

BRIEF REPORT

Appearance of Platelet-Clumping Substance in Plasma of Rabbits after Intravenous Injection of Agar-Solution, Bacterial Endotoxin or Adrenaline

By HIROH YAMAZAKI, TATSUO SHIMAMOTO, HIROSHI MURASE AND
TAKIO SHIMAMOTO

IN 1958, Shimamoto et al.¹ reported platelet aggregation induced by heparinized plasma of rabbits treated with bacterial endotoxin, and concluded that this was due to an appearance of platelet-clumping substance in plasma. These results were confirmed in vitro by Des Prez, Horowitz and Hook² in 1961. Since then, many investigators have reported adenosine diphosphate (ADP), thrombin, adrenaline and several other substances which aggregated platelets in platelet-rich plasma. The present paper describes the appearance of a platelet-clumping substance in plasma, which may differ from ADP and others, after intravenous injection not only of bacterial endotoxin, but also of agar-solution or adrenaline.

MATERIALS AND METHODS

Sixty-five male rabbits, weighing 1.8 to 2.5 Kg., were used. Twenty-nine rabbits were intravenously injected with 5 ml./Kg. of 0.5 per cent agar-saline solution (Agar noble, Nihon Eiken Co., Ltd.), or 100 µg./Kg. of bacterial endotoxin prepared from *Escherichia coli* O-1 (supplied by Dr. S. Iwahara, Department of Bacteriology, National Hygienic Laboratory), or 10 µg./Kg. of adrenaline (Sankyo Co., Ltd.). Before each challenge—and then 5 minutes, 30 minutes, and 1, 2 and 24 hours after challenge—2 ml. of spontaneously flowing blood were collected from the cut end of a marginal ear vein into a siliconized tube containing 100 units of heparin sodium (Takeda Pharmacol. Co., Ltd.). Immediately after the collection, the platelet-poor plasma was taken from the blood by centrifugation. At the same time the platelet count was carried out by Olef's method.

About 50 ml. of blood was drawn from the carotid artery of the remaining 36 rabbits into a siliconized tube containing EDTA-Na₂ (0.1 per cent in final concentration). The collection of platelets was performed by centrifugation at 4 C. Platelets in the sediment layer of EDTA-plasma were washed with physiologic saline twice and resuspended in saline at one-half the volume of the original blood sample. The pH of the saline was adjusted from 7.4 to 7.6 with NaHCO₃. The washed platelet suspension (7×10^8 - 10×10^6 /mm.³) showed no spontaneous clumping under microscopic examination.

One drop of the platelet-poor plasma was mixed with one drop of the normal platelet-saline suspension in a paraffin cup by gently sucking and blowing 10 times with a siliconized Pasteur-pipette at room temperature. The mixture was immediately transferred to a

From the Institute for Cardiovascular Diseases, School of Medicine, Tokyo Medical and Dental University, Yushima, Tokyo.

First submitted April 4, 1967; accepted for publication July 5, 1967.

HIROH YAMAZAKI M.D.: Associate Professor, Institute for Cardiovascular Diseases, Tokyo Medical and Dental University School of Medicine, Tokyo, Japan. TATSUO SHIMAMOTO, M.D.: Tokyo Medical and Dental University School of Medicine, Tokyo, Japan. HIROSHI MURASE, M.D.: Tokyo Medical and Dental University, Tokyo, Japan. TAKIO SHIMAMOTO, M.D.: Professor and Director of Institute for Cardiovascular Diseases, Tokyo Medical and Dental University School of Medicine, Tokyo, Japan.

siliconized slide and covered gently with a siliconized coverslip; presence of clumping of platelets was observed under a regular light microscope ($\times 450$). The test to observe clumping of platelets was carried out over 3 times, using the platelets collected from different rabbits. The plasma, which induced clumping of platelets, was found to contain the platelet-clumping substance.

RESULTS

In the heparinized plasma of 13 rabbits collected before the intravenous injection of agar-solution, the platelet-clumping substance appeared only 2 out of 13 cases (15.4 per cent). On the contrary, 5 minutes after the injection, the platelet-clumping substance appeared in 10 out of 13 cases (76.9 per cent). It was observed in 11 cases (84.6 per cent) after 30 minutes and in 9 cases (69.2 per cent) after 1 and 2 hours of the injection. The statistical significant difference ($p < 0.01$) was found in comparison between the numbers of cases, which showed the platelet-clumping substance, before and after the injection during the experimental time of up to 2 hours. At the same time, the circulating platelet count decreased to $59.5 \pm 3.5 \sim 66.6 \pm 0.7$ per cent of the preinjection count with the statistical significance ($p < 0.01 \sim 0.05$).

The appearance of the platelet-clumping substance and the decrease of the circulating platelet count were also observed 5 minutes to 2 hours after the intravenous injection of bacterial endotoxin or adrenaline. In the case of bacterial endotoxin, the platelet-clumping substance was observed in 1 out of all 7 cases (14.3 per cent) before the injection. One and 2 hours after the injection, the platelet-clumping substance was found in 4 out of 7 cases (57.1 per cent), with the statistical significance ($p < 0.05$), while the platelet count decreased to 46.0 ± 1.0 and 50.3 ± 4.1 per cent of the pre-injection count ($p < 0.05$). In the case of adrenaline, the platelet-clumping substance was observed in 1 out of all 9 cases (11.1 per cent) before the injection. One hour after the injection, the platelet-clumping substance was found in 6 out of 9 cases (66.7 per cent) ($p < 0.05$), while the platelet count decreased to 59.5 ± 4.1 per cent ($p < 0.05$). In the above 3 cases, these changes were not observed after 24 hours of the injections.

The platelet-clumping substance caused a similar degree of clumping of various platelets collected from the different rabbits, at least quantitatively. The clumping of platelets induced by the platelet-clumping substance appeared not only in platelet-saline suspension but also in citrated platelet-rich plasma.

The platelet-clumping substance was not identified in the serum nor in the citrated, oxalated or EDTA-plasma, regardless of the challenges. After the addition of calcium or magnesium ions (0.1, 1, 10 or 20 mM) to the citrated or EDTA-plasma, which already contained heparin (50 units/ml.), the platelet-clumping activity did not appear in the plasma. On the other hand, after the addition of sodium citrate (0.38 per cent in final concentration) or EDTA- Na_2 (0.1 per cent in final concentration) to the heparinized plasma, which contained the platelet-clumping substance, the platelet-clumping activity in the plasma did not disappear.

The platelet-clumping activity in the plasma was not related to the pH of the plasma, at least from 7.8 to 8.6. It was stable during storage for 5 weeks at

4 C. and was not inactivated by dialysis for 24 to 72 hours against Ringer's solution at pH 4.0 to 8.6.

After the addition of ADP (Sigma Chemical Co.), serotonin (Upjohn Co.), adrenaline, 1-noradrenaline (Winthrop Lab.), or thrombin (bovine thrombin, NBC Co.) in a dose of 0.001, 0.01, 0.1, 1.0, 10 or 100 $\mu\text{g./ml.}$ or units/ml. to the platelet-saline suspension, no clumping occurred. Although the medium also contained 50 per cent (v/v) the normal heparinized platelet-free plasma, the above substances did not cause clumping of platelets.

DISCUSSION

It was reported^{1,3-5} that several bacterial endotoxins, high molecular weight substances or adrenaline caused clumping of platelets and decrease of circulating platelet count. The decrease of the circulating platelet count may be partially due to the adhesion of platelets to the vessel wall undergoing edematous changes,⁵ or it may be related to the clumping of platelets induced by the platelet-clumping substances in plasma.

Marcus and Zucker⁶ suggested that the platelet-clumping substance described by the authors¹ might be ADP. However, ADP does not cause clumping of washed platelets.⁷⁻⁸ According to Born and Cross,⁸ ADP induced clumping of washed platelets when platelet-free plasma was added. In our experiments, rabbit platelets suspended in saline were not clumped by the addition of ADP, serotonin, adrenaline or thrombin in a dose of 0.001~100 $\mu\text{g./ml.}$ or units/ml. with or without the presence of the normal heparinized plasma. Moreover, the platelet-clumping substance in the plasma did not disappear after dialysis or storage for 5 weeks at 4 C. Free form of ADP may be not present after the dialysis or the storage. These results suggest that our platelet-clumping substance differs from ADP, although the possibility is not to be negated at present that the plasma activity may be an ADP cofactor.⁸

The presence of calcium or magnesium ions were not related to the appearance of the clumping of platelets induced by our platelet-clumping substance in vitro. These results suggest that our platelet-clumping substance differs from TAg or TAg'.⁹

SUMMARY

The platelet-clumping substance appeared statistically significant ($p < 0.01 \sim 0.05$) in the heparinized plasma of 29 rabbits collected 5 minutes, 30 minutes, and 1 and 2 hours after the intravenous injection of either 5 ml./Kg. of 0.5 per cent agar-saline solution, 100 $\mu\text{g./Kg.}$ of bacterial endotoxin derived from *Escherichia coli*, or 10 $\mu\text{g./Kg.}$ of adrenaline, as compared with that in the platelet-saline suspension from the other 36 rabbits. At the same time, the circulating platelet count decreased significantly ($p < 0.01 \sim 0.05$). Twenty-four hours after the injection these changes disappeared. The platelet-clumping substance was detected only in heparinized plasma, and not in citrated, oxalated, and EDTA-plasma or serum. The addition or removal of calcium or magnesium ions did not affect the appearance of clumping of platelets. It is stable for storage at 4 C. and is not dialyzable. It induced clumping of platelets not only

in the platelet-saline suspension, but also in the platelet-rich plasma, while ADP, serotonin, catecholamine or thrombin (0.001~100 μ g./ml. or units/ml.) did not induce clumping of washed rabbit platelets even under the presence of the normal platelet-free heparinized plasma.

SUMMARIO IN INTERLINGUA

Le substantia plachetto-aggregante appareva in un concentration statisticamente significativa ($p < 0,01$ e $0,05$) in le heparinisate plasma de 29 conillos, colligite 5 minutas, 30 minutas, 1 hora, e 2 horas post le injection intravenose de (1) 5 ml/kg de 0.5 pro cento de agar in solution salin, (2) 100 μ g/kg de endotoxina bacterial derivate ab *Escherichia coli*, o (3) 10 μ g/kg de adrenalina, in comparation illo in le caso de le 36 conillos normal. Simultaneamente, le numeration del plachettas circulante monstrava un declino significative ($p < 0,01$ e $0,05$). Vinti-quattro horas post le injection, iste alterationes habeva disparite. Le substantia plachetto-aggregante esseva detegibile solo in plasma heparinisate sed non in plasma tractate con citrato, oxalato, o EDTA e non in le sero. Le addition o remotion de calcium o magnesium non afficeva le apparition del effecto aggregatori. Le substantia es stabile in preservation a 4 C e non es dializabile. Illo induceva le aggregation del plachettas non solo in le suspension de plachettas in solution salin sed etiam in plasma ric in plachettas. ADP, serotoninina, catecholamina, o thrombina (0,001 a 100 μ g o unitates per ml) non induceva aggregation de lavate plachettas de conilio, mesmo in le presentia de normal heparinisate plasma libere de plachettas.

REFERENCES

1. Shimamoto, T., Yamazaki, H., Sagawa, N., Iwahara, S., Konishi, T., and Maezawa, H.: Effect of bacterial endotoxins on platelets and release of serotonin: II. Appearance of platelet agglutinating substance and serotonin in the plasma of rabbit by administration of bacterial endotoxin. *Proc. Japan. Acad.* 34:450, 1958.
2. Des Prez, R. M., Horowitz, H. I., and Hook, E. W.: Effects of bacterial endotoxin on rabbit platelets. I. Platelet aggregation and release of platelet factors in vitro. *J. Exp. Med.* 114:857, 1961.
3. Stetson, C. A.: Studies on the mechanism of the Shwartzman phenomenon. Certain factors involved in the production of the local hemorrhagic necrosis. *J. Exp. Med.* 93:489, 1951.
4. McKay, D. G., and Shapiro, S. S.: Alterations in the blood coagulation system induced by bacterial endotoxin. I. In vivo (generalized Shwartzman reaction). *J. Exp. Med.* 107:353, 1958.
5. Shimamoto, T.: The relationship of edematous reaction in arteries to atherosclerosis and thrombosis. *J. Atheroscler. Res.* 3:87, 1963.
6. Marcus, A. J., and Zucker, M. B.: *The Physiology of Blood Platelets.* New York, Grune & Stratton, Inc. New York, 1965, p. 60.
7. Mitchell, J. R. A., and Sharp, A. A.: Platelet clumping in vitro. *Brit. J. Haemat.* 10:78, 1964.
8. Born, G. V. R., and Cross, M. J.: Effects of inorganic ions and of plasma proteins on the aggregation of blood platelets by adenosine diphosphate. *J. Physiol.* 170:397, 1964.
9. Brinkhous, K. M., LeRoy, E. C., Cornell, W. P., Brown, R. C., Hazlehurst, J. L., and Vennart, G. P.: Macroscopic studies of platelet agglutination; Nature of thrombocyte agglutinating activity of plasma. *Proc. Soc. Exper. Biol. Med.* 98:379, 1958.