

# Study of Platelet-Mediated Neurotoxicity in Rat Brain

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**Background and Purpose:** The mechanism of ischemic neuronal injury is not fully resolved. The present view is that vascular occlusion per se does not fully account for the extent of neurological dysfunction. We previously hypothesized that platelet secretory products might contribute to neuronal injury in the central nervous system. Our preliminary studies using organotypic rat spinal cord cultures exposed to human platelet and its secretory products revealed that platelet products had neurotoxic properties.

**Methods:** Further studies, using the same methods, were conducted, with the addition of several refinements such as use of gel-filtered platelets (as opposed to washed platelets) and adding additional relevant controls including platelet membranes, red blood cells, and washed rat platelets.

**Results:** Exposure of spinal cord explant cultures to platelet secretory products resulted in reduced number of neurons per ventral horn compared with control.

**Conclusions:** Our findings suggest that platelet secretory products have neurotoxic properties. This effect was seen with platelet secretion obtained from physiological platelet concentrations. It appears possible that more abundant release of platelet products at the site of thrombus formation could have pathological significance in vivo. (*Stroke* 1992;23:394–398)

**KEY WORDS** • blood platelets • neuronal damage • rats

Blood platelets contain and secrete a number of active biological substances such as serotonin, thromboxane, and catecholamines.<sup>1</sup> Among them, serotonin has been convincingly shown to suppress central nervous system function.<sup>2</sup> Arterial thrombi, a major cause of cerebral infarction, consist largely of platelets, constituting >50% by volume. This platelet concentration in thrombi amounts to 190 times the concentration in whole blood.<sup>3</sup> We reasoned that a high concentration of platelet secretory products released during thrombus formation may contribute to ischemic neuronal injury.<sup>4–7</sup> At a more systemic level, we had shown that platelets of patients with acute ischemic stroke are more prone than those of control subjects to secrete the contents of their organelles.<sup>8,9</sup> The aim of this study was to determine whether platelet secretory products have morphologically demonstrable neurotoxicity. We used organotypic culture of rat spinal cord as a substrate of central nervous system tissue. This method is believed to be more representative of the in vivo condition than are dissociated culture preparations.

## Materials and Methods

Explants of spinal cord from 4–9-day-old rats were used as a model for the study of mammalian central nervous system neurons. Unlike cerebral tissue, these explants have relatively well-defined neuroanatomical

landmarks that permit better morphometric evaluation. Explants were prepared using modifications of the method described by Delfs et al.<sup>10</sup> Lumbosacral spinal cord of postnatal Sprague-Dawley rats were dissected out under aseptic conditions and the spinal cord cut serially into twelve 400- $\mu$ m thick sections from the thoracolumbar junction downwards. Each section (explant) was transferred to 20  $\mu$ l chicken plasma (Cocalico Biologicals, Reamstown, Pa.) placed on a coverslip. The plasma was then clotted with an equal volume of thrombin (500 units/ml). The coverslip with the adherent explant was placed in a 10-ml-volume tissue culture tube containing 1.2 ml serum-containing medium (25% heat-inactivated horse serum [GIBCO, Grand Island, N.Y.], 50% basal Eagle's medium with Earle's salts, 25% Hank's balanced salt solution, 0.5% L-glutamine, and 6.4 mg/ml glucose). The tubes were capped and incubated at 36°C in a roller drum assembly rotating at 10 revolutions/hr. The medium was changed on a weekly basis at the time of evaluation under a dissecting microscope. The explants that had recognizable and flattened dorsal and ventral horns were retained. These amounted to approximately 60–70% of the total number of explants plated. Experiments were carried out only on explants that had survived intact for  $\geq 1$  week to reduce the influence of the natural high rate of explant loss in the first week of incubation.

At the end of the experiments, the explants were stained for acetylcholinesterase.<sup>11</sup> The explants were fixed for 1 hour in 4% paraformaldehyde kept at room temperature, washed with distilled water, exposed to chilled 5% dimethylsulfoxide for 30 minutes, washed again, and then incubated overnight (12 hours) in a solution containing sodium sulfate 662 mM, acetylthiocholine iodide 4 mM, glycine 10 mM, cupric sulfate 2

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mM, sodium acetate 83 mM, and ethopropazine 0.2 mM. The explants were again washed and exposed for 2.5 minutes to sodium sulfide (178 mM, made up in 0.1N HCl). The specimens were washed, dehydrated, and mounted on slides with Permount.

Specimens that had recognizable central canal and ventral horns were again matched for age of animal, duration of incubation in culture medium before the experiment, and spinal level. Only specimens that matched for all these parameters in the test and control groups were subjected to morphometric analysis. A line was drawn along the dorsal margin of the central canal parallel to the outer ventral margins of both ventral horns. All acetylcholinesterase-positive neuronal somata ventral to this line were outlined using camera lucida at 25 $\times$  magnification. In our previous studies relating to the effect of platelet products on the neurons of spinal explants, we had quantified by morphometric techniques the number and the size distribution of neurons in the ventral horns. We found, as reported by Delfs et al,<sup>10</sup> that >90% of the neurons in the ventral horns, both in the treated and control groups, were <500  $\mu\text{m}^2$ , and the major portion of this was in 100–200  $\mu\text{m}^2$  range. Although the number of neurons per ventral horn in the explants varied depending on the age of the animal, spinal level, and duration in culture before the experiment, these were strictly controlled for within each experiment. In our present analysis, we chose the number of neurons per ventral horn as the parameter to estimate the effect of agents used in the related experiments, as we found this to be more reliable.

Gel-filtered platelets were prepared by a modification of a method we have previously used.<sup>12</sup> Venous blood (120 ml) from healthy volunteers was drawn into sterile acid-citrate-dextrose in a 1:9 dilution, centrifuged at 185g for 15 minutes, and the supernatant platelet-rich plasma collected. The functional integrity of the platelets in the platelet-rich plasma was confirmed by stimulation with thrombin (final concentration 1 unit/ml) and collagen (final concentration 10  $\mu\text{g}/\text{ml}$ ), and by observing aggregation and ATP release. Thereafter, platelet-rich plasma was treated with prostaglandin  $\text{E}_1$  (final concentration 1  $\mu\text{M}$ ), centrifuged at 1,160g for 15 minutes, and the supernatant discarded. The platelet pellet was resuspended in *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered Tyrode's solution (NaCl 129 mM,  $\text{NaHCO}_3$  8.9 mM, KCl 2.8 mM,  $\text{KH}_2\text{PO}_4$  0.8 mM,  $\text{MgCl}_2$  0.8 mM, dextrose 5.6 mM, HEPES 10 mM, pH 7.3) containing prostaglandin  $\text{E}_1$  (1  $\mu\text{M}$ ) and centrifuged (1,160g for 15 minutes) again. The resultant platelet pellet was resuspended in 2 ml serum-free medium (50% basal Eagle's medium with Earle's salts, 25% Hank's balanced salt solution, 0.5% L-glutamine, 6.4 mg/ml glucose) containing 100 units penicillin/ml and 100  $\mu\text{g}$  streptomycin/ml. This suspension was divided into two equal fractions, and each was filtered through a 10-ml bed volume of Sepharose 2B previously primed with 30–40 ml of the same serum-free medium. The eluent, gel-filtered platelets, was collected from each column in four fractions, and the desired platelet count of 2–3 $\times 10^8/\text{ml}$  (a physiological concentration in humans) obtained by serial dilution with the same serum-free medium. The functional integrity of the platelets in this final suspension was also checked as in the earlier phase of preparation, and the

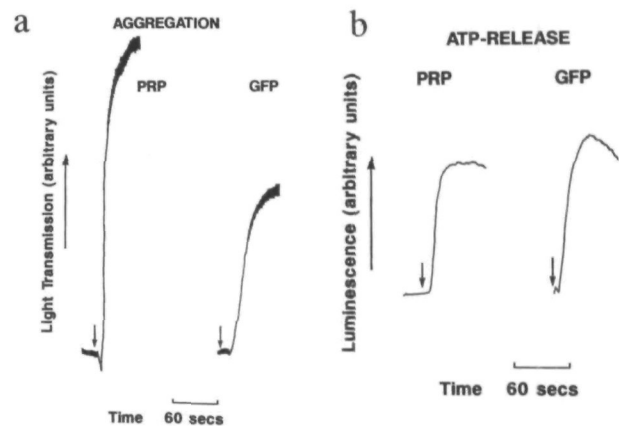


FIGURE 1. Panel a: Tracings comparing the aggregation of platelets in platelet-rich plasma (PRP) and gel-filtered platelets (GFP) to demonstrate normal response of GFP to collagen (10  $\mu\text{g}/\text{ml}$ ) stimulation. Reduced extent of aggregation in the latter is due to absence of plasma proteins, particularly fibrinogen. Panel b: Tracings of ATP release, an index of platelet secretion, from PRP and GFP in response to stimulation with collagen (10  $\mu\text{g}/\text{ml}$ ). Responses are comparable.

two responses were comparable. The aggregation in gel-filtered platelets is known to be lower than that in platelet-rich plasma due to the absence of plasma proteins, particularly fibrinogen (Figure 1).

The following related experiments were carried out on the explant cultures that had stabilized with recognizable ventral and dorsal horns: 1) effect of gel-filtered platelets, 2) effect of collagen-stimulated gel-filtered platelets, 3) effect of thrombin-stimulated gel-filtered platelets, 4) effect of filtrate of stimulated gel-filtered platelets, 5) effect of washed platelet membranes, 6) effect of washed red blood cells, and 7) effect of washed rat platelets.

For experiment 1, we used suitable explants that met the requirements described above and matched for age of the animal, duration of incubation of the explants before use in the experiment, and spinal levels of the sections. There were 18 in the test group and 18 in the control group. In the test group, the serum-containing medium was replaced with an equal volume of serum-free medium that contained gel-filtered platelets in a concentration of  $2.1 \times 10^8/\text{ml}$ ; in the control group, the serum-free medium did not contain platelets. The explants in the two groups were incubated for a period of 72 hours, the same duration of exposure that others have used to demonstrate the neurotoxicity of *N*-methyl-D-aspartic acid using a similar culture system.<sup>13</sup> The explants were stained for acetylcholinesterase. Specimens that had recognizable central canal and ventral horns were again matched for age of animal, duration of incubation in culture medium before the experiment, and spinal level; only such matched pairs were subjected to morphometric analysis.

The rationale for treating the explants with collagen-stimulated gel-filtered platelets (experiment 2) is that the activated platelets, along with their secretions, would be exposed directly to the specimens. This experiment controls for the possibility that gel-filtered platelets may become activated by collagen in the spinal tissue. Twenty-four suitable explants were divided equally into the treated and control groups. The culture

medium in the treated group was replaced with serum-free medium containing collagen-stimulated, gel-filtered platelets in a concentration of  $2.9 \times 10^8$ /ml. The collagen concentration in the serum-free medium added to the treated and the controls was 10  $\mu$ g/ml. The explants, as in the other related experiments, were further incubated for 72 hours and processed for evaluation as described above.

The experiment with thrombin-stimulated, gel-filtered platelets (experiment 3) was carried out to serve as an adjunct to experiment 2, as thrombin is another important platelet activator *in vivo*. The method was similar to that in experiment 2. The concentration of thrombin in the treated and the control groups was 1 unit/ml, and the concentration of gel-filtered platelets in the treated group was  $2.9 \times 10^8$ /ml. There were 10 explants in the treated group and 9 in the control group.

For experiment 4, gel-filtered platelets ( $2.7 \times 10^8$ /ml) in serum-free medium were stimulated with thrombin (1 unit/ml) and agitated for 5 minutes. The medium was then passed through a 0.45- $\mu$ m sterile Millipore filter and the filtrate added to the test group. The serum-free medium used for the controls had been prepared in a similar manner but without platelets. There were seven explants in the treated group and seven in the control group.

Experiment 5 was performed to examine the effect of platelet membranes, inevitably present in the preparation used in experiments 1, 2, and 3, and it serves to identify the components of platelets responsible for neuronal damage. Gel-filtered platelets ( $3.5 \times 10^8$ /ml) in serum-free medium were stimulated with collagen (10  $\mu$ g/ml), centrifuged at 1,160g for 15 minutes, and the supernatant removed. The sediment was washed three times with HEPES-Tyrode's buffer containing prostaglandin  $E_1$  (1  $\mu$ M). After the final wash, the sediment was reconstituted in serum-free medium so that the amount of platelet membrane per unit volume was equal to that in the original platelet concentration. There were 16 explants, eight in the test and eight in the control group.

The rationale for experiment 6 was to compare and contrast the effect of an alternative anuclear blood element on the explants with that of platelets. Washed red cells were prepared by centrifuging 120 ml anticoagulated (1:9 acid-citrate-dextrose) whole blood at 185g for 15 minutes, then removing the supernatant (platelets), the buffy coat (leucocytes), and the top 2 ml of red cells. The sediment was washed three times with HEPES-Tyrode's buffer containing prostaglandin  $E_1$  (1  $\mu$ M) and the sediment (washed red blood cells) resuspended in serum-free medium to a concentration of  $3 \times 10^8$ /ml to match the concentration of the two blood elements. There were 19 explant cultures in the test group and 19 in the control group.

Experiment 7 was conducted to address the question of interspecies mismatch: human platelets on rat spinal explants. The washed rat platelets, obtained from adults of the same species, were prepared in a manner similar to that for human platelets before gel-filtration. The latter procedure was not practical with the limited supply of rat blood. We felt that this shortcoming could be compensated for by adding a calculated amount of rat plasma to the serum-free medium used in the control group. The concentration of rat platelets used in

the experiment was  $3 \times 10^8$ /ml (a physiological human platelet concentration, but lower than physiological in rats). There were 14 explants in the test and 14 in the control group.

Analysis of variance was performed to test for differences between control and test groups where there were sufficient numbers of explants. The analysis of variance F test used to compare the two groups was adjusted for slice (which accounts for the matching) and side (left or right) factors. In the analysis, it is assumed that the correlation structure of slices within any particular rat is the same as the correlation structure of slices between different rats. This is important because of the possibility that a particular animal may be represented more than once in the data. More than one of the slices from a particular animal may be included in the same group (control or test) or across groups. Because a factor for the correlation between different observations on the same animal cannot be included in the analysis model, it is assumed that the correlation between slices of the same animal is the same as that across animals. Descriptive statistics are reported for experiments with small numbers of ventral horns per group.

## Results

In the experiment exposing spinal explants to gel-filtered platelets, the number of surviving neurons in the treated group was approximately 70% less than that in the control group (Figure 2A). The results of exposing platelets stimulated with collagen or thrombin to the explants were similar to the results from exposure to unstimulated platelets (Figure 2B). There was no difference using either platelet agonist (data not shown). Further, the number of surviving neurons in the explants treated with a filtrate from stimulated platelets was also reduced (Figure 2C). The results of several control experiments that were carried out appear to validate the above findings. There was only minimal relative decrease in the number of neurons in explants treated with platelet membranes (Figure 2D) and washed human red blood cells (Figure 2E). The experiment with washed rat platelets to address the interspecies mismatch showed that rat platelets, similar to human platelets, also caused a reduction in the number of neurons (Figure 2F).

## Discussion

The work carried out after our initial reports<sup>5-7</sup> on the possibility of platelet secretion having neurotoxicity has provided further supportive evidence. We conducted a more comprehensive range of related experiments to confirm that platelet secretory products had a damaging effect on central nervous system neurons. The consistent pattern of reduction in the number of surviving neurons in the experiments using platelet and its secretory products appears to confirm their neurotoxicity. As the neurotoxic effect of platelet secretion is similar to that of platelets, we believe that the effect in the latter is due to secretory products released after platelet activation by collagen in the explants. The concentrations of platelets and their secretory products used in the experiments were in the range that would normally be present in a unit volume of whole blood before and after platelet activation. Considering that

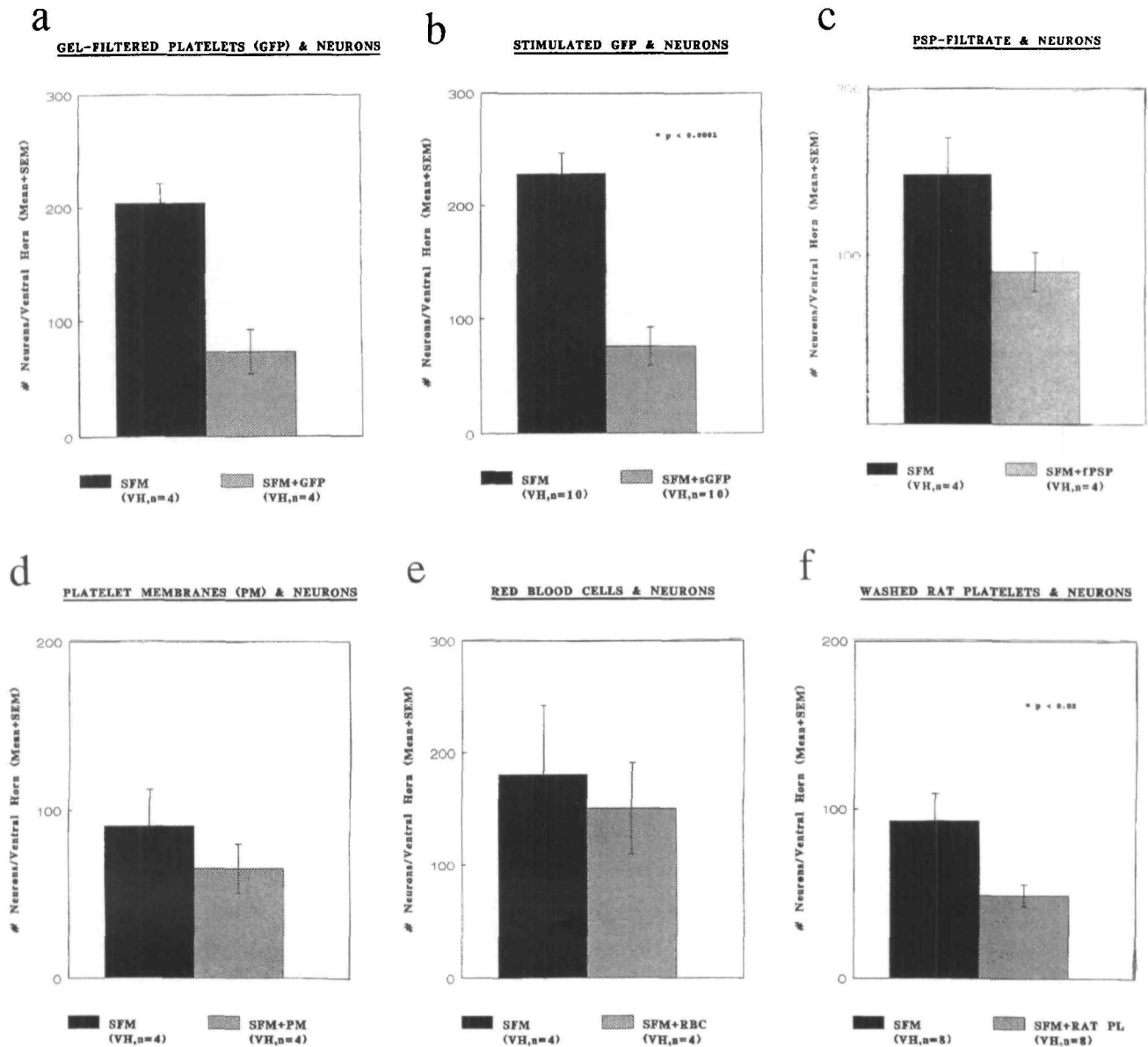


FIGURE 2. Bar graphs showing analyses of results in the related experiments (see text for details in "Materials and Methods"). Results are indicated in terms of number of neurons per ventral horn. Solid bars, control group; hatched bars, test group. Panel a: Effect of gel-filtered platelets (GFP). SFM, serum-free medium; VH, ventral horn. Panel b: Effect of stimulation by collagen or thrombin. sGFP, stimulated gel-filtered platelets. Panel c: Effect of filtrate derived from activated platelets. PSP, platelet secretory products; fPSP, filtrate of platelet secretory products. Panel d: Effect of platelet membrane (PM). Panel e: Effect of human red blood cells (RBC). Panel f: Effect of washed rat platelets (PL).

the damaging effect was seen with these low concentrations, possibly greater or more widespread damage to neurons in tissue contiguous to an acute thrombus may take place. It is possible that even a short exposure to a high concentration of platelet products could result in neurotoxicity, as was demonstrated with glutamate *in vitro*.<sup>14</sup> Our finding, and the postulate based on it, could have implications in both the pathophysiology and therapy of neuronal injury.

We see several positive points in this *in vitro* study. Maintaining central nervous system neurons, as we have in our study, in organotypic culture, is viewed by most as being closer to the *in vivo* condition than is dissociated neuronal culture systems.<sup>10</sup> The use of spinal cord explants had the added advantages of having recogniz-

able neuroanatomical landmarks with intact cross-sectional outlines that were crucial during analysis. The specimens were strictly matched for age, spinal level of explants, and duration of incubation before exposure to platelets or their secretory products. This inevitably reduced the number of suitable matching explant pairs for analyses. The relatively small size of samples for analysis in some of the experiments may be considered a shortcoming, but, in our view, the consistent pattern of neuronal damage seen in all the experiments may further strengthen the value of the observations. Although other formed elements or plasmatic factors in normal blood may have neurotoxicity, our experiments with washed human red blood cells and platelet membranes have not demonstrated such an effect.

In conclusion, the results of the present study suggest that platelet secretory products have toxic effects on central nervous system neurons. The results of this in vitro study could form the basis of future studies in vivo that may shed further light on the role of platelet and its secretion in neuronal injury.

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