Flow-Induced Platelet Activation in Mechanical Heart Valves

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Background and aim of the study: **A study was conducted to measure in vitro the procoagulant properties of platelets induced by flow through mechanical heart valves.**
Methods: **The procoagulant activity of platelets was measured using a real-time assay of platelet activation state (PAS), which was based on a modification of the prothrombinase method. Acetylated prothrombin was used instead of normal prothrombin in this assay in order to eliminate the positive feedback effect of thrombin. This enabled a direct comparison between thrombin generation rates in the assay and the flow stresses that induce platelet activation. Gel-filtered platelets (10^5 per μl) were circulated through a left ventricular assist device with two Björk-Shiley monoleaflet mechanical heart valves mounted in opposition, and platelet activation state was measured over 30-min time courses. The results were compared with two configurations in which the leaflet motion of one of the valves was restricted (severely restricted and mildly restricted), mimicking defective function of a compromised valve in vivo, and with a control lacking valves.**
Results: **The severely restricted valve activated the platelets at a rate eight-fold higher than with unrestricted valves, and three-fold higher than with mildly restricted valves. Both restricted valves activated platelets at rates significantly higher than either the control (no valves) or the unrestricted valve.**
Conclusion: **Flow through compromised mechanical heart valves causes platelet activation, which can be measured with a modified prothrombinase assay system. The ability to perform sensitive quantitative measurements in cardiovascular devices in vitro may have a significant impact on the design and development of these devices.**

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The development of implantable vascular devices has provided life-saving solutions to patients with severe cardiovascular diseases. Mechanical heart valves are already routinely used for replacing diseased heart valves. Recently, it was reported in the REMATCH study (1) that left ventricular assist devices (LVADs) are superior to drug therapy, paving the way for their ultimate use as a long-term heart replacement therapy for patients not eligible for heart transplant. However, cardiovascular devices share a common problem, causing a significant risk of complications such as hemolysis, platelet destruction and thromboembolism (2,3). Thromboembolism and the attendant risk for cardioembolic stroke remains a significant impediment to the long-term use of these devices. The mandatory life-long anticoagulant drug regimen they require, which induces vulnerability to hemorrhage and is not a viable therapy for some patients, does not eliminate this risk (4).

One of the major culprits in the genesis of cardiovascular disease is the formation of non-physiological (pathological) flow patterns that cause a thrombogenic response (5). Elevated flow stresses that are present in the non-physiological geometries of devices enhance their propensity to initiate thromboembolism. Mechanically induced blood trauma has been almost exclusively studied with respect to erythrocyte damage (hemolysis), yet platelets have long been regarded as the pre-eminent cell involved in hemostasis and thrombosis. Indeed, in recent years it has been demonstrated that chronic flow-induced platelet activation and the initiation of thrombus formation are the salient aspects of this blood trauma (6-8).

Cardioembolism is the most serious complication of mechanical heart valves, and may lead to local occlusion of the valve or to the formation of thromboemboli that can cause critical obstruction to blood flow in distant organs. Although thromboembolic complication

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rates have been reduced by developing new valve designs, the incidence of thromboembolic complications (2-4) remains relatively high (1.5 to 3 per 100 patient-years). Valve thrombogenicity is related to both hemodynamic design directed at mitigating the non-physiological flow characteristics and the materials to which the blood is exposed (3). The thrombogenicity of all mechanical heart valves is primarily due to platelet activation (4). In patients with either mechanical or bioprosthetic heart valves, platelets are constantly activated, platelet survival is reduced, thromboxane A_2 is synthesized and released, and the release of β-thromboglobulin and other α-granule proteins is increased (3,4,6). Although the foregoing problems are generally agreed to be intimately related to the flow characteristics of the valves, very little quantitative information is actually available regarding the platelet-activating potential of prosthetic valves.

Current valve designs still generate areas of high shear stress, turbulent fluctuations, and regions of recirculation and stasis, which enhance the risk of thromboembolism by inducing platelet activation, aggregation and deposition. These fluid mechanical factors have been implicated in platelet activation, aggregation, and subsequent deposition (5,9,11). Hellums et al. (10) depicted a locus of shear exposure time versus platelet serotonin release, which is commonly used as a standard for a platelet activation threshold, while the present authors have studied the cumulative effect of varying flow stresses and exposure times along individual platelet paths (12-14). Heart valve leaflets also generate a wake of shed vortices that play a pivotal role in activation and damage to blood constituents. These vortices have been observed experimentally and simulated computationally in the wake of mechanical heart valve leaflets (15-17), and shown to entrap potentially activated platelets (13,14). Conditions are therefore optimal for the generation of cerebrovascular microemboli that carry with them the acute risk associated with mechanical heart valves, namely cardioembolic stroke.

Herein is reported the application of a method which was developed to quantify the platelet activating potential induced by flow stresses, and subsequently tested in platelets subjected to circulation through prosthetic heart valves in a plasma-free milieu. Other primary platelet functions, such as adhesion-dependent activation and platelet-platelet aggregation, were largely prevented because the necessary plasma proteins - particularly von Willebrand factor and fibrinogen - were absent. Additionally, red cells, which are known to play modulatory roles in platelet function, were absent. Thus, the study focused as far as possible specifically on the direct platelet-activating potential of the devices under investigation.

Materials and methods

Platelets

For each daily platelet preparation, four measurements of platelet activation rate were made in an LVAD-based flow loop of approximately 200 ml volume. Each measurement required about 2×10^{10} gel-filtered platelets. Outdated (80 h) plasmapheresis units of platelet-rich plasma (PRP) containing ca. 3×10^{11} platelets were obtained from the Stony Brook University Hospital blood bank or Long Island Blood Services, Melville, NY, USA. Gel filtration of PRP was performed in a column of coarse Biogel A50M 2.7% agarose beads (Bio-Rad, Hercules, CA, USA), which included all proteins of M_r $<5 \times 10^6$ Da. The only likely relevant contaminant of gel-filtered platelets was traces of highly multimeric von Willebrand factor (M_r $>10^7$ Da). (Biogel A50M is no longer manufactured; a close equivalent is Sepharose 2B (2% agarose), available from Sigma Chemical, St. Louis, MO, USA.) PRP (100 ml) was gel filtered in a 55 × 550 mm silanized (Sigmacote; Sigma Chemical) column at a flow rate of 13 ml/min in a platelet buffer consisting of a HEPES-modified Ca^{2+}-free Tyrodes buffer containing 0.1% fatty-acid-free bovine serum albumin (18). The platelets eluted at about 25 min. To minimize the risk of contamination with plasma proteins, only the leading two-thirds of the platelet peak was collected. The pool was counted (Coulter Z1 counter; Beckman Coulter, Hialeah, FL, USA) and adjusted with platelet buffer to a count of 10^5 per µl. Platelets were maintained with gentle rocking at room temperature, and used within 6 h of gel filtration.

Platelet activation state (PAS) assay

Timed samples (ca. 50 µl) were removed from the circulation loop (with no replacement of removed volume), and PAS was measured by a modification of the prothrombinase method of Jesty and Bluestein (19), using exactly 10 µl of platelet sample in each assay. Significant improvements over the original method have recently been reported (20). Essentially, acetylated prothrombin is incubated with factor Xa and a platelet sample in the presence of 5 mM Ca^{2+} for 10 min at 37°C. The amount of acetylated thrombin generated depends on the activation state of the platelet sample. Positive controls (maximally activated) platelet samples are obtained by treatment with Ca^{2+} ionophore A23187, and this provides a reference value for the PAS activity of maximally activated platelets. PAS determinations for the results presented were normalized to this ionophore-generated maximum activity (measured individually at the end of every circulation experiment) and are presented as normalized ratios. The PAS assay has previously been shown to correlate with

annexin V binding measured by flow cytometry of platelets subjected to activation by varying concentrations of ionophore (20). The present method routinely reported base PAS activities of fresh gel-filtered platelets of 0.5-2.5% of the maximum attainable, and of outdated plasmapheresis platelets 1-7% of maximum (20).

Recirculation studies of platelets

Platelet activation measurements were performed in a circulation loop based on an LVAD, which was the implantable part of a pneumatic heart-assist system developed by one of the authors (K.A.). The LVAD chamber (70-mm diameter diecast PVC, containing two valves and a 0.6 mm-thick polyurethane diaphragm with a blood-compatible inner coating) has a 65-ml stroke volume, approximating that of a typical adult left ventricle, and a total volume of about 100 ml. Two opposed mechanical heart valves (23-mm Björk-Shiley monoleaflet) control the direction of circulation, the output and input being connected by a compliance reservoir (Figs. 1 and 2) made of 12.5-cm (5-inch) clinical grade surgical Penrose tubing (Bard Inc., Covington, GA, USA). The diaphragm is driven by an external reciprocating pump (Harvard Apparatus model 1423) capable of producing quasi-physiological flow curves and regulation of both stroke volume and stroke rate (Fig. 2). In order to ensure minimal compliance and accurate definition of the flow characteristics, water, rather than air, is used to drive the LVAD diaphragm. The pump rate used is 4.7 l/min with a stroke rate of 72 per min and a systolic/diastolic ratio of 0.375, representing a normal cardiac output. The lifetime of platelets in the normal circulation is about seven days, and on average a platelet passage through the left ventricle occurs every 90 s, corresponding to ca. 6,700 valve passages during the average platelet lifetime. The time chosen for the recirculation experiments was 30 min, corresponding (for two valve passages per LVAD cycle) to 2,800 passages. Taking into account the 6-h usage limit of the platelet preparation, this duration allowed (on any one day) use of the same platelet batch for duplicate measurements of platelet activation rate with each of two valve configurations. The circulation loop was operated in an incubator at 37 ± 2°C. Timed aliquots were removed from the loop every 3 min, and assayed immediately for PAS activity. Between experimental runs, the LVAD and mounted valves were cleaned by treatment with: (i) a 10% solution of commercial bleach (Clorox); (ii) water; (iii) 10 mm HCl (to ensure that the alkalinity of the bleach was fully neutralized); (iv) water; and (v) platelet buffer. Between days, the valves were dismounted and together with the LVAD were cleaned with 0.5% sodium dodecyl sulfate in addition to the bleach-water-HCl process described above.

A set of control experiments was performed in an LVAD without valves. The platelet suspension was then passed back and forth between the LVAD chamber and the compliance tubing. Circulation experiments, with two valves mounted in the LVAD, were carried out with the outflow valve in three configurations: a free valve; a mildly restricted valve; and a severely restricted valve. In all cases the inflow valve in the LVAD remained unmodified in order to maintain unidirectional circulation. The restriction method limited the leaflet trajectory so that the leaflet motion

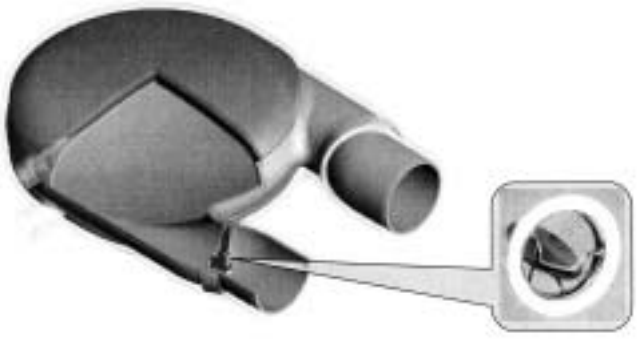

Figure 1: The left ventricular assist device (LVAD), showing the mounting of one of the two 23-mm Björk-Shiley mechanical heart valves and the location of the driven diaphragm. In the LVAD used here, the second valve (not shown) is mounted in the other port in the same geometry, but in the opposing direction.

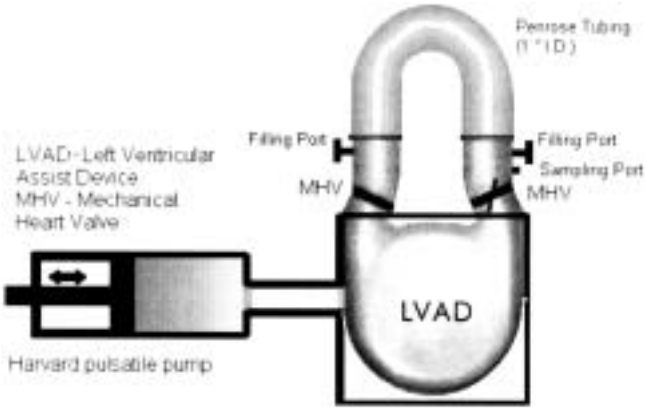

Figure 2: Schematic of the left ventricular assist device (LVAD) circulation loop. On the pump's drive stroke the water compresses the LVAD diaphragm, forcing the platelet suspension through the output valve into the compliance reservoir, the opposed valve remaining closed. On the return pump stroke, the diaphragm is under negative pressure, drawing the platelet suspension from the reservoir through the opposing input valve into the left ventricular assist device body. Each stroke is 65 ml, with a stroke rate of 72 per min.

towards its fully closed or fully open position was impaired. This was achieved by placing a surgical suture around one of the two arms of the inlet support strut (Fig. 3). For the mildly restricted valve, only leaflet closing was impaired, enhancing regurgitant flow conditions in the diastolic phase. For the severely restricted valve, both opening and closing were impaired, generating abnormal flow conditions in the forward and regurgitant flow phases. Both conditions were analogous to conditions that can occur when a thrombus forms on the struts. Geometric characteristics resulting from the corresponding valve configurations are listed in Table I. For each configuration, the reproducibility of leaflet restriction was estimated at ±5°.

Platelet variability; statistical analysis

A number of effects combine to limit the precision of measurement of platelet activation rate. First, individual gel-filtered platelet preparations vary in both their base activation state and their sensitivity to activation, and no standard platelet preparation exists. Second, because reproducible suturing of the valve struts was time-consuming, the duplicate experiments with modified valves were performed in sequential pairs, rather than in random or palindromic order. Third, gel-filtered platelets activate slowly while maintained at room temperature, introducing the complication of a slowly shifting baseline during the course of a series of runs. To reduce interference from these effects, individual (daily) sets of circulation experiments were restricted to two valve conditions, each performed in duplicate. (For the control experiments lacking valves, all data sets were collected together, and not interspersed with valve data.)

Each combined data set was analyzed as follows. PAS data of individual experimental runs were fitted by linear regression to obtain the platelet activation rate (PAR). Since PAS is a normalized, dimensionless value (see PAS assay, above), the units of platelet activation rate are min^{-1}. Mean (± SD) PAR values were then calculated for each valve condition, and these values were used to calculate probabilities for pair comparisons, using Student's *t*-test. The use of linear regression assumes that platelet activation is linear with time or, more accurately, that it cannot be demonstrated to be non-linear. Single time courses cannot be analyzed for linearity because the variance of the single data points is unknown. However, Gaussian analy-

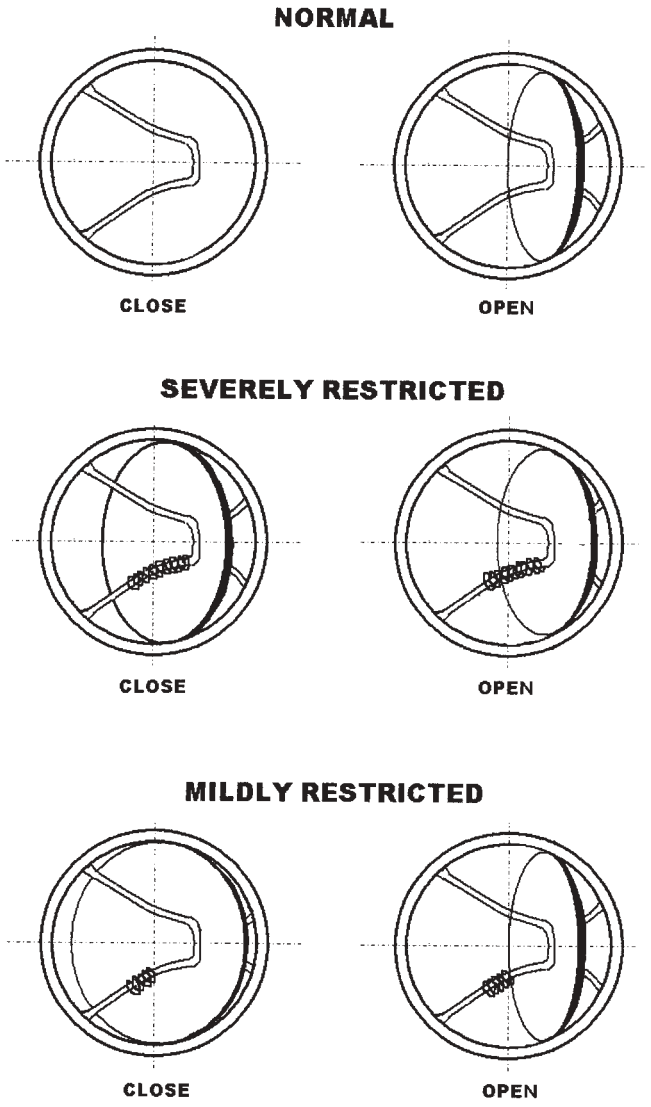

Figure 3: Schematic of normal, severely restricted, and mildly restricted valves, showing the valves in their corresponding open and closed positions. The restriction was achieved by placing a surgical suture around one of the two arms of the inlet support strut.

Table I: Open and closed angles of the mechanical heart valve leaflet in unmodified and modified valves.

Leaflet position	Unmodified		Mild restriction		Severe restriction	
	Angle of leaflet (°)	Open area (%)	Angle of leaflet (°)	Open area (%)	Angle of leaflet (°)	Open area (%)
Open	80	100	80	100	75	93
Closed	5	0	20	20	60	73

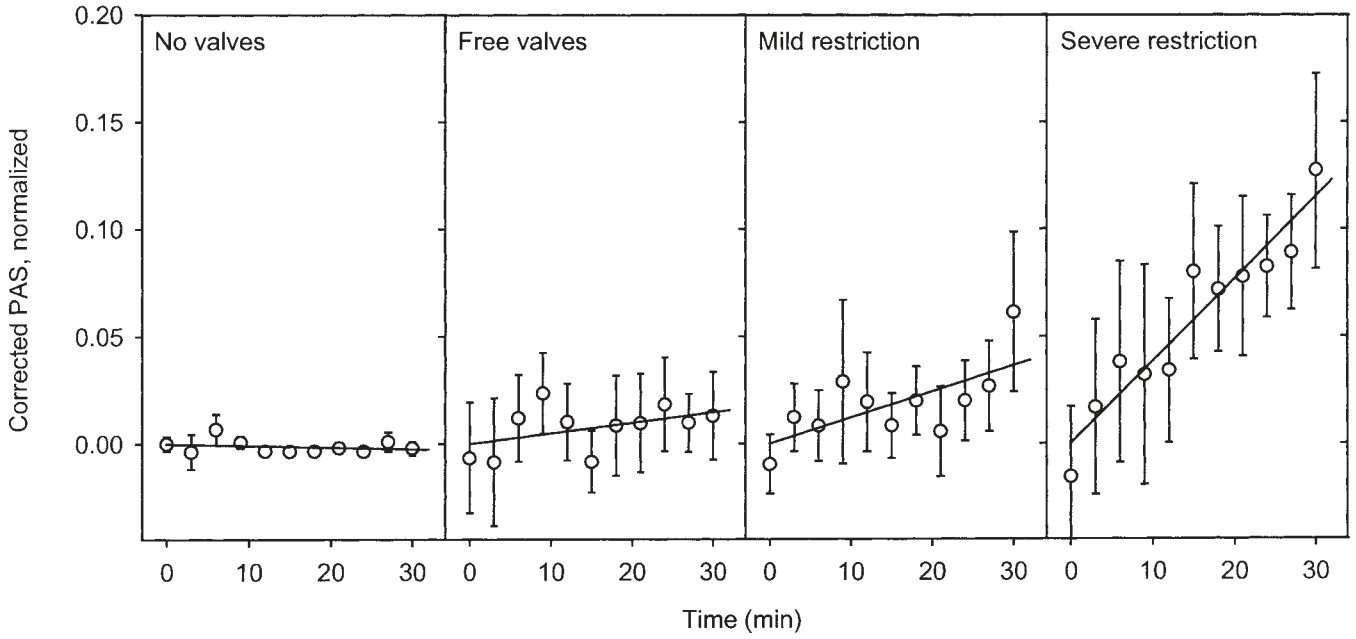

Figure 4: Activation of platelets during circulation in the left ventricular assist device. Platelets (10^5/μl in platelet buffer) were circulated at 37 ± 2°C, and timed samples were removed for the measurement of platelet activation state (PAS). Each panel shows the collected PAS data of all experimental runs (± SEM), normalized to the activity of ionophore-activated suspension (n = 6). The mean zero-time intercept for each set is subtracted to enable visual comparison across panels. Individual runs in each experimental set were fitted by linear regression to obtain individual rates of platelet activation. The lines shown are the mean slopes of the collected experimental runs.

sis of variance of the linear regression of the combined data sets for each condition, as recommended and described by Colquhoun (21), provides no evidence for non-linearity in the generation of platelet activity for any condition ($p > 0.1$).

Results

Figure 4 shows platelet activation during: (i) non-circulating passage back and forth in a LVAD lacking valves (see Materials and methods); (ii) circulation through two unmodified valves; (iii) circulation through an unmodified and a mildly restricted valve; and (iv) circulation through an unmodified and a severely restricted valve. As described in Materials and methods, the data from each individual circulation run (n = 6) were normalized to the maximum activity attainable, determined by activation with Ca^{2+} ionophore A23187, and measured on the platelet suspension at the end of every run. It is emphasized that the large error bars on each data point were not caused by ill-determined rates of individual experimental runs, and were not used in the statistical analysis. The variability was chiefly the result of: (i) wide variation in the initial activation state among individual platelet preparations; and (ii) lesser variation in the rates of platelet activation under flow conditions. The method of analysis, using the rates alone, is detailed in Materials and methods.

The mean rates of platelet activation are shown in Figure 5, the data providing the following statistical results. The mean (± SEM) rate of platelet activation in the control series with no valves was $-0.08 \pm 0.12 \times 10^{-3}$ min^{-1}, a rate that was not significantly different from zero ($p > 0.2$). This result showed that flow and contact with the LVAD alone did not cause measurable platelet activation over the 30-min time course studied. On introducing circulation through two normal, unrestricted valves, the mean rate of platelet activation was increased to $0.49 \pm 0.37 \times 10^{-3}$ min^{-1}, but the rate of activation was still not - at least in this small series - significantly different from zero ($p > 0.2$).

Upon introduction of a restricted valve into the LVAD circuit, significant platelet activation occurred. In the case of a valve with a mild leaflet restriction (Fig. 3; Table I), with the other valve remaining unrestricted, the platelet activation rate rose to $1.22 \pm 0.40 \times 10^{-3}$ min^{-1}, which was significantly higher than either the control series or the unrestricted series ($0.02 < p < 0.05$). The degree of restriction of this valve was fairly minor, preventing full opening, but not affecting the closing of the leaflet.

When a more severe restriction was introduced, preventing both full opening and closing, the effect on

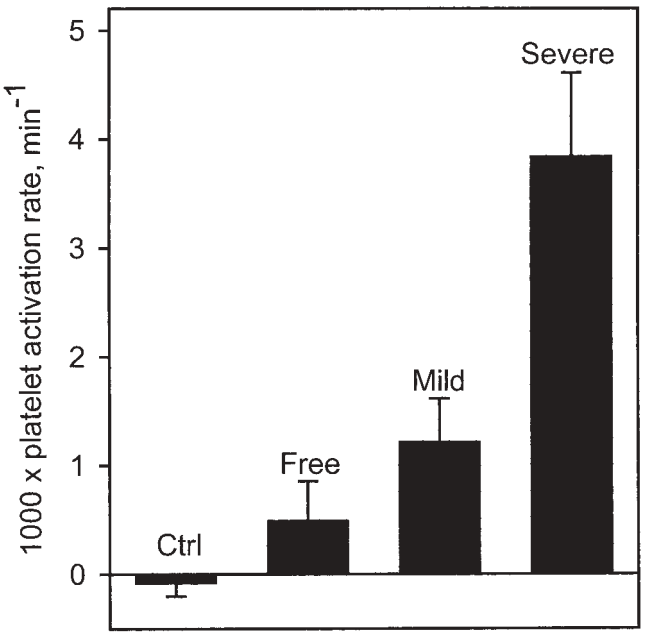

Figure 5: Mean (± SEM) rates of platelet activation in each left ventricular assist device configuration, derived from the data of Figure 4. These data were analyzed using Student's t-test to calculate the probabilities given in the Results section.

platelets was much greater. Under these conditions, with one severely restricted valve in the LVAD loop, the rate of platelet activation, at $3.83 \pm 0.77 \times 10^{-3}$ min^{-1}, was three-fold higher than with a mild restriction ($0.01 < p < 0.02$), and almost eight-fold higher than with normal unrestricted valves ($p < 0.005$).

Discussion

Studies of the behavior of gel-filtered platelets in large recirculation loops are decidedly problematic in several respects. One problem relates to the unfeasibility of using freshly drawn platelets because of the large volumes required on a daily basis. A second problem is that gel-filtered platelets have a maximum working life of about 5-6 h, and even over this period their activation state increases to a small degree. A third difficulty results from the substantial variability among individual platelet units in both base activation state and susceptibility to activation. With the LVAD system described herein, a confounding limitation is the number of valve passages that can be attained. Platelets in vivo have a life span of about seven days, during which they will pass through the left ventricle about 7,000 times. In comparison, running the LVAD at a normal heart rate of 4.7 l/min, only 1,400 passages are attained through each valve, and it was also noted in these studies that only one of the two valves in the LVAD was restricted.

On moving from systems of defined shear-stress properties, such as cone-and-plate devices (22) or the present capillary-based circulation system (20) to the study of real prosthetic devices, the stress history of the platelets is clearly more difficult to define. In particular, just as is true of implanted mechanical heart valves in vivo, in any single valve passage only a small fraction of the platelets are exposed to high shear stress. Given this very limited exposure, the extent of bulk platelet activation reported here for the severely restricted valve is very striking. As an approximate reference, these data can be compared with recently acquired PAS data on platelets subjected to defined intermittent shear in a capillary circulation loop (20). Under the most severe conditions in that study, platelets were exposed to a time-integrated shear stress of 120 dyne·min/cm^2 (10 min shear-exposure time in a 30-min experiment at a shear stress of 12 dyne/cm^2). Such conditions caused approximately the same extent of platelet activation as was observed with 1,400 passages through a severely restricted mechanical heart valve. However, the average valve transit time per passage is less than 0.1 s, and thus the integrated platelet valve-exposure time is less than 2.5 min. Additionally, as noted, the large majority of platelets pass through the valve far from the wall or the leaflet, and under conditions of low shear stress (14). Taking these two facts into account, the extent of platelet activation produced by the severely restricted valve implies that, in any given passage through such a valve, the small proportion of platelets subjected to local stress are exposed to shear stresses that must be orders of magnitude higher than in the previous study - that is, in the range of hundreds or even thousands of dyne/cm^2. In comparison, the rate of platelet activation by the unrestricted valve is about one-eighth that produced by the severely restricted valve, and in fact the mean platelet activation rate observed for the unmodified valve in this small study was not significantly different from zero.

Those mechanisms which are conducive to platelet activation in the severely restricted valve occur during both systole and diastole. During systole (the forward flow phase), the leaflet was restricted to approximately 60° opening rather than the normal 80°. In a monoleaflet valve the flow field generated by such a configuration is characterized by the formation of a recirculation zone and enhanced vortex-shedding behind the valve, and computational studies have suggested that this can generate local turbulent stresses of the order of several thousand dyne/cm^2 (14,16). During diastole, the restriction prevents the valve from fully closing, resulting in retrograde flow characterized by strong regurgitant jets. These also induce strong shear and turbulent stresses capable of activating platelets (8). While a free valve may contain a small degree of retrograde flow, the artificial restriction

imposes a much higher retrograde flow rate. The present experimental data go a long way to demonstrating directly that flow abnormalities can indeed cause very substantial extents of platelet activation.

Similar flow mechanisms induce platelet activation in the mildly restricted valve, albeit only during diastole, because the smaller suture prevents full closure of the valve but does not restrict its full opening. Thus, it was expected that the mildly restricted configuration will be less activating to platelets, as indeed was established by the present measurements (Fig. 5).

Study limitations

The inability to devise a proper valve-free control circulation loop was a minor limitation. Without valves, the system is no longer a flow loop because the valves control the fluid circulation; and in the control series (Fig. 4, left panel) the platelet suspension passed back and forth through both ports without circulation. Nonetheless, the observed mean rate of platelet activation in this case was essentially zero (Fig. 5). Another limitation was the use of the LVAD without a proper afterload on the valves. In mock circulation studies of valves, a pulse-duplicator system with resistance elements is often used to generate the afterload. Resistance devices, however, such as windkessels or flow restrictions, would inevitably cause more platelet activation than the valves themselves. Despite these constraints, the present compact model served as a good approximation, and came much closer to the conditions to which platelets are exposed in flow through prosthetic valves than any other in-vitro device of which the present authors are aware.

In conclusion, flow-induced platelet activation was measured in a plasma-free platelet preparation circulated through a LVAD fitted with monoleaflet Björk-Shiley mechanical heart valves. Moreover, evidence was presented that the modified prothrombinase assay used provides a quantitative measure of the platelet activation produced by mechanical heart valves. The ability to perform sensitive measurements of platelet activity in flow through cardiovascular devices in vitro may have a major impact on the design of these and other vascular devices, potentially allowing prior examination and prediction of their propensity to induce platelet-dependent thrombotic events.

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