# SALIVARY GLAND SECRETION OF THE LEECH Hirudo medicinalis INHIBITS ADP-INDUCED HUMAN PLATELET ADHESION ON A COLLAGEN-COATED SURFACE

I. P. Baskova, F. Misselwitz,
G. I. Nikonov, I. D. Novikov,
V. L. Leitin, and V. S. Repin

UDC 615.339.015.4:612.111.7

KEY WORDS: secretion of Hirudo medicinalis; platelet aggregation, adhesion and migration.

The leech Hirudo medicinalis has been used since olden times as an agent for removing blood. Leeches are used in the cerebral form of essential hypertension, in prestroke states, angina pectoris and myocardial infarction, venous thrombosis and thrombophlebitis, conditions associated with local stasis, headaches, and other diseases. Secretion of the salivary glands produced by leeches during biting promotes maintenance of the liquid state of the blood during blood sucking and also for a short time after removal of the leech [4]. The secretion of leeches can be used as a source of inhibitors of proteolytic enzymes – thrombin, trypsin, and chymotrypsin [2].

In the present experiments the action of the secretion on the blood clotting system was studied. It was shown that the secretion possesses marked antithrombin activity, due to the presence of a highly specific natural thrombin inhibitor, hirudin; it inhibits the contact stage of blood clotting and also inhibits the formation of protein deposits on the surface of a glass rod inserted into the rat jugular vein. These observations show that the secretion of leeches acts on the coagulation stage of hemostasis by inhibiting blood clotting factors leading to the formation of a fibrin clot.

In this investigation the action of the secretion on the platelet component of hemostasis was studied. Two model systems were used: 1) The action of the secretion on platelet aggregation in suspension, induced by ADP, a natural platelet activator, was investigated by a photometric method [5]; 2) the effects of the secretion on platelet adhesion to a surface coated with fibrillary collagen, a connective-tissue component of the blood vessel wall, which is exposed on the side of the lumen of the vessel during injury and which is the adhesive substrate for circulating platelets, were investigated by scanning electron microscopy (SEM). The secretion was shown

TABLE 1. Effects of Different Doses of Secretion on Adhesion of Gel-Filtered Platelets on Collagen Substrate: Morphometric Counting of Adherent Platelets by SEM (M ± m)

| Dose of secretion vols. % | n | Adherent platelets · $10^{-3}$/mm$^2$ | | | |
|---|---|---|---|---|---|
| | | A | $U_c$ | $S_c$ | $U_s$ |
| 0 | 5 | 6,68±0,71 | 3,80±1,30 | 1,86±0,50 | 1,02±0,57 |
| 0,1 | 6 | 2,68±0,47 | 1,58±0,35 | 0,76±0,28 | 0,34±0,23 |
| 1 | 6 | 2,13±0,36 | 1,36±0,22 | 0,61±0,28 | 0,16±0,06 |
| 10 | 6 | 0,75±0,19 | 0,62±0,17 | 0,08±0,03 | 0,05±0,02 |

Legend. Here and in Table 2, n denotes number of investigations.

Laboratory of Physiology and Biochemistry of Blood Clotting, Biological Faculty, M. V. Lomonosov Moscow University. Laboratory of Cell and Tissue Culture and Department of Computer Techniques and Mathematical Methods, All-Union Cardiologic Scientific Center, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR S. E. Severin.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 97, No. 6, pp. 696-699, June, 1984. Original article submitted May 3, 1983.

 0007-4888/84/9706-0768$08.50 

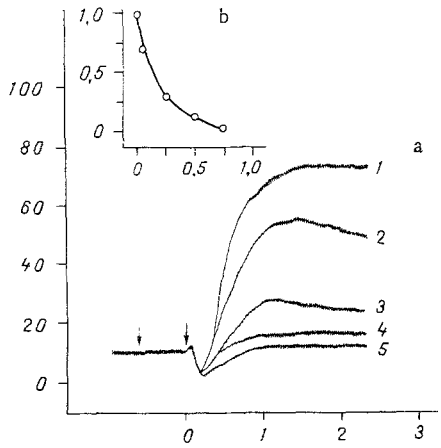


Fig. 1. Inhibitory action of secretion of the leech on aggregation of gel-filtered human platelets induced by ADP. a: Abscissa, time (in min) after addition of 10 $\mu$M ADP to cuvette of aggregometer (arrow on right); ordinate, light transmission (T, %). 0.15 M NaCl or different concentrations of secretion added to cuvette 40 sec before ADP (arrow on left): 1) 0; 2) 0.05 vol. %; 3) 0.25 vol. %; 4) 0.50 vol. %; 5) 0.75 vol. %. b: Abscissa, concentration of secretion (in vols. %; ordinate, relative coefficient of platelet aggregation $R_{ad}=\frac{T^+}{T^-}$, where $T^+$ and $T^-$ are values of transmission of the platelet suspension in the presence of different doses of secretion and in the absence of secretion respectively, 140 sec after addition of ADP.

TABLE 2. Effects of Different Doses of Secretion on Relative Average Coefficients of Platelet Adhesion (M ± m)

| Dose of secretion, vols. % | n | $R_{at}$ | $R_{ac}$ | $R_s$ | $R_{as}$ |
|---|---|---|---|---|---|
| 0 | 5 | 1,00 ± 0,11 | 1,00 ± 0,22 | 1,00 ± 0,35 | 1,00 ± 0,53 |
| 0,1 | 6 | 0,40 ± 0,07** | 0,41 ± 0,08* | 0,72 ± 0,21 | 0,89 ± 0,34 |
| 1 | 6 | 0,31 ± 0,06** | 0,35 ± 0,06* | 0,67 ± 0,21 | 0,91 ± 0,52 |
| 10 | 6 | 0,12 ± 0,03*** | 0,13 ± 0,03** | 0,31 ± 0,11 | 0,79 ± 0,28 |

Legend. Values of coefficients $R_{at}$, $R_{ac}$, $R_s$, $R_{as}$ calculated by equations (1-4) from individual values of A, $U_c$, $U_s$, and $S_c$. Significance of differences between means in experiments with secretion and experiments without secretion calculated by Welch's criterion [1]. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$. Numbers without asterisks – differences not significant ($P > 0.05$).

to inhibit aggregation and adhesion of platelets, from which it can be deduced that it contains components preventing interaction of circulating platelets with one another and with the injured vessel wall.

## EXPERIMENTAL METHOD

Blood was collected into an anticoagulant consisting of acid citrate–dextrose–apyrase [3]. Platelets were isolated by gel-filtration through sepharose 2B [8], and the column was equilibrated with Tyrode solution without $Ca^{++}$ and $Mg^{++}$, containing 1 mg/ml of dextrose and 3.5 mg/ml of bovine serum albumin [7]. Platelet aggregation

was carried out in a two-channel aggregometer (Payton, USA) [5]. The suspension of gel-filtered platelets was made up with Tyrode solution to a concentration of $1.5 \cdot 10^8$ cells/ml, and $CaCl_2$, $MgCl_2$, and human fibrinogen (Sigma, USA) were added up to final concentrations of 2 mM, 1 mM, and 0.4 mg/ml respectively. The secretion of the leech was diluted fourfold with 0.15 M NaCl and added to the cuvette of the aggregometer in a volume of 1-15 μl (0.05-0.75 vol. %). Platelet adhesion was carried out in wells 16.4 mm in diameter in multiwell cultural plates (Falcon, USA), coated with fibrillary collagen from calf skin [3, 6]. To each well was added 0.25 ml of a suspension of gel-filtered platelets ($3 \cdot 10^7$ cells in Tyrode solution containing $Ca^{++}$, $Mg^{++}$, dextrose, and albumin) and 0.25 ml of secretion, diluted with Tyrode solution to the required concentration. Incubation was carried out for 40 min at 37°C, the multiwell plates being rotated in the horizontal plane at a speed of 36 rpm. Nonadherent platelets were removed and specimens were prepared for SEM [3, 6]. Spread ($S_c$) and unspread ($U_c$, $U_s$) platelets were counted separately, and the total number of adherent platelets (A) and relative coefficients of platelet adhesion (see below) were calculated.

## EXPERIMENTAL RESULTS

During realization of the hemostatic function of the platelet two types of interactions take place: adhesion to the damaged vessel wall, and linking of one platelet to another (aggregation). To study the possible effects of the secretion of the leech on the platelet component of hemostasis, the action of the secretion was studied on ADP-induced adhesion of platelets in suspension and their adhesion to a surface coated with collagen.

Platelet aggregation was studied by a photometric method [5]. The data in Fig. 1a show that the secretion itself caused no change in amplitude of the oscillations or in the value of light transmission and, consequently, it did not induce any change in shape or aggregation of the platelets. At the same time, the secretion is a powerful inhibitor of ADP-induced platelet aggregation. The magnitude of the inhibitory effect depended on the dose of secretion, 50% inhibition was observed with secretion in a concentration of 0.13 vol. %, 97% in a concentration of 0.75 vol. % (Fig. 1b).

To study the effects of secretion on platelet adhesion, interaction between platelets and a collagen-coated surface of calf skin was investigated. This substrate does not induce the formation of large superficial platelet aggregates [3, 7], so that the process of platelet adhesion can be studied in a pure form – without any accompanying aggregation. It was shown by SEM that adhesion of platelets to this substrate consists of several successive stages: 1) Discoid and spherical platelets from suspension adhere to the collagen substrate and form surface outgrowths or pseudopodia; 2) a certain proportion of the nonadherent platelets spreads out on the collagen substrate; 3) platelets from suspension adhere to the top surface of the spread-out platelets [3, 6]. Under the influence of secretion the number of unspread platelets of discoid and spherical shape ($U_c$) and the number of spread-out platelets ($S_c$) adherent to collagen-coated areas of substrate, the number of unspread platelets adherent to the upper surface of platelets spread out on collagen ($U_s$), and the total number of adherent platelets (A) are all reduced (Table 1).

On the basis of these results it is possible to calculate relative coefficients characterizing the effects of secretion on the various stages of interaction of platelets with the collagen substrate:

$$R_{at} = \frac{A^+}{\frac{1}{n} - \sum A^-} \tag{1}$$

$$R_{ac} = \frac{U_c^+ + S_c^+}{\frac{1}{n} - \sum \left(U_c^- + S_c^-\right)} \tag{2}$$

$$R_s = \frac{\frac{S_c^+}{U_c^+ + S_c^+}}{\frac{1}{n} - \sum \frac{S_c^-}{U_c^- + S_c^-}} \tag{3}$$

$$R_{as} = \frac{U_s^+ / S_c^+}{\frac{1}{n} - \sum \left(U_s^- / S_c^-\right)} \tag{4}$$

where $R_{at}$, $R_{ac}$, and $R_s$ are relative coefficients of total adhesion, initial adhesion, and spreading out of platelets on the collagen substrate respectively; $R_{as}$ is the relative coefficient of adhesion of platelets from suspension to the upper surface of the spread-out platelets, + and – are indices of experiments conducted in the presence of different doses of secretion and in the absence of secretion; n denotes the number of corresponding experiments.

Table 2 gives data characterizing the effects of different doses of secretion on the relative mean coefficients of adhesion. These data show that the secretion reduced the coefficients of total adhesion ($R_{at}$) and of initial adhesion ($R_{as}$) of platelets to the collagen substrate statistically significantly. Although the mean values of the coefficient of spread of the platelets on the collagen substrate ($R_s$) and of adhesion of platelets to the upper surface of the spread-out platelets ($R_{as}$) also were reduced by the action of the secretion, these effects were not statistically significant.

It was shown previously that secretion of the medical leech inhibits activation of blood clotting factor XII on the surface and inhibits conversion of fibrinogen into fibrin in solution [2]. The present investigation showed that the secretion inhibits both aggregation of platelets in suspension (Fig. 1) and adhesion of platelets to a collagen-coated surface. In conjunction with data in the literature [2], the results suggest that the secretion of Hirudo medicinalis contains components which inhibit the coagulation and platelet components of hemostasis.

## LITERATURE CITED


1. A. Afifi and S. Azen, Statistical Analysis. A Computer-Oriented Approach, Academic Press (1979).
2. I. P. Baskova, V. V. Mosolov, and G. I. Nikonov, Vopr. Med. Khim., No. 1, 64 (1984).
3. V. L. Leitin and D. D. Sviridov, in: The Blood Vessel Wall in Atherogenesis and Thrombogenesis [in Russian], Moscow (1983), p. 155.
4. G. G. Shchegolev and M. S. Fedorova, The Medical Leech and Its Use [in Russian], Moscow (1955).
5. G. V. Born, Nature, 194, 927 (1962).
6. V. L. Leitin (V. L. Leytin), E. V. Lyubimova, D. D. Sviridov, et al., Thromb. Res., 20, 509 (1980).
7. A. V. Mazurov, V. L. Leitin (V. L. Leytin), V. S. Repin, et al., Thromb. Res., 30, 183 (1983).
8. O. Tangen, H. J. Berman, and P. Marfey, Thromb. Diath. Haemorrh., 25, 268 (1971).