untreated) and controls remains unknown except for one study reporting that most RA patients had an enhanced platelet sensitivity to collagen and epinephrine.²⁸ Obviously, comparative sensitivity to IgG aggregates/immune complexes would be relevant.

This study indicates that there may be a role for antiplatelet agents in vasculitis prone patients. Aspirin in high dosage, as is often administered to RA patients, may detrimentally alter the balance of prostacyclin/thromboxane A₂ production to favour platelet aggregation. Sulphinpyrazone may be beneficial in inhibiting primary or secondary involvement of platelets in the vasculitic process. Agents reducing the permeability of the endothelial barrier such as hydroxyethylrutinosides²⁹ may modify the initial events leading to vasculitis.

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