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ADHESION OF PLATELETS TO MICROCAPSULES AND THE ROLE OF COMPLEMENT

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Rabbit platelets rapidly adhered to carboxylated polyamide microcapsules through the adsorbed layer of plasma components. These components were found to be heat-labile proteins, which exist in fresh serum, and to demand calcium ions to function. These findings ascribed the components to the complement system. In fact, a good correlation was obtained between platelet adhesion to, and complement fixation by, the microcapsules. Moreover, the activation of the complement system by the microcapsules was assumed to proceed via the classical pathway. It was concluded that adhesion of platelets to the microcapsules is brought about by immune adherence.

Introduction

In the course of the development of microcapsule-type artificial red blood cells [1], the disadvantage was noted that platelets rapidly adhere to these cells [2–4]. Moreover, these artificial cells join together through the adhered platelets to form large aggregates [3] which may give rise to thrombosis should they be actually introduced into the blood stream.

It is generally accepted that plasma proteins are adsorbed to foreign substances as soon as the latter come in contact with blood, and that the adsorbed proteins control the biocompatibility of the substances [5–8]. There are many papers on the compatibility of synthetic polymer films with blood, directing attention to the adsorption of such major blood proteins as albumin, γ-globulin and fibrinogen [7–12]. However, our earlier papers indicated that these three proteins can hardly be regarded as the plasma factors responsible for controlling adhesion of platelets to the microcapsules, but suggested instead an important role for complement [4,13]. The complement system is well known for its protective response to exogenous cells such as bacteria at the initial stage of infection. Therefore, this work was aimed at verifying the role of complement in controlling adhesion of platelets to microcapsules.

Experimental

Preparation of microcapsules. Polyamide microcapsules containing 5% (w/v) dextran solution (Pharmacia, $\overline{M}_r$ 500000) as core material were prepared by the interfacial polycondensation between terephthaloyl dichloride and diamine according to the procedure described earlier [2]. The diamine used here was a mixture of L-lysine and piperazine in the molar ratio of 0.4. This ratio was chosen for the microcapsules obtained to have an electrophoretic mobility similar to that of rabbit erythrocytes (approximately $-1.4\ \mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$ in a medium of pH 7.4 and $I = 0.01$). The microcapsules so prepared were thoroughly dialyzed against water and were finally dispersed

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in Ca^{2+}- and Mg^{2+}-free Tyrode buffer solution (pH 7.4). The mean diameter of microcapsules was found 10.2 ± 4.0 μm, and the concentration of the microcapsule suspension was then adjusted to present a total surface area of about $3 \cdot 10^{10}$ $\mu m^2/ml$.

Preparation of platelet suspension. Nine volumes of blood were withdrawn from the auricular artery of a rabbit into a syringe containing 1 vol. of an anticoagulant solution, 130 mM sodium citrate or 100 mM disodium EDTA solution. This anticoagulated blood was immediately centrifuged at about $100 \times g$ for 10 min to give platelet-rich plasma. The platelet-rich plasma was further centrifuged at about $1000 \times g$ for 20 min to yield platelet-free plasma and platelet pellet. The pellet was washed three times with anticoagulant-citrate-dextrose solution and was finally dispersed in Ca^{2+},Mg^{2+}-free Tyrode buffer solution (pH 7.4). The concentration of platelets in platelet-rich plasma or washed-platelet suspension was always adjusted to be $(1.5–2.0) \cdot 10^8$ platelets/ml. Separately, rabbit blood was collected without any anticoagulant and was allowed to stand for 3 h at 37°C. After centrifuging the coagulated blood, the supernatant was used as fresh serum.

Determination of adhesion of platelets to microcapsules. The principle and the method of measuring platelet adhesion have been described in detail in our earlier paper [2]. In brief, 200 μl of the microcapsule suspension were added to 800 μl of the platelet suspension. Aliquots of 20 μl each were pipetted out of the mixture at given time intervals, and the number of platelets in each sample remaining unadhered to the microcapsules was counted with a Coulter counter, model ZB-1 (Coulter Electronic Inc.).

Coating of microcapsules with plasma or serum. An aliquot of the microcapsule suspension was mixed with an equal volume of plasma or serum. The mixture was incubated for 30 min at 37°C and was washed twice on the centrifuge. The coated microcapsules were finally dispersed in Ca^{2+}, Mg^{2+}-free Tyrode buffer solution (pH 7.4).

Assay of complement titer. Complement titers in the samples were measured according to a modification of Mayer's procedure [14]. Namely, 0.4 ml of sheep erythrocyte suspension ($5 \cdot 10^8$ cells/ml) sensitized with rabbit anti-sheep hemolysin (purchased from Ishizu Pharm. Co., Japan) was added to 2.6 ml each of serially diluted samples. The mixtures were incubated for 1 h at 37°C and centrifuged. Then the degree of hemolysis in each sample was determined colorimetrically at 541 nm, and the quantity of complement which hemolysed 50% of erythrocytes (CH_{50}) was calculated.

Results and Discussion

In Fig. 1 are shown the reductions in platelet number with time caused by the addition of microcapsule suspension to citrate-anticoagulated and to EDTA-anticoagulated platelet-rich plasmas. When citrate was used as an anticoagulant, the number of free platelets rapidly decreased after a short lag time, showing a time-course quite similar to that obtained previously [2–4]. On the contrary, EDTA almost completely inhibited adhesion of platelets to the microcapsules. This difference would be brought about by the difference in the amounts of divalent cations (presumably Ca^{2+} and/or Mg^{2+}) in the plasma, since the effectiveness of EDTA as a chelator is far stronger than that of citrate under the conditions employed here; divalent cations should be a requisite for platelets to adhere to the microcapsules.

It is well known that, when foreign substances

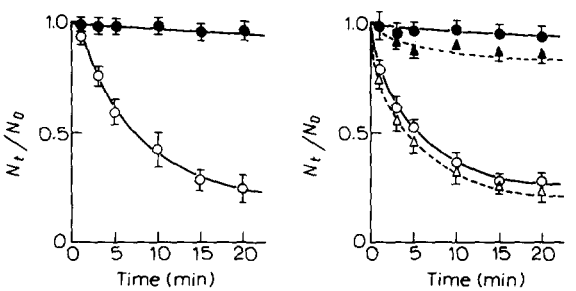

Fig. 1. Effects of anticoagulants on adhesion of platelets in platelet-rich plasma to microcapsules. Anticoagulant: citrate (○), EDTA (●); Vertical bars denote S.D. of the mean for six separate determinations. The ordinate represents the ratio of the number of single platelets remaining not adhered at a given time, N_t, to those before those adding microcapsules, N_0.

Fig. 2. Adhesion of platelets to plasma-coated microcapsules. Microcapsules: Coated with citrate-anticoagulated plasma, added to EDTA-anticoagulated platelet-rich plasma (○) and washed-platelet suspension (△); coated with EDTA-anticoagulated plasma, added to EDTA-anticoagulated platelet-rich plasma (●) and washed-platelet suspension (▲). Vertical bars and ordinate are as in Fig. 1.

come in contact with blood, plasma components are rapidly adsorbed to their surfaces in advance of platelet adhesion [5–8]. Therefore, the microcapsules were precoated with citrate- or EDTA-anticoagulated plasma, the coated microcapsules were added to EDTA-anticoagulated platelet-rich plasma, and platelet adhesion was measured. The results are given in Fig. 2. As in the case of the microcapsules coated with EDTA-anticoagulated plasma, platelets hardly adhered to the microcapsules. However, although practically no divalent cations are assumed to exist in EDTA-anticoagulated platelet-rich plasma, an immediate and rapid decrease in free platelets was observed when the microcapsules coated with citrate-anticoagulated plasma were added to the platelet-rich plasma. In order to exclude additional effects of plasma, these coated microcapsules were added to the washed-platelet suspension and the platelet adhesion was compared with that in EDTA-anticoagulated platelet-rich plasma (Fig. 2). This clearly indicates that it is the adsorbed plasma layer on the microcapsules and not the microcapsule surface itself which determines platelet adhesiveness. The formation of the layer accessible to platelet adhesion requires divalent cations, but once it is formed on the microcapsules, divalent cations in the medium are no longer necessary for platelet adhesion. In addition, the short lag time revealed in the case of adhesion of platelets to the bare microcapsules in citrate-anticoagulated platelet-rich plasma (Fig. 1) may be assigned to the period necessary for the microcapsules to acquire such a plasma layer accessible to platelet adhesion.

The formation of the adsorbed plasma layer may be related to blood coagulation activity, since divalent cations strongly affect the activity. The effects of serum coating were therefore investigated. Fig. 3 shows that the adhesiveness of washed platelets to the serum-coated microcapsules is quite similar to that to the citrate-anticoagulated plasma-coated microcapsules, although the former is slightly higher than the latter. The figure also reveals that washed platelets hardly adhere, either, to the microcapsules coated with the serum containing 10 mM of EDTA. Therefore, it seems that there is very little connection bteween blood coagulation activity and adhesion of platelets to the microcapsules, and that certain components exist in serum which are adsorbed to the microcapsules to control platelet adhesiveness in the presence of divalent cations.

In an attempt to find out what kind or kinds of serum components affect platelet adhesion, fresh serum was treated with α-chymotrypsin (100 μg/ml serum) (Bovine Pancreas, P-L Biochemicals Inc.) for 30 min at 37°C. The microcapsules were then coated with the enzyme-treated serum, and adhesion of platelets to the coated microcapsules was measured. In addition, the microcapsules precoated with fresh serum were similarly treated with α-chymotrypsin and adhesion of platelets to these microcapsules was examined. The results are given in Fig. 4, in which the effect of coating with heat-inactivated serum (fresh serum heated at 56°C for 30 min) is also presented. These modes of treatment gave rise to considerable inhibition of adhesion of washed-platelets to the microcapsules. As α-chymotrypsin is a typical proteolytic enzyme, the components which affect platelet adhesion would be proteins that are liable to be digested by α-chymotrypsin even after being adsorbed. Moreover, these proteins seem to be less resistant to heat.

The preceding findings, that the components which are adsorbed to the microcapsules to control

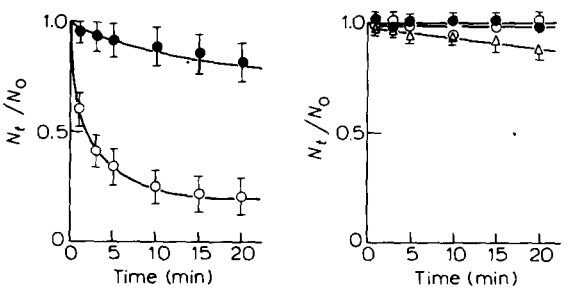

Fig. 3. Platelet adhesion in washed platelet suspension to serum-coated microcapsules. Microcapsules: Coated with intact serum (○) and EDTA-containing serum (●). Vertical bars and ordinate as in Fig. 1.

Fig. 4. Washed-platelet adhesion to microcapsules coated with enzyme-treated sera. Microcapsules: Coated with α-chymotrypsin-treated serum (○); coated with heat-inactivated serum (△); treated with α-chymotrypsin after coated with intact serum (●). Vertical bars denote S.D. of the mean for three separate determinations. The ordinate is the same as that in Fig. 1.

platelet adhesiveness are heat-labile proteins in fresh serum and demand divalent cations, would indicate complement to be the most probable candidate. Complement is well known to participate in the initial protective response of a living body in case of bacterial infections through immune adherence [15]. Therefore, fresh serum was mixed with heat-aggregated γ-globulin (10 mg/ml serum) or zymosan (2 mg/ml serum), and incubated for 30 min at 37°C. Heat-aggregated γ-globulin (rabbit γ-globulin heated at 63°C for 30 min and freeze-dried) and zymosan (from *Saccharomyces cerevisiae*, Sigma) are known to activate complement and fix it on their surfaces [16–18].

The titers remaining in the sera treated with heat-aggregated γ-globulin and zymosan were found to be 49% and 43% of the titer for the fresh serum, respectively. Fig. 5 shows that the adhesion of washed platelets to the microcapsules coated with these modified sera is less pronounced than that to those coated with the fresh serum, presumably because the amount of complement available for platelet adhesion to the microcapsules is diminished to some extent by the action of these activators. Furthermore, in order to ensure that the microcapsules activate and fix complement on their surfaces, the microcapsules were coated with fresh serum and adhesion of washed platelets to the coated microcapsules as a function of the latters' concentration was examined. In addition, suspensions of the microcapsules of different concentrations were mixed with fresh serum and the mixtures were incubated for 30 min at 37°C. The incubated mixtures were then centrifuged and the

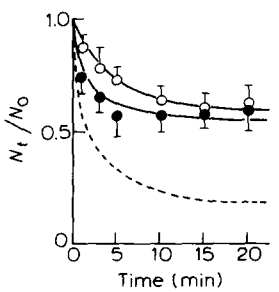

Fig. 5. Effects of complement activators on washed-platelet adhesion to microcapsules. Microcapsules: Coated with serum mixed with heat-aggregated γ-globulin (○) and zymosan (●); Coated with intact serum (broken line). Vertical bars and the ordinate represent the same as those in Fig. 4.

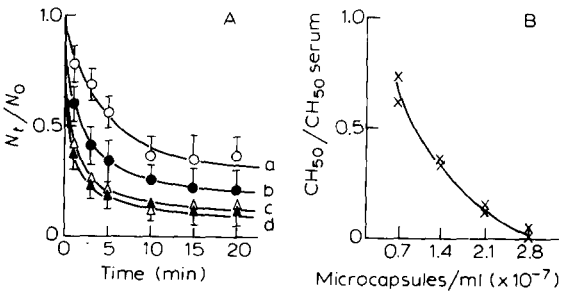

Fig. 6. (A) Adhesion of washed platelets to fresh serum-coated microcapsules. (B) reduction of complement titer. Concentrations of serum-coated microcapsule suspension: (a) $0.7 \cdot 10^7$; (b) $1.4 \cdot 10^7$; (c) $2.1 \cdot 10^7$; (d) $2.8 \cdot 10^7$/ml. Vertical bars and ordinate in A are as in Fig. 1. The ordinate in B represents the ratio of the titer for treated sera (CH_{50}) to that for intact serum (CH_{50} serum).

complement titers remaining in the supernatants was assayed. Adhesion of platelets to the serum-coated microcapsules increased in proportion to the amount of the coated microcapsules added (Fig. 6A) and the complement titer remaining was also dependent on the amount of the microcapsules (Fig. 6B). A good correlation can be observed between the adhesion of platelets and the diminution of complement. These results suggest that the microcapsules really activate and fix complement on their surfaces, and platelets recognize the adsorbed and activated complement to adhere to the microcapsules. Among the components of complement, the activated third component, C3b, will be the most probable one, since rabbit platelets are known to have C3b receptors on their membrane [19]. Moreover, it is reported that the binding activity of C3b is extremely short-lived [20,21], and that the C3b is easily degraded by C3b inactivator, β1H, and other proteolytic enzymes in serum, unless it binds to the activators [22–24].

In order to verify that C3b is the most probable candidate for platelet adhesion to the microcapsules, fresh serum was premixed with the cobra venom factor (*Naja naja kaouthia*, Cordis) for 60 min at 30°C, which is well known to activate and deplete selectively the third component, C3 [25]. In fact, 10 U of the cobra venom factor were found to reduce the complement titer of fresh serum to about 13%. Then, the microcapsules were coated with the treated serum and adhesion of the washed platelets to the coated microcapsules was mea-

sured. The results are shown in Fig. 7. Compared to the fresh serum coating (dashed line), the cobra venom factor-treated serum coating greatly inhibited platelet adhesion.

There are two pathways of complement activation, the classical and the alternative [26,27]. The former demands Ca^{2+} and Mg^{2+}, but the latter requires Mg^{2+} alone. In an attempt to discover by which pathway the microcapsules activate complement, they were coated with serum containing 10 mM Mg^{2+}-EGTA and adhesion of washed platelets to the coated microcapsules was examined, since EGTA chelates Ca^{2+} selectively. Furthermore, reductions of the complement titer caused by the additions of the microcapsules to EDTA serum and to Mg^{2+}-EGTA serum were assayed. At the same time, the effect of zymosan on the titer was examined, since zymosan is a typical activator through the alternative pathway. Fig. 8 indicates that platelet adhesiveness to the microcapsules coated with Mg^{2+}-EGTA serum is slightly higher than that to those coated with EDTA serum, but is much lower than that to those coated with intact serum. Meanwhile, reduction of the complement titer was not so pronounced for the microcapsules mixed with EDTA serum (82% of the value for intact serum) and with Mg^{2+}-EGTA serum (71% of the value for intact serum) as for zymosan mixed with Mg^{2+}-EGTA serum (28% of the value for intact serum). Thereofre, it is likely that the microcapsules activate complement predominantly through the classical pathway, although a slight activation through the alternative one cannot be ruled out.

In summary, the process of adhesion of platelets to the microcapsules is assumed to be as follows. The first component of complement, C1, is adsorbed and activated on the microcapsule surface in the presence of Ca^{2+} as soon as the microcapsules come in contact with blood. Then, the serial activation steps proceed on the microcapsule surface and, when the activated third component, C3b, is formed, platelets recognize it and adhere to the microcapsules through the C3b receptors on the platelet membrane. As for the initial step of C1 adsorption and activation, it is not clear at present to which sites of the microcapsules the C1 component is adsorbed or by what mechanism it is activated. However, in view of the previous finding that platelets in plasma adhered more to the γ-globulin-coated microcapsules than to the bare ones [28], adsorption of γ-globulin to the microcapsules may be a prerequisite to C1 adsorption and activation, as in the initial bactericidal step of complement.

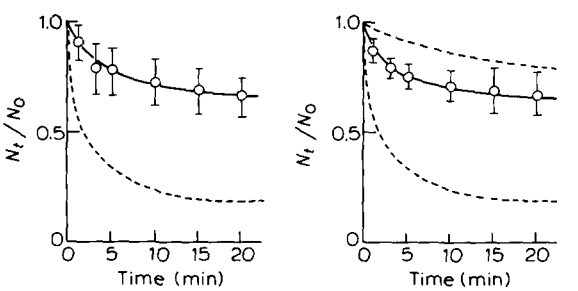

Fig. 7. Adhesion of washed platelets to the microcapsules coated with cobra venom factor-treated serum. Vertical bars denote S.D. of the mean for three separate determinations. Ordinate in Fig. 1. The dashed line shows the results for the fresh serum-coated microcapsules.

Fig. 8. Effect of calcium ions on adhesion of washed platelets to microcapsules. Microcapsules: Coated with mg^{2+}-EGTA serum (solid line); coated with EDTA serum (upper broken line); coated with intact serum (lower broken line). Vertical bars and ordinate as in Fig. 1.

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