The sensitivity of tests to detect *in vivo* platelet activation induced by the removal of arterial endothelium of the baboon (*Papio ursinus*)

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Summary

The relative sensitivities for the various *in vivo* and *in vitro* tests for platelet activation are unknown. This was studied in a baboon model where limited and more substantial injury to the vascular endothelium was inflicted. The endothelium of a segment of the right carotid artery was removed with a balloon catheter on day 0 (limited de-endothelialisation), and that of the left carotid artery, abdominal aorta and left femoral artery on day 7 (substantial de-endothelialisation). Eight baboons (*Papio ursinus*) were used. Baseline tests for platelet activation (platelet volume, platelet density, platelet aggregate ratio, and platelet and plasma levels of platelet factor 4 [PF4] and β-thromboglobulin [β-TG]) were performed 7 days before de-endothelialisation and repeated on days 1, 9 and 16. The kinetics of indium-111-labelled platelets were measured after substantial de-endothelialisation. Sham operations were done on 3 animals exactly as in the test, except that the balloon injuries were not inflicted. No influence on the results of the platelet function tests was found. The only test capable of detecting limited injury to the endothelium was the measurement of plasma PF4. The mean platelet life-span (MPLS) shortened, mean platelet density decreased, the circulating platelet aggregate ratio decreased, and plasma levels of PF4 and β-TG increased ($P < 0,05$ in all instances) after the substantial endothelial injury. The mean platelet volume, intraplatelet PF4 and β-TG, and the *in vivo* distribution and sites of sequestration of labelled platelets were poor tests for *in vivo* platelet activation. Nine days after the substantial de-endothelialisation, the only remaining evidence of platelet activation was an increase in the number of circulating platelet aggregates. It is concluded that the most sensitive tests for *in vivo* platelet activation are the measurement of the circulating platelet aggregate ratio (sensitivity 0,63) and plasma β-TG (sensitivity 0,63), and the estimate of the MPLS (sensitivity 0,50).

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In clinical medicine platelet function tests are helpful for the diagnosis of platelet abnormalities as a possible cause of a bleeding diathesis, but they may also be used to determine whether the platelets have been activated *in vivo*. Provided that due attention is paid to the influence of various technical factors,[1] it is usually relatively simple to diagnose qualitative platelet defects.

The detection of *in vivo* platelet activation is, however, more difficult. The usual *in vitro* tests of platelet function are not of much value in the evaluation of thrombo-embolism.[2] Attention has therefore recently focused on the direct measurement of the *in vivo* processes of platelet activation and consumption. The measurement of the mean platelet life-span (MPLS) is considered the most sensitive of these tests.[3] However, to our knowledge, the relative sensitivities of the MPLS and the other tests for *in vivo* platelet activation have not been compared.

A study was undertaken to compare the relative sensitivities of a selection of readily available *in vivo* and *in vitro* tests of platelet function for the detection of limited and more extensive injury to the vascular endothelium. The effects of concomitant arteriosclerosis on platelet function tests were excluded by using baboons with no evidence of arterial disease for the experiments.

Materials and methods

Eight healthy, juvenile baboons (*Papio ursinus*), weighing 8 - 12 kg (mean 10,4 ± 1,7 kg), were used in a project approved by the Ethical Committee of the Provincial Administration and the University of the Orange Free State. They did not receive antiplatelet drugs before or during the study. Handling was accomplished through anaesthesia with intramuscular ketamine hydrochloride 10 mg/kg body mass.

Reference values for the MPLS, the *in vivo* distribution and the sites of sequestration of indium-111-labelled platelets had earlier been determined in our laboratory on a group of 8 baboons with similar physical characteristics.[4,5] We employed the same standardised methods, reagents and internal and external quality control procedures in this study.

Experimental protocol

Tests for *in vivo* platelet activation were done on blood samples taken 7 days before vascular endothelium was removed (baseline values). The endothelium was then removed on two separate occasions with a balloon catheter. On day 0 only the endothelium of the right carotid artery was removed. Seven days later (day 7), a more substantial area involving the left carotid artery, the abdominal aorta and the femoral artery was denuded. We tested for platelet activation 1 day after de-endothelialisation of the right carotid artery (day 1), and 2 (day 9) and 9 (day 16) days after the more substantial de-endothelialisation. The MPLS, the *in vivo* distribution of platelets at equilibrium, and the sites of sequestration of platelets at the end of their life-span were determined after the substantial de-endothelialisation (starting on day 7).

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De-endothelialisation of arteries with a balloon catheter

Baboons were sedated with ketamine hydrochloride, intubated and anaesthetised with a mixture of halothane, O_2 and N_2O. The carotid artery or femoral artery was exposed to ensure the introduction of a balloon catheter with minimal injury to the vessel and the surrounding tissue. The catheter was advanced into the artery through a small cut-down. The balloon was then inflated with air and gently retracted. This procedure was repeated within 30 seconds. The arterial wall was sutured with 6-0 Prolene, and the skin wound closed with a silk suture. The arteries, as observed at autopsy on day 16, remained patent in all cases.

The catheter passed easily into the vessel lumen. After inflation with air, the vessel was visibly distended and there was palpable resistance when the catheter was retracted. The efficacy of the procedure to remove the endothelium was assessed by scanning electron microscopy in preliminary studies done on 8 baboons. In the final study, removal of endothelium was confirmed at the end of the investigation.

A 3 cm length of endothelium of the carotid artery was removed with a No. 6 balloon catheter inflated with 1,5 ml air. The endothelium of the abdominal aorta and femoral artery, extending from the site of entry in the femoral artery to the diaphragm, was removed with a similar catheter inflated with 2 ml of air.

Sham experiments without endothelial injury

Three baboons, similar in size to the experimental animals, were subjected to exactly the same procedures (anaesthetic, exposure of blood vessels) at the same time intervals but without the balloon injuries. The tests for *in vivo* platelet activation were done at the same time intervals as in the test protocol. The *in vivo* distribution of the labelled platelets and their final sites of sequestration were, however, not quantitated. The platelet concentrations of platelet factor 4 (PF4) and β-thromboglobulin (β-TG) were also not determined.

Platelet labelling and measurement of platelet kinetics

This was performed in 6 of the baboons. Blood was collected on day 7, within 30 minutes of de-endothelialisation. Autologous platelets were isolated[4,6] and labelled with indium-111-tropolone.[7] A platelet population, representing 94 ± 16% of that in the whole blood sample, was isolated with differential centrifugation and repeated washing of the red blood cell layer. Labelling efficiency was 78 ± 12%. A total of 6,8 ± 1,7 × 10^9 platelets, labelled with 20 ± 3 MBq ^{111}In, were re-injected within 4 hours of venesection. The labelled platelets were considered viable in view of their adequate *in vitro* aggregation response to adenosine diphosphate (ADP)[8] and normal recovery in the circulation.[4]

The rate of disappearance of labelled platelets from the circulation was determined by measuring changes in the radioactivity in blood samples collected 15 minutes after re-injection of the labelled platelets, and then twice daily for 6 days. The MPLS was estimated by fitting a γ function[9] to the data describing the removal of labelled platelets from day 1 to day 6. Recovery of labelled platelets in the circulation at equilibrium was calculated by back extrapolation of the platelet survival curve to zero time.[4] The data of the test, sham and reference platelet survival curves were analysed in identical fashion.

Anterior and posterior images of the whole body were acquired daily for 7 days with a large-field-of-view scintillation camera and an A² MDS data processing system. The *in vivo* distribution of the labelled platelets was quantified by the geometric mean method, and the distribution at equilibrium and the sites of sequestration of platelets at the end of MPLS estimated as described.[5,6] Images (128 × 128 word mode) of the abdomen were taken for 30 minutes to detect the deposition of labelled platelets onto the de-endothelialised segment of abdominal aorta.

Tests for *in vivo* platelet activation

The mean volume of the platelets was measured according to the method of Corash *et al.*[10] with an electronic particle counter interfaced with a multichannel analyser and logarithmic amplifier (Coulter Electronics, Hialiah, Fla, USA).

The density distribution of the platelets was determined on a non-linear Percoll (Pharmacia AB, Uppsala, Sweden) density gradient[11] calibrated with density marker beads. Percoll was made isosmotic with a buffered saline-glucose-citrate solution[10] by adding 9 parts of Percoll to 1 part of a 10 × concentrated buffer-solution. Uniform mixtures of the isosmotic Percoll (4,5 ml) and buffer (5,5 ml) were poured into polycarbonate tubes. The density gradient was formed by centrifuging the tubes at 10 000 *g* for 30 minutes in a fixed angle rotor. Platelet-rich plasma (PRP), containing a fully representative platelet population (96 ± 6% of those present in the blood), was prepared by repeated washing of platelets from the red cell layer.[4] PRP 2 ml was gently layered onto the gradient. The platelets were separated by centrifuging at 400 *g* for 30 minutes in a swing-out rotor. The gradients were fractionated by carefully removing 1 ml aliquots from the top of the gradient.

Platelets in each aliquot were counted with the electronic particle counter, and the density of each aliquot determined by light refraction. The concentration of platelets in an aliquot was expressed as a fraction of the total number of platelets layered onto the gradient. A cumulative frequency of the platelets in the aliquots (representing the different densities) was calculated. The mean density of the platelet population was estimated at the 50th percentile

Circulating platelet aggregates, expressed as an aggregate ratio, were determined according to Wu and Hoak.[12] Blood was collected into either formalin, which fixes aggregates, or ethylenediaminetetra-acetic acid (EDTA), which disperses them.

PF4 and β-TG. Blood (5 ml) was collected in precooled Thrombotect tubes (Abbott Laboratories, Chicago, Ill., USA).[13] The tubes were centrifuged at 2 500 *g* for 20 minutes, the platelet-free plasma aspirated and frozen at −20°C until assayed with commercial kits (PF4 — Abbott Laboratories; β-TG — Radiochemical Centre, Amersham, UK). To measure intraplatelet PF4 and β-TG, platelets in PRP were lysed with Triton X-100, 1% final concentration. Cell debris was removed by centrifuging at 4 000 *g* for 30 minutes and PF4 and β-TG of the supernatant assayed. There is no evidence that these proteins show species-related antigenic differences.

Statistical analysis. Paired data were compared by Student's *t*-test if the distribution of data was normal or with the two-tailed Wilcoxon signed-rank test. The possible relationship between the MPLS and the results of the other tests for *in vivo* platelet activation was assessed with linear correlation analyses. Mean values are given with 1 SD.

The sensitivity of each test was assessed:[14]

$$\text{sensitivity} = \frac{\text{No. of positive tests*}}{\text{No. of baboons studied}}$$

*A test was regarded as positive if the test result was outside 2 standard deviations of the mean baseline value.

Results

Sham experiments

The results of the sham experiments are given in Table I. Anaesthesia and the surgical procedures had no significant effect on the *in vivo* and *in vitro* platelet function tests.

Baseline values

The peripheral blood platelet counts and the results of the tests for *in vivo* platelet activation are given in Table II.

TABLE I. EFFECT OF SHAM EXPERIMENTS, WITH NO BALLOON INJURY TO THE VASCULAR ENDOTHELIUM, ON TESTS OF PLATELET FUNCTION

Platelet function tests	Baseline	Anaesthesia and exposure	
		Carotid artery	Femoral artery
Platelet			
Count ($\times 10^9$/l)	**346 ± 49**	**359 ± 46**	**382 ± 59**
Volume (fl)	**7,8 ± 0,3**	**8,1 ± 0,3**	**7,2 ± 0,8**
Density (g/ml)	**1,0446 ± 0,0014**	**1,0468 ± 0,0027**	**1,0470 ± 0,0009**
Dense bodies	**6,6 ± 1,0**	**6,6 ± 0,5**	**6,5 ± 0,5**
Mean life-span (h)	**135 ± 19**	—	**145 ± 11**
Plasma			

TABLE II. RESULTS OF THE PLATELET FUNCTION TESTS AT BASELINE, AFTER LIMITED (DAY 1) AND SUBSTANTIAL (DAY 9) DE-ENDOTHELIALISATION OF THE ARTERY WALL, AND AFTER HEALING (DAY 16)

Platelet function test	Baboon 1	2	3	4	5	6	7	8	Mean ± 1 SD
Baseline									
Platelet count ($\times 10^9$/l)	**446**	**525**	**447**	**561**	**644**	**425**	**452**	**530**	**504 ± 75**
Platelet volume (fl)	**4,64**	**5,40**	**5,25**	**4,08**	**4,25**	**5,37**	**5,74**	**5,49**	**5,03 ± 0,62**
Platelet density (g/ml)	**1,0489**	**1,0496**	**1,0476**	**1,0487**	**1,0470**	**1,0472**	**1,0472**	**1,0487**	**1,0481 ± 0,001**
Platelet PF4 (μg/10^8)	**2,55**	**3,82**	**0,57**	**4,16**	**3,99**	**1,75**	**2,60**	**1,87**	**2,60 ± 1,26**
Platelet β-TG (μg/10^8)	**2,18**	**3,54**	**2,89**	**2,53**	**6,82**	**1,07**	**2,14**	**1,63**	**2,85 ± 1,77**
Platelet aggregate ratio	**0,88**	**0,73**	**1,00**	**0,99**	**1,00**	**0,97**	**0,88**	**0,65**	**0,89 ± 0,13**
Plasma PF4 (ng/ml)	**5**	**7**	**15**	**10**	**21**	**12**	**29**	**26**	**16 ± 9**
Plasma β-TG (ng/ml)	**13**	**10**	**28**	**10**	**13**	**14**	**23**	**35**	**18 ± 9**
β-TG : PF4 ratio	**2,6**	**1,4**	**1,9**	**1,0**	**0,6**	**1,2**	**0,8**	**1,4**	**1,4 ± 0,6**
Limited de-endothelialisation									
Platelet count ($\times 10^9$/l)	**517**	**451**	**618**	**565**	**684**	**295**	—	**510**	**523 ± 75**
Platelet volume (fl)	**4,23**	**4,83**	**5,79**	**4,68**	**4,29**	**5,26**	—	**4,60**	**4,81 ± 0,55**
Platelet density (g/ml)	**1,0479**	**1,0486**	**1,0494**	**1,0447**	**1,0470**	**1,0472**	—	**1,0486**	**1,0479 ± 0,001**
Platelet PF4 (μg/10^8)	**2,85**	**2,40**	**4,09**	**1,52**	**2,30**	**3,12**	—	**7,24**	**3,36 ± 1,89**
Platelet β-TG (μg/10^8)	**2,74**	**1,65**	**4,77**	**6,06**	**2,34**	**3,47**	—	**6,32**	**3,91 ± 1,84**
Platelet aggregate ratio	**0,87**	**0,57**	**1,00**	**0,62**	**0,58**	**0,57**	—	**1,00**	**0,75 ± 0,22**
Plasma PF4 (ng/ml)	**18**	**33**	**18**	**74**	**65**	**13**	—	**41**	**37 ± 24***
Plasma β-TG (ng/ml)	**24**	**25**	**17**	**74**	**56**	**15**	—	**32**	**35 ± 22**
β-TG : PF4 ratio	**1,3**	**0,8**	**0,9**	**1,0**	**0,9**	**1,2**	—	**0,8**	**1,0 ± 0,2**
Substantial de-endothelialisation									
Platelet count ($\times 10^9$/l)	**495**	**459**	**508**	**634**	**619**	**419**	**346**	**571**	**506 ± 99**
Platelet volume (fl)	**5,03**	**4,40**	**4,85**	**4,25**	**5,79**	**4,90**	**4,25**	**4,80**	**4,79 ± 0,51**
Platelet density (g/ml)	**1,0466**	**1,0467**	**1,0472**	**1,0452**	**1,0438**	**1,0465**	**1,0465**	**1,0460**	**1,0462 ± 0,001***
Platelet PF4 (μg/10^8)	**4,94**	**4,53**	**4,51**	**4,69**	**1,74**	**3,03**	**3,25**	**2,58**	**3,66 ± 1,17**
Platelet β-TG (μg/10^8)	**4,36**	**3,85**	**4,54**	**6,59**	**2,62**	**2,34**	**2,89**	**1,97**	**3,65 ± 1,52**
Platelet aggregate ratio	**0,76**	**0,82**	**0,74**	**0,30**	**0,24**	**0,58**	**0,55**	**0,33**	**0,54 ± 0,23***
Plasma PF4 (ng/ml)	**58**	**21**	**28**	**32**	**35**	**8**	**46**	**25**	**32 ± 15***
Plasma β-TG (ng/ml)	**56**	**19**	**42**	**46**	**22**	**16**	**65**	**42**	**39 ± 18***
β-TG : PF4 ratio	**1,0**	**0,9**	**1,5**	**1,4**	**0,6**	**2,0**	**1,4**	**1,7**	**1,3 ± 0,5**
Effect of healing									
Platelet count ($\times 10^9$/l)	**585**	**535**	**650**	—	**719**	**634**	**764**	**504**	**622 ± 88***
Platelet volume (fl)	**5,54**	**4,64**	**4,29**	—	**4,25**	**4,81**	**4,81**	**4,44**	**4,68 ± 0,44**
Platelet density (g/ml)	**1,0490**	**1,0476**	**1,0475**	—	**1,0455**	**1,0472**	**1,0463**	**1,0462**	**1,0467 ± 0,001**
Platelet PF4 (μg/10^8)	**3,83**	**2,81**	**2,00**	—	**1,66**	**3,43**	**3,10**	**3,53**	**2,88 ± 0,79**
Platelet β-TG (μg/10^8)	**3,48**	**3,94**	**4,36**	—	**1,48**	**2,70**	**2,32**	**5,36**	**3,38 ± 1,32**
Platelet aggregate ratio	**0,58**	**0,64**	**0,584**	—	**0,52**	**0,55**	**0,80**	**0,73**	**0,63 ± 0,10***
Plasma PF4 (ng/ml)	**8**	**76**	**19**	—	**10**	**11**	**54**	**44**	**32 ± 27**
Plasma β-TG (ng/ml)	**4**	**42**	**41**	—	**15**	**23**	**52**	**27**	**29 ± 27**
β-TG : PF4 ratio	**0,5**	**0,5**	**2,2**	—	**1,5**	**2,1**	**1,0**	**0,6**	**1,2 ± 0,7**

*$P < 0,05$ versus baseline; Wilcoxon signed-rank test.

Changes induced by limited de-endothelialisation

The results are summarised in Table II. The mean blood platelet count did not change significantly from that at baseline ($P > 0,05$). Minimal endothelial damage increased the plasma concentration of PF4 significantly ($P < 0,05$). Evidence of *in vivo* platelet activation could not be detected by the other tests.

Changes induced by substantial de-endothelialisation

Tests for *in vivo* platelet activation. The mean platelet count, the mean platelet volume, the mean intraplatelet PF4 and β-TG and the plasma β-TG:PF4 ratio remained unchanged. Changes in the mean platelet density, the mean circulating platelet aggregate ratio, and the mean plasma concentrations of PF4 and β-TG did, however, provide evidence that the platelets were activated *in vivo* (Table II).

Platelet kinetics. The mean disappearance curves of the labelled platelets are given in Fig. 1. The results on platelet kinetics are summarised in Table III. The MPLS was significantly shorter than normal, and the mean platelet turnover increased significantly.

The *in vivo* distribution of the labelled platelets at equilibrium and their sites of sequestration at the end of platelet life-span, did not differ significantly from normal. Deposition of labelled platelets onto the abdominal aorta could not be detected by scintigraphy. Quantification of ^{111}In-deposition in this region was therefore not attempted.

Effect of healing of the injured vessel

The blood platelet count on day 16 was significantly higher than baseline ($P < 0,05$). Except for the increased numbers of circulating platelet aggregates, none of the other tests reflected residual platelet activation (Table II).

Sensitivity of the platelet function tests

The results are given in Table IV. The circulating platelet aggregate ratio, the plasma β-TG and MPLS were the most sensitive of these tests.

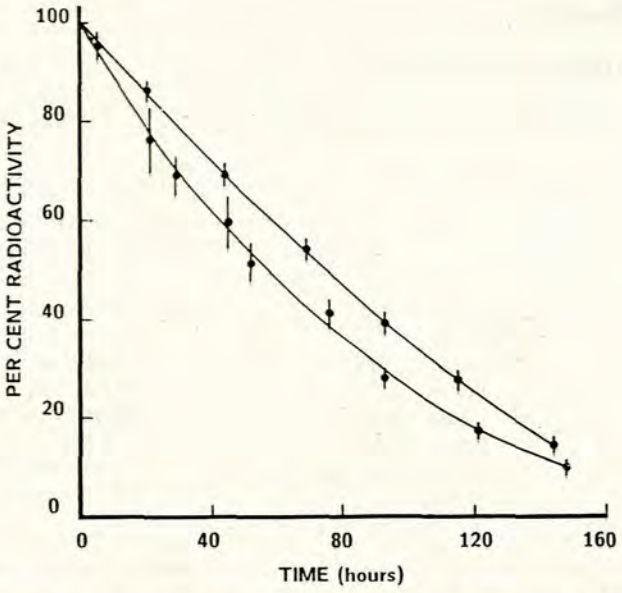

Fig. 1. The mean disappearance of ^{111}In-labelled platelets from the circulation. The top curve represents normal baboons. The bottom curve is the disappearance of labelled platelets after the more substantial injury. The values represent the mean % radioactivity ± 1 SEM of the radioactivity/ml injected, i.e. the recovery.

TABLE IV. SENSITIVITY OF THE PLATELET FUNCTION TESTS IN RESPONSE TO INJURY OF THE ARTERIAL ENDOTHELIUM

	Sensitivity of tests after		
Platelet function test	Limited injury	Substantial injury	7d healing
Platelet MPLS	—	**0,50**	—
Platelet volume	**0,00**	**0,00**	**0,00**
Platelet density	**0,17**	**0,38**	**0,17**
Platelet dense bodies	**0,00**	**0,13**	**0,14**
Platelet PF4	**0,14**	**0,00**	**0,00**
Platelet β-TG	**0,00**	**0,13**	**0,00**
Platelet aggregate ratio	**0,57**	**0,63**	**0,57**
Plasma PF4	**0,43**	**0,38**	**0,43**
Plasma β-TG	**0,29**	**0,63**	**0,43**

TABLE III. EFFECT OF DE-ENDOTHELIALISATION OF THE LEFT CAROTID ARTERY AND THE ABDOMINAL AORTA ON THE KINETICS OF ^{111}IN-LABELLED PLATELETS

	Platelet			Distribution of labelled platelets*			
	Recovery		Turnover	At equilibrium		At end of MPLS	
Baboon	(%)	MPLS (h)	(× 10^9/l/h)	Spleen	Liver	Spleen	Liver
1	**67**	**132**	**3,4**	**16,5**	**17,1**	**22,6**	**33,2**
2	**94**	**113**	**4,3**	**16,4**	**16,2**	**21,6**	**19,6**
3	**90**	**87**	**5,1**	**13,5**	**17,0**	**19,6**	**36,9**
6	**99**	**111**	**3,1**	**17,4**	**19,1**	**22,4**	**26,8**
7	**71**	**148**	**2,8**	**19,2**	**15,6**	**23,6**	**17,9**
8	**71**	**130**	**3,3**	**18,4**	**19,9**	**22,0**	**25,9**
Mean ± SD	**82 ± 14**	**120 ± 21**	**3,7 ± 0,8**	**16,9 ± 2,0**	**17,5 ± 1,7**	**21,9 ± 1,4**	**39,0 ± 3,2**
Reference	**85 ± 9**	**146 ± 13**	**2,8 ± 0,9**	**16,0 ± 1,9**	**15,8 ± 2,9**	**23,3 ± 4,6**	**37,6 ± 6,0**
***P* value†**	**0,56**	**0,02**	**0,03**	**0,30**	**0,20**	**0,46**	**0,59**

*Organ radioactivity expressed as % of *in vivo* whole-body radioactivity.
† Student's *t*-test for unpaired data.

Linear correlation analyses

The results of the linear correlation analyses between MPLS and the other tests for *in vivo* platelet activation induced by the substantial injury to the vessel wall, were: platelet volume $r = -0{,}3768$; platelet density $r = -0{,}6922$; platelet PF4 $r = 0{,}3451$; platelet β-TG $r = -0{,}4217$; platelet aggregate ratio $r = -0{,}4267$; plasma PF4 $r = 0{,}5542$; plasma β-TG $r = 0{,}5842$; and β-TG:PF4 ratio $r = 0{,}1589$. None of these correlation coefficients were statistically significant ($P > 0{,}05$ in all instances).

Autopsy results in baboons

None of the animals had any macroscopic or microscopic evidence of atherosclerosis or systemic disease. As expected, the de-endothelialisation had induced hyperplasia of the intima of the arteries evident at the time of sacrifice.[15,16]

Discussion

These experiments were performed to determine the relative sensitivities of *in vivo* and *in vitro* tests of platelet function in response to *in vivo* platelet activation. The latter was accomplished by exposing circulating platelets to a site or sites of injured vascular endothelium. A limited and a more substantial lesion was used to determine the sensitivity of the various tests. Healthy juvenile baboons, with no evidence of degenerative arterial disease, were considered a prerequisite for the experiment to exclude the added effects of concomitant arteriosclerosis on the platelet function tests.

In animals the removal of the vascular endothelium with a balloon catheter is followed by rapid accumulation of platelets on the exposed sub-endothelium. Once the surface is covered by a layer of platelets, few platelets continue to interact with the surface.[15,16] This may explain why de-endothelialisation of the aorta does not shorten the MPLS in rabbits.[15] This reaction may be species-dependent, because in man and non-human primates there is evidence that platelets are activated *in vivo* if the endothelium is damaged by homocysteine,[17] cigarette smoking[18] or hypercholesterolaemia.[19,20]

De-endothelialisation of the artery of a healthy young primate was, in the light of the above, considered an appropriate model. The baboons had no evidence of arterial disease at autopsy, and the results of the baseline platelet function tests were also normal. The sham experiments demonstrated that the anaesthesia, surgical exposure of the arteries and the stress of handling had no effect on platelet function (Table I). It is therefore reasonable to ascribe the evidence of platelet activation that was observed to the interaction of the platelets with the damaged arterial wall. We assessed the influence of limited and more substantial endothelial damage on platelets and determined the relative sensitivities of a group of tests to diagnose *in vivo* platelet activation.

The baseline values of all the measurements were as expected and the animals showed no evidence of *in vivo* platelet activation. It should be noted that in this study the β-TG:PF4 ratio, contrary to the finding in another study,[21] did not indicate platelet activation. In these animals the baseline β-TG:PF4 ratio approximated unity, whereas in humans the ratio is approximately 6,3.[13] We have no ready explanation for this discrepancy. It is possible that the difference in the half-lives of these platelet-specific proteins in man and non-human primates may play a role. In man the half-life of β-TG is 100 minutes[22] and in non-human primates 8,1 minutes;[23] that of PF4 is unmeasurably brief in man[22] and in Rhesus monkeys there is a slow component with a half-life of 92 minutes.[23] Our finding can also probably not be ascribed to the use of an assay for human β-TG and PF4 in our experiments with baboons; there is no species-related antigenic differences between β-TG and PF4 of humans and Rhesus monkeys.[23]

The only test result that increased significantly after de-endothelialisation of a segment of one carotid artery was the mean plasma PF4 level. This increase was slight and individual results varied widely. It is therefore unlikely that the measurement of this platelet-specific protein will prove clinically useful for the diagnosis of *in vivo* platelet activation. Although the platelet aggregate ratio, indicative of circulating platelet aggregates, decreased in 5 of the 7 baboons, this change was not of statistical significance. The other tests for platelet activation clearly could not detect the effect of such a limited degree of endothelial injury on platelets. It should be noted that we did not measure the sensitivity of the MPLS because it was technically not feasible to perform this test on the same animal within 2 weeks.

More of the tests were able to detect de-endothelialisation of a substantial segment of the vascular system: the MPLS shortened, there was a decrease in the circulating platelet aggregate ratio, mean platelet density changed and plasma PF4 and β-TG levels increased.

Although this was seen in only 3 of the 6 animals, the MPLS shortened moderately, but significantly. Baboon 3 had a short platelet survival time of 87 hours. Removing the data from this animal does not influence the statistical comparison with the control group and therefore does not affect the conclusions. Mean platelet turnover also increased, compensating for the shortened platelet life-span, thus maintaining a normal blood platelet count. The decrease in the MPLS could not be linked to the visualisation of the deposition of platelets on the injured areas. If there was only slight deposition, the sensitivity of imaging will clearly be hampered by the relatively high concentration of radioactivity associated with platelets in the blood pool compared with those reacting with the lesion of the vessel wall.

It should be noted that this study was not performed to assess the effect of endothelial injury on the MPLS. It is well known that platelets will adhere to injured endothelium. We argue that it will be of more clinical value to know what the effect of an established, but not old, injury to the endothelium will be on the platelet life-span. Also, platelet kinetic measurements assume a steady state, therefore it is difficult to interpret the curve reflecting platelet life-span if labelled platelets are re-injected before the endothelial damage.

The interaction of platelets with the sub-endothelium had no influence on the sequestration pattern of the labelled platelets in the reticulo-endothelial system. This finding was not unexpected, since in patients with aortic aneurysms[24] or with arterial prostheses[25] labelled platelets are not permanently retained in the thrombogenic areas but are sequestered in the spleen, liver and bone marrow. The quantification of the organ distribution of labelled platelets at the end of their life-span is clearly not a useful test of *in vivo* platelet activation.

The most sensitive test for *in vivo* platelet activation was the determination of the circulating platelet aggregate ratio; this was low in 7 of the 8 baboons. One should, however, recognise that the test of Wu and Hoak[12] for platelet aggregates may reflect the propensity for platelets to aggregate *in vitro*.[26] The result of this test should therefore be interpreted to reflect hyperreactive platelets rather than a chronic state of micro-embolisation.

Plasma β-TG increased in all of the baboons; the mean increase was significant and the sensitivity of the test was high. However, the extent of the increase of the β-TG levels was slight and varied widely in individual animals. The finding that the plasma PF4 levels increased twofold is of some interest. It has been stated that the plasma concentration of PF4 should be low in absence of renal disease[21] because the

half-life of PF4 in the circulation is very short.[23] According to this view, elevated PF4 in the plasma reflects *in vitro* platelet activation during blood sampling and manipulation.[21,23] This may, however, not always be so; for instance, plasma PF4-levels may be elevated in patients with vascular disease.[13] The results of our study strengthen the view that elevated PF4 levels may reflect *in vivo* platelet activation. The sensitivity of the measurement of PF4 for this purpose was, however, low.

The density of platelets is considered to be dependent on their α-granule content[27] and their densities decrease after the release reaction.[28] Such a test for an acquired storage pool deficit may therefore be a sensitive indicator of *in vivo* platelet activation. The validity of this approach was confirmed in this study in which it was found that the mean platelet density does decrease after *in vivo* platelet activation. This result is consistent with some, but not all, published reports. Some of these discrepancies may be related to species-differences. It is generally agreed that in the rabbit increased platelet consumption may be reflected by an increase in numbers of platelets of decreased density.[29] In humans, results are conflicting and it is not clear whether active vascular disease is associated with a decrease in mean platelet density[29] or not.[30] Clearly, further studies are needed to resolve this question.

We could not determine the cause of the decrease in platelet density induced by platelets reacting with a more substantial area of de-endothelialisation. Intraplatelet PF4 and β-TG were not altered by platelet activation. The concomitant increase in *plasma* PF4 and β-TG therefore suggests that these platelet-specific proteins are released mainly, or only, from those platelets that are rapidly removed from the circulation. Furthermore, our results do not support the view that the density of platelets are determined only by the β-TG and PF4 content of the α-granules.[27] A plausible explanation of the small decrease in platelet density may be that, because of the increased platelet turnover and shortened MPLS, the number of young platelets in the circulation increases.[31]

After the endothelium was allowed to heal for 16 days, almost no evidence of continuing *in vivo* platelet activation remained. Again, measurement of the circulating platelet aggregate ratio proved to be the most sensitive, and indeed the only, indicator of residual *in vivo* platelet activation.

It is evident from the results of this study that there are important discrepancies between the results of the various tests for *in vivo* platelet activation. It is clear that there is no ideal test to demonstrate such platelet activation. If the MPLS is accepted as the most specific and the most sensitive procedure,[3] it is noteworthy that there is no relationship between the result of this 'gold standard' and those of other tests. The limitations of these tests as indicators of *in vivo* platelet activation become even more apparent if results in individual animals are noted. For instance, after substantial de-endothelialisation β-TG remained normal in 3 baboons and the platelet aggregate ratio only decreased in 4; there was no consistent pattern from animal to animal.

We acknowledge that these data cannot readily be extrapolated to those pertaining in humans. However, injury to the endothelium is basic to diseases affecting the vascular system and, as such, central to the process of *in vivo* platelet activation. It therefore seems reasonable to expect that results similar to those observed in this study are likely to be encountered when the process of *in vivo* platelet activation is measured in humans. It should therefore be noted that the results of this study indicate that the measurement of the circulating platelet aggregate ratio, the assay of plasma β-TG levels and the determination of the MPLS are the most sensitive indicators of *in vivo* platelet activation. However, because of notable discrepancies between the results in tests it would be prudent to use these tests in combination, and to interpret results with caution.

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