Agents and Actions, vol. 25, 3/4 (1988)

0065-4299/88/040401-06 $ 1.50+0.20/0

Anti-bacterial activity mediated by human platelets

G. Miragliotta, M. Lafata and E. Jirillo

Institute of Medical Microbiology, University of Bari, Bari, Italy

Abstract

Platelet-mediated antibacterial activity against gram-negative microorganisms has been investigated. Data show that human washed platelets possess the ability to partially inhibit growth of *Salmonella typhi* Ty-2 (a smooth strain) but not of two rough strains of *Salmonella minnesota* (R345-Rb and R595-Re). On the other hand, no antibacterial activity was noted in the presence of the gram-positive *Staphylococcus aureus*. This activity is dependent on the length of incubation of platelets with bacteria and apparently is not mediated by platelet soluble factors. Finally, lysis of platelets with Triton X-100 following their incubation with bacteria gives rise to the same degree of antibacterial activity observed in the untreated samples. This implies that the described platelet function does not rely on the possible trapping of bacteria in platelet aggregates.

Introduction

Gram-negative bacteria can exert multiple biologic effects when in contact with the blood of animals and man. Among blood cells, platelets are important in the response of the host to the microorganisms. Indeed, platelets interact with pathogens and participate in their trapping [1, 2]. As far as platelet antibacterial activity is concerned, several controversial reports have appeared in the literature. In this respect, Clawson and White [3] have shown that bacteria remain viable in spite of their sequestration within platelet aggregates. On the other hand, Kahn and Flinton [4] have demonstrated that, among seven bacteria tested, platelets exhibited a bactericidal activity only against *Bacillus subtilis* and *Sarcina*. Furthermore, although the release of a listericidin from rat platelets has been described by Czuprynski and Balish [5], these authors were unable to evidence a direct killing of *Listeria monocytogenes* in the presence of rat platelet rich plasma (PRP) [5].

In the light of the above concepts, it was of interest to reassess this putative platelet-mediated antibacterial activity, focusing our studies on the interaction between human platelets and gram-negative organisms. Results will show that human platelets are able to inhibit the growth of a smooth strain of *Salmonella* (*Salmonella typhi* Ty-2) but not of rough mutants (R 345-Rb and R 595-Re) of *Salmonella minnesota*.

Materials and methods

Microorganisms

The gram-negative bacteria used in our experiments were all stock plate culture strains of a smooth (S)

Correspondence address: Giuseppe Miragliotta, MD, Istituto di Microbiologia, Head: D. Fumarola, Policlinico, Piazza G. Cesare, I-70124 Bari, Italy.

Salmonella typhi Ty-2 (0:9, 12, Vi:d; ATCC 19430) and *Salmonella minnesota* rough (R) mutants, R345 (Rb) and 595 (Re). The stock cultures were maintained on trypticase soy agar tubes at room temperature and transferred weekly. Before experiments, bacteria were grown at 37 °C for 24 hr in trypticase broth, washed twice with sterile pyrogen-free saline and adjusted at the desired concentration. Before each step of the diluting process, samples were mixed vigorously on a vortex; the number of bacteria per milliliter was determined spectrophotometrically. The gram-positive *Staphylococcus aureus* was used as a control.

Blood collection and platelet preparation

Venous punction of blood was done from healthy donors who had not taken any drug for at least two weeks prior to donating blood. Trisodium citrate was used as anticoagulant (0.015 *M* final concentration). Blood was centrifuged at room temperature at 100 g for 15 min. After removal, platelet rich plasma (PRP) was adjusted to 3×10^8 platelet/ml. Washed platelets were prepared by albumin density gradient separation using the method described by Walsh [6]. After their separation platelets were washed three times with calcium-free Tyrode's solution and were not damaged as seen by morphological and functional criteria. Indeed, immediately before their use, washed platelets were observed by light microscopy and were investigated, after suspension in autologous plasma, in their aggregation response to adenosinediphosphate (ADP) which was found to be identical to that of the corresponding PRP.

Platelet mediated antibacterial activity

In order to investigate the effect of platelets on bacteria, portions of PRP and bacteria (*S. typhi* Ty-2, *S. minnesota* Rb, *S. minnesota* Re, and *S. aureus*) were incubated at different ratios for 1 hr at 37 °C on a rocking platform. Afterwards, equal aliquots of the incubation mixtures were streaked in triplicate on the entire surface of trypticase soy agar plates. The plates were incubated at 37 °C for 18–24 hr, and the number of colonies formed was evaluated. Any plate count that was less than 50% or over 150% of the average for the other two was not used. The evaluation of the antibacterial activity (AA) of platelets was performed according to the formula:

$$\%AA = 100 - 100 \times \frac{\text{No of CFU of experimental plates}}{\text{No of CFU of control plates without platelets}}$$

As a control, plates were streaked with corresponding amounts of bacteria alone and with autologous platelet poor plasma (PPP). Similar experiments were performed with washed platelets to avoid the possible interference of plasma factors. When washed platelets were used, control plates were overlaid with the corresponding volume of buffered solution in which platelets had been previously suspended. AA was calculated according to the above formula.

In another series of experiments platelet-bacteria mixtures, after 1 hr incubation at 37 °C, were divided into halves before plating. One portion was plated just after division, while the other was plated after lysis of platelets in 1% Triton X-100 (Sigma Chemical Company, St. Louis, Mo, USA). At this concentration of Triton X-100 bacterial growth was not affected. After overnight incubation at 37 °C, the antibacterial activity was evaluated.

Results

In preliminary experiments, *Salmonella typhi* Ty-2 and *Salmonella minnesota* Rb and Re respectively were incubated with human PRP and with autologous PPP. In both cases bacterial growth was markedly inhibited ($95 \pm 3\%$). This finding is in accordance with the well known antibacterial activity of human plasma [7]. When the S strain *Salmonella typhi* Ty-2 was incubated with washed platelets, the antibacterial activity was maximally expressed by the 0.05 washed platelet-microorganism ratio. On the contrary, no antibacterial activity was seen when either R strains (*Salmonella minnesota* Rb and *Salmonella minnesota* Re) or the typical gram-positive *Staphylococcus aureus* were incubated at different ratios with washed platelets (Fig. 1).

To further characterize the antibacterial activity exhibited by platelets, we have utilized *Salmonella typhi* Ty-2 in its exponential growth phase. In this experimental condition, the growth of bacteria preincubated with washed platelets was initially depressed, while in control bacteria organisms

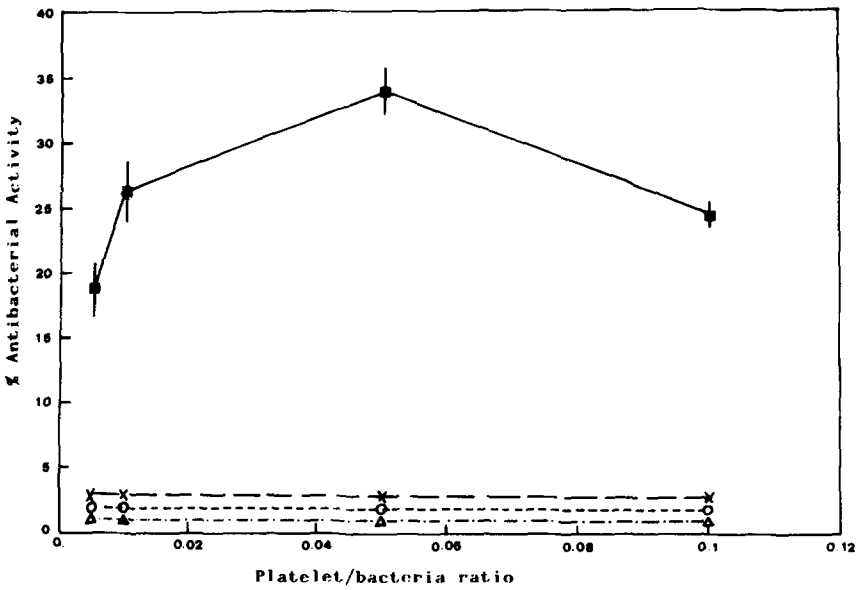

Figure 1
Effect of washed human platelets on bacterial growth expressed as % of antibacterial activity (see Materials and methods). Results are the mean of six separate experiments ±SEM. ■ *Salmonella typhi* Ty-2; × *Salmonella minnesota* R345 (Rb); ○ *Salmonella minnesota* R595 (Re); △ *Staphylococcus aureus*.

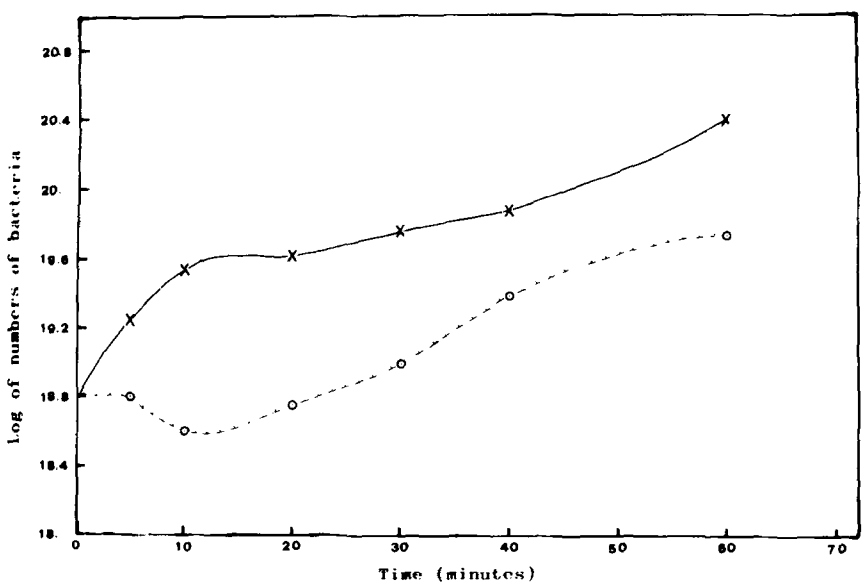

Figure 2
Salmonella typhi Ty-2 growth with (○) and without (×) preincubation with human washed platelets.

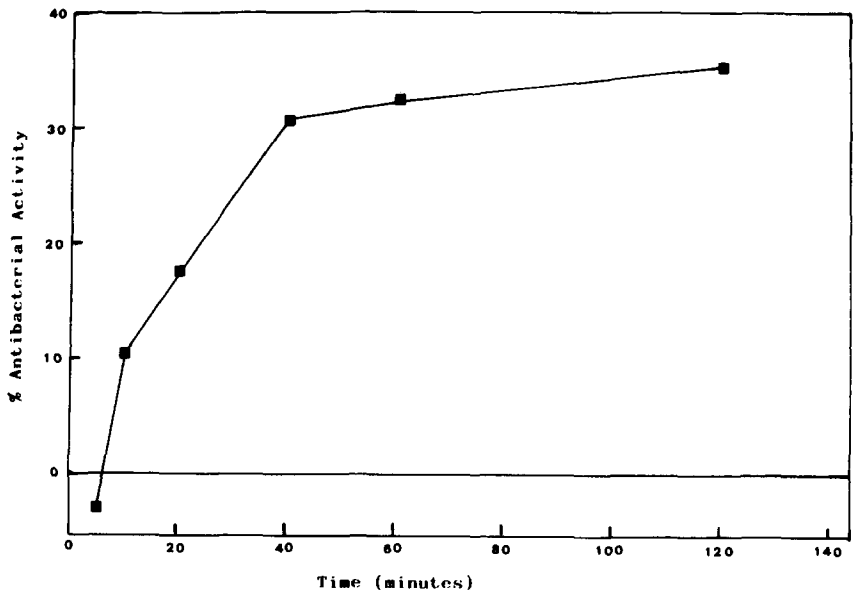

Figure 3
Effect of human washed platelets on *Salmonella typhi* Ty-2 growth at different intervals of incubation time (platelet/bacteria ratio 0.05:1).

Table 1
Pretreatment of washed human platelets with Triton X-100 and effects on antibacterial activity.

Samples	% Antibacterial activity*
Washed platelets-bacteria**	40 ± 1.2
Washed platelets incubated with bacteria and treated with Triton-X 100	36 ± 0.9
Washed platelets treated with Triton-X 100 and incubated with bacteria	0

* Values are computed as the mean ± SEM of 5 separate experiments.
** Experiments were run at the optimal ratio (Platelet/Bacteria 0.05/1).

grew according to a classical pattern. A typical experiment is illustrated in Fig. 2.
In another series of experiments platelet-mediated antibacterial activity at the optimal ratio 0.05/1 was evaluated at different intervals of incubation time. Fig. 3 shows that the antibacterial activity increased with the length of incubation.
In order to rule out the possibility that bacteria were only trapped within platelets and in such a way inhibited in their growth, washed platelet-bacteria mixtures were incubated with Triton-X 100 at a concentration which is lytic for platelets only (see Materials and Methods). No significant difference was found between samples pretreated with Triton-X 100 and untreated samples in terms of antibacterial activity. Moreover, when platelets were treated with Triton-X 100 before their incubation with bacteria, they could not exhibit any antibacterial activity (Table 1).
Finally, to investigate whether any antibacterial agent was released from platelets during their incubation with *Salmonella typhi*, the supernatant of washed platelet-bacteria incubation mixtures was added to a suspension of *Salmonella typhi* Ty-2. In repeated experiments, bacterial growth was not influenced by the addition of supernatants from different platelet-bacteria ratio incubation mixtures (data not shown).

Discussion

In preliminary experiments, we have studied the interaction of bacteria with PRP in order to evaluate platelets in their physiological environment. In these experimental conditions, bacterial growth is

inhibited as well as in the presence of PPP, this implying the role of plasma factor(s) in bacterial killing [7]. In subsequent experiments, the use of washed platelets allows the elimination of plasma factors, this precluding the formation of fibrin clots which might interfere with the recovery of bacteria. In fact, plasma proteins (i.e. immunoglobulins and components of the classic and alternate complement system) are able to interact with bacteria [8] and the interaction of human fibrinogen with staphylococci, leading to formation of clumps, has been described by Hawiger et al. [9]. Moreover, in our washed platelet suspensions contamination with neutrophils which are capable to engulf and kill bacteria [10] is negligible (less than 1%) as assessed by light microscopy. Our data clearly show that human washed platelets possess an antibacterial activity against gram-negative bacteria which is restricted to smooth strains. The fact that this activity is not directed to rough strains may indicate the requirement for the O-polysaccharide chain for its expression. On the other hand, the circumstance that gram-positive organisms are unaffected is consistent with the observation by Clawson and White [3] who found that *Staphylococcus aureus* and *Streptococcus faecalis,* although agglutinated, were not killed by washed human platelets. This finding should not be surprising since only gram-negative bacteria have onto their surface endotoxin (lipopolysaccharide) which can interact both *in vivo* or *in vitro* with receptors on platelets of different species [11–14]. To define the antibacterial activity of platelets and exclude that bacteria were only sequestered within platelet aggregates or incorporated into them, the bacterial survival was evaluated after lysis of platelets in Triton-X 100. Indeed, this experiment rules out the possibility of a bacterium entrapment within platelets since the recovery of bacteria in terms of CFU is similar either in samples pretreated with Triton X-100 or in untreated samples (Table 1). This would suggest that bacterial growth is actually inhibited by the contact with washed platelets and bacteria are definitively damaged. In addition, when triplicate samples of identical incubation mixtures of human washed platelets and *Salmonella typhi* Ty-2 (at the optimal ratio P/B 0.05:1) were taken at different intervals of time, the antibacterial activity appears to be dependent on the lenght of incubation. This would suggest that antibacterial activity increases with a more prolonged contact between the two components of the reaction.

An indirect evidence of platelet antibacterial activity emerges from experiments in which *Salmonella typhi* Ty-2 in its exponential growth phase has been preincubated with washed platelets. In fact, the pattern of bacterial growth is different from that observed in controls since the addition of platelets causes an initial retardation of the growth.

Finally, another important point was to rule out the possibility that following bacteria-washed platelet contact, intraplatelet factors with antibacterial activity might be released. Our experiments support the notion that a close contact between platelets and bacteria, as suggested by Clawson [15], is necessary for the occurrence of the described activity since all cell-free bacteria-platelet incubation media have no effect on bacterial growth.

Taken together, these data suggest a further contribution of platelets in the host protection to gram-negative bacteria. The effects of antiplatelet drugs and antienzymatic molecules in order to clarify the mechanisms by which platelets exert their antibacterial activity are in progress.

Acknowledgements

We wish to thank Dr. G. De Gaetano, Istituto di Ricerche Farmacologiche "Mario Negri", Milan, Italy, for his critical revision of the present paper. This research was supported in part by Consiglio Nazionale delle Ricerche, Rome, Italy.

Received 24 August 1987; accepted 2 May 1988

References

[1] C. C. Clawson, G. H. R. Rao and J. G. White, *Platelet interaction whit bacteria. IV. Stimulation of the release reaction.* Am. J. Pathol. *81,* 411–419 (1975).

[2] S. B. Smith, *Platelet in host resistance: in vitro interaction of platelets, bacteria, and polymorphonuclear leukocytes.* Blut *25,* 104–107 (1972).

[3] C. C. Clawson and J. G. White, *Platelet interaction with bacteria. II. Fate of bacteria.* Am. J. Pathol. *65,* 381–395 (1971).

[4] R. A. Kahn and L. J. Flinton, *The relationship between platelets and bacteria.* Blood *44,* 715–721 (1974).

[5] C. J. Czuprynski and E. Balish, *Interaction of rat platelets with Listeria monocytogenes.* Infect. Immun. *33,* 103–108 (1981).

[6] P. N. Walsh, *Platelet washing by albumin density gradient separation (ADGS).* In *Platelet function and thrombosis. A*

review of methods (Ed. P.M. Mannucci and S. Gorini). Plenum Press, New York-London 1982.

[7] D. C. Morrison and R. J. Ulevitch, *The effects of bacterial endotoxins on host mediation systems*. Am. J. Pathol. *93*, 527–617 (1978).

[8] I. Roitt, *Essential Immunology* (ed. 2). Blackwell. Oxford (1974).

[9] J. Hawiger, D. K. Hammond, S. Timmons and A. Z. Budzynski, *Interaction of human fibrinogen with staphylococci: presence of a binding region on normal and abnormal fibrinogen variants and fibrinogen derivatives*. Blood *51*, 799–812 (1978).

[10] S. J. Klebanoff, *Antimicrobial mechanisms in neutrophilic polymorphonuclear leukocytes*. Seminar. Hemat. *12*, 117–142 (1975).

[11] G. F. Springer and J. C. Adye, *Endotoxin binding substances from human leucocytes and platelets*. Infect. Immun. *12*, 978–986 (1975).

[12] G. F. Springer and J. C. Adye, *Endotoxin interaction with phosphatides from human leukocytes and platelets and with lymphoid cells of susceptible and resistant mice*. In *Microbiology* (Ed. D. Schlessinger). Am. Soc. Microbiol., Washington 1977.

[13] D. C. Morrison and Z. G. Oades, *Mechanisms of lipopolysaccharide-initiated rabbit platelet responses. II. Evidence that lipid A is responsible for binding of lipopolysaccharide to the platelet*. J. Immunol. *122*, 753–758 (1979).

[14] J. Hawiger, A. Hawiger, S. Steckley, S. Timmons and C. Cheng, *Membrane changes in human platelets induced by lipopolysaccharide endotoxin*. Br. J. Haematol. *35*, 285–299 (1977).

[15] C. C. Clawson, *Platelet interaction with bacteria. III. Ultrastructure*. Am. J. Pathol. *70*, 449–464 (1973).