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Effects of Plasma Components on Platelet Adhesion to Microcapsules

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Adhesion of rabbit platelets to microcapsules having different numbers of carboxyl groups was examined. The platelets adhered more easily to microcapsules having higher negative charges than to those having lower ones. It was strongly suggested that this trend was caused by plasma components adsorbed on the microcapsules; the difference in surface negative charge caused a difference in adsorption of plasma components, which would in turn result in the difference in platelet adhesion.

Among various plasma components, the major proteins, albumin, γ-globulin and fibrinogen, were all found to inhibit platelet adhesion to the microcapsules, while fresh rabbit serum facilitated it, irrespective of surface negative charge. On the other hand, heat-inactivated rabbit serum was found to inhibit platelet adhesion almost completely. These findings led us to conclude that certain complement adsorbed on the microcapsule surface facilitate platelet adhesion, and the difference in platelet adhesion, which is dependent on the surface negative charge of the microcapsules in the presence of plasma, can be explained in terms of competitive adsorption of the complement and fibrinogen.

Keywords——microcapsule; platelet adhesion; surface charge; plasma protein; complement adsorption

One promising application of microcapsules in the medical field is as artificial red blood cells to enclose mammalian hemolysate or hemoglobin solution within their ultrathin polymer membranes.[1] However, earlier studies[2] on platelet adhesion have shown that platelets adhered easily to the surfaces of carboxylated and sulfonated poly(1,4-piperazinediyl-terephthaloyl) membranes. These findings suggest that these sorts of artificial red blood cells may cause a serious disease such as thrombosis or angiostenosis if they are actually introduced into the blood stream. Moreover, it was also found[2] that platelet adhesion was greater on the surfaces of high negative charge than on those of low negative charge. Difference in adsorption of plasma components was assumed to be the primary cause of the difference in platelet adhesion which was dependent on the surface negative charge of the microcapsules. However, it is still not clear what kinds of plasma components affect platelet adhesion.

Therefore, in this work, microcapsules with different numbers of carboxyl groups on their surfaces were prepared and platelet adhesion on these microcapsules was examined with a view to identifying the plasma components affecting platelet adhesion.

Experimental

Preparation of Microcapsules——Three kinds of microcapsules having different numbers of carboxyl groups on their surfaces were prepared by interfacial polycondensation between terephthaloyl dichloride and diamines according to the method described earlier.[2] The diamines used here were mixtures of piperazine and L-lysine. When the ratio of L-lysine to piperazine in the diamine mixture was fixed at 0, 0.2 and 0.6, the microcapsules obtained were termed MC-1, MC-2 and MC-3, respectively. All kinds of MC prepared were thoroughly dialyzed against water and were finally dispersed in Tyrode solution (pH 7.4). The size and number of microcapsules in each MC suspension were measured and each suspension was diluted with the Tyrode solution to have a total surface area of

Table I. Size and Electrophoretic Mobility of MC

	L-Lysine content in diamine mixture (M/M)	Mean diameter (μm)	Electrophoretic mobility[a] ($\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$)	
			Bare	Plasma coated
MC-1	0	10.6 ± 3.8	-0.88 ± 0.08	-0.88 ± 0.02
MC-2	0.2	10.5 ± 4.0	-1.13 ± 0.10	-0.93 ± 0.08
MC-3	0.6	10.2 ± 4.0	-1.32 ± 0.06	-0.92 ± 0.07

a) Electrophoresis was carried out at 25 °C in medium (pH 7.4 and ionic strength 0.01) containing 10% w/v sucrose to prevent the MC sedimenting during the measurements.

approximately 2×10^{10} μm^2/ml. Electrophoresis of each MC was carried out using a microelectrophoresis apparatus (Rank Brothers Inc.) under conditions of pH 7.4 and ionic strength 0.01, and the mobilities obtained are listed in Table I.

Preparation of Platelet Suspension——Citrate-anticoagulated rabbit blood was centrifuged at 1000 rpm for 10 min to give platelet-rich plasma (PRP). The PRP was further centrifuged at 3000 rpm for 20 min to yield platelet-free plasma and the platelet pellet. Then, the pellet was gently redispersed into ACD (acid–citrate–dextrose) solution, and the suspension obtained was again centrifuged at 3000 rpm for 20 min. This washing procedure was repeated three times and the resultant pellet was finally dispersed in the Tyrode buffer solution.

Determination of Platelet Adhesion to MC——The principle and the method of measuring platelet adhesion were described in detail in our earlier paper.[2] In short, 200 μl of the MC suspension was added under constant speed stirring (800 rpm) to 800 μl of the platelet suspension containing $1.5—2.0 \times 10^8$ platelets/ml, aliquots of 20 μl each were pipetted out of the mixture, and the numbers of platelets remaining not adhered to the MC were counted with a Coulter counter, model ZB-I (Coulter Elec. Inc.), at suitable time intervals.

Coating of MCs with Plasma, Serum and Proteins——An aliquot of each MC suspension was mixed with an equal volume of plasma, serum or protein solutions. The mixtures were incubated for 30 min at 25 °C and centrifuged at 2000 rpm for 15 min to settle the MC. This procedure was repeated three times and the MC precipitates were finally dispersed with the Tyrode solution to give the original microcapsule concentrations. The proteins, albumin, γ-globulin and fibrinogen, were all separated from rabbit blood by acid–ethanol fractionation,[3] a salting-out technique using ammonium sulfate[4] and Cohn's ethanol fractionation,[5] respectively. These proteins were dissolved in the Tyrode solution and their concentrations were adjusted approximately to those in plasma.

Scanning Electron Microscopic Observation of Adhering Platelets——Each of the MC membranes was dissolved in *m*-cresol, the solution was spread on aluminum foil, and *m*-cresol was evaporated off in a vacuum. The foil was washed with ethanol until no smell of *m*-cresol was detectable. The platelet suspension was left in contact with the foil for 10 min and then washed away. After several washings with the Tyrode buffer, the adhering platelets were fixed with 1 w/v% glutaraldehyde-Tyrode solution. The fixed samples were dried by serial ethanol dehydration, and observed with a scanning electron microscope (SEM) (JSM-T20, JEOL).

Results and Discussion

The decrease of platelets with time after mixing each of the MC suspensions and PRP is shown in Fig. 1. This figure indicates that the platelets adhered more readily to MC having higher surface negative charge than to MC having lower one, the trend being much the same as that observed previously.[2] It is well known that, when foreign surfaces come in contact with blood, plasma proteins are rapidly adsorbed onto the surfaces in advance of platelet adhesion.[6] Therefore, each of the MC suspensions was premixed with plasma, and the electrophoretic mobility of and platelet adhesion to the plasma-coated MCs were measured. The results are given in Table I and Fig. 2. The plasma coating was found to reduce the difference in mobility among the bare MCs and to give a nearly identical mobility value, suggesting that considerable amounts of plasma components were adsorbed on the MC surfaces to shield the original negative charges. The decrease curves of platelets in Fig. 2 are quite similar to those in Fig. 1, although no appreciable difference in mobility was observed among the plasma-coated MCs. These findings imply that surface negative charge of MC

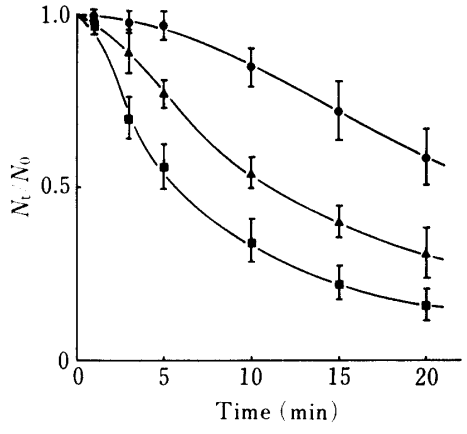

Fig. 1. Platelet Adhesion to MC in PRP

The ordinate represents the ratio of platelet number (N_t) at a given time to the initial value (N_0): ●, MC-1; ▲, MC-2; ■, MC-3. Each point shows the mean value of 6 experiments and each bar, the standard deviation.

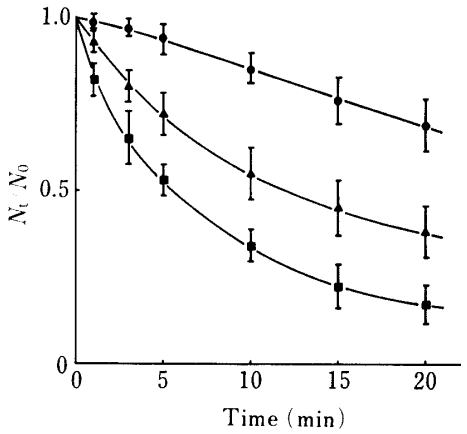

Fig. 2. Platelet Adhesion to Plasma-Coated MC in PRP

The ordinate and the symbols used here are the same as those in Fig. 1. Each point shows the mean value of 6 runs and each bar, the standard deviation.

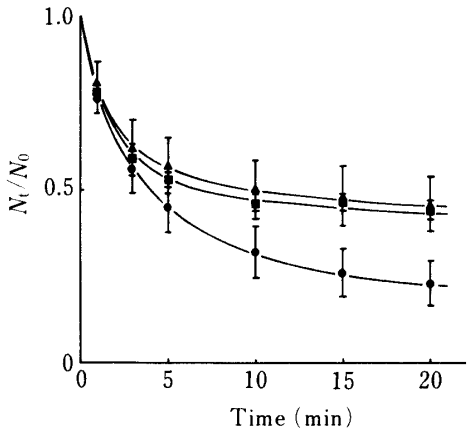

Fig. 3. Adhesion of Washed Platelets to MC

The ordinate and the symbols used here are the same as those in Fig. 1. Each point shows the mean value of 6 runs and each bar, the standard deviation.

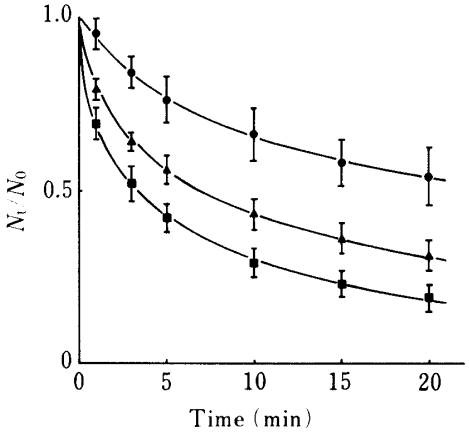

Fig. 4. Adhesion of Washed Platelets to Plasma-Coated MC

The ordinate and the symbols used here are the same as those in Fig. 1. Each point shows the mean value of 6 runs and each bar, the standard deviation.

affects platelet adhesion not directly, but through the adsorbed plasma layer.

In order to make clearer the role of plasma components, PRP was centrifuged to wash away the plasma components, and adhesion of the washed platelets to the MCs was examined. Figures 3 and 4 show the results of adhesion of the washed platelets to the bare and plasma-coated MCs, respectively. The washed platelets adhered rapidly to the bare MCs and no clear dependency of adhesion on the surface negative charge of MCs was observed. However, in the case of adhesion of the washed platelets to the plasma-coated MCs, the decrease of the number of platelets was again found to depend on the surface negative charge of the bare MCs. Scanning electron micrographs of the platelets adhering to the MC membrane are shown in Fig. 5. Here, only the platelets adhering to the MC-3 membrane are shown, since no definite differences in morphology could be discerned among the platelets adhering to the membranes of all MCs. However, morphological differences are evident between the adhering platelets in the presence (Fig. 5a) and absence (Fig. 5b) of plasma. It is supposed that these changes in morphology apparent in Fig. 5b were brought about not by the washing process but by adhesion to the MC membrane and the adsorbed layer served as a

kind of cushion between the platelets and the MC membrane. This is supported by Fig. 5c, from which it can be seen that the washed platelets undergo no serious morphological deformation on adhering to the plasma-coated MC membrane, although the shape of adhering platelets differs a little from that observed in Fig. 5a. These SEM observations and comparison of Fig. 4 with Fig. 2 indicate that the adsorbed plasma components exert a primary influence on platelet adhesion. As the washing process was found to cause no serious damage to the platelets, the following experiments were all conducted using washed platelets.

In an attempt to elucidate what kinds of plasma components affect platelet adhesiveness, adhesion of the washed platelets to serum-coated MCs was measured. The results are shown in Fig. 6, in which the abscissa represents the percentage of the adhering platelets 20 min after mixing suspensions of the coated MCs and the washed platelets. This figure shows that the serum coating facilitates platelet adhesion irrespective of the surface charge of MC, indicating that certain components in the serum are adsorbed on the MC and affect platelet adhesion. To identify the components, the major serum proteins, albumin and γ-globulin, were separated from serum and used to coat each of the MCs. Adhesion of the washed platelets to the coated MCs is shown in Fig. 7. Contrary to our expectation, both albumin and γ-globulin exhibited an inhibitory effect on the platelet adhesion. In this figure, it can be seen that albumin shows a lesser effect than γ-globulin. However, it was revealed in the previous paper[2b] that albumin was adsorbed on the microcapsules to a lesser extent than γ-globulin under the same conditions as those employed here, and albumin is reported[7] to be adsorbed reversibly on the synthetic polymer film, thus allowing the adsorbed albumin to exchange easily with that in the bulk solution. In fact, SEM observation reveals that the morphology of the platelets adhering to the albumin-coated MC membrane is similar to that on the bare MC membrane, suggesting

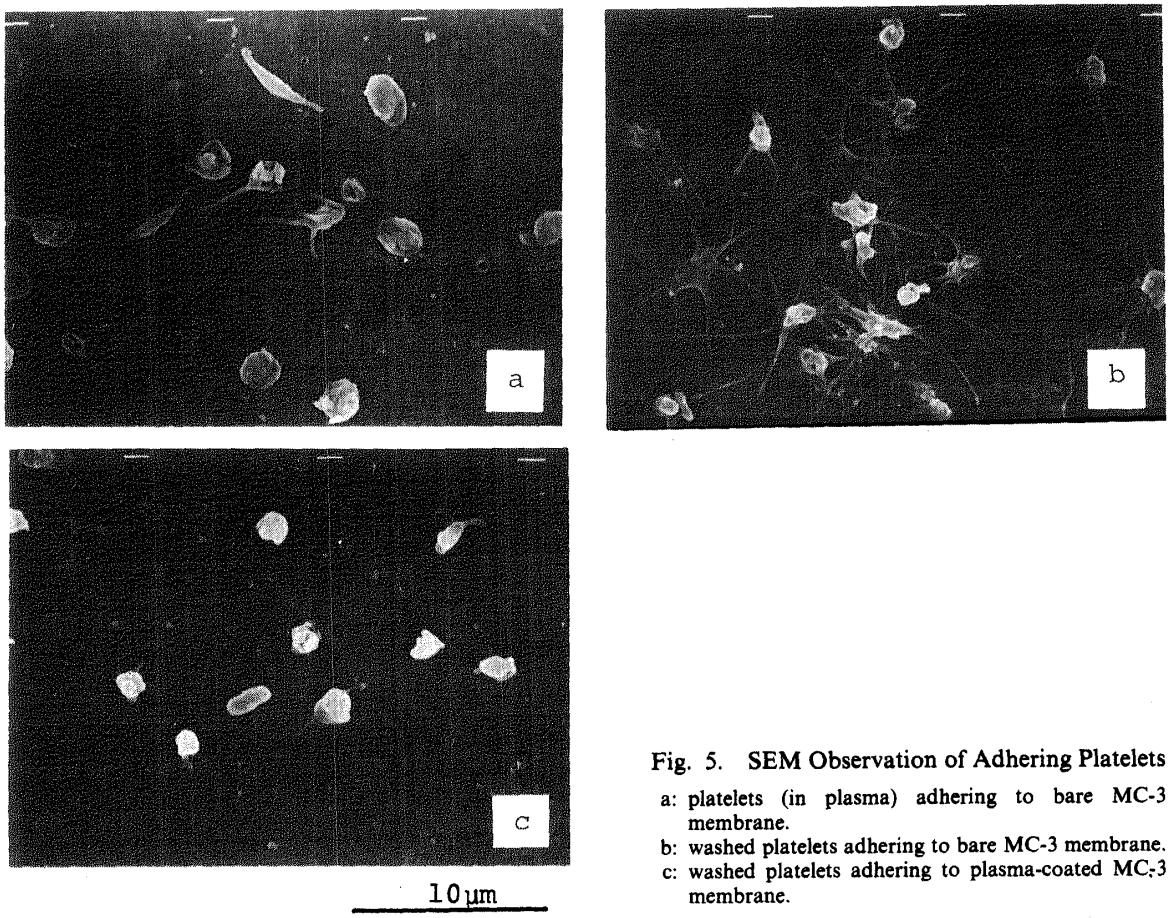

Fig. 5. SEM Observation of Adhering Platelets

a: platelets (in plasma) adhering to bare MC-3 membrane.
b: washed platelets adhering to bare MC-3 membrane.
c: washed platelets adhering to plasma-coated MC-3 membrane.

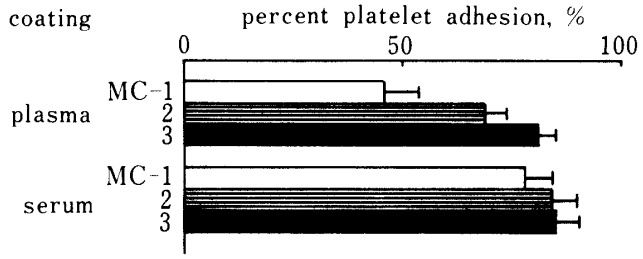

Fig. 6. Effects of Coating on Adhesion of Washed Platelets

The abscissa represents the percentage of adhering platelets 20 min after mixing washed platelets and coated MC.

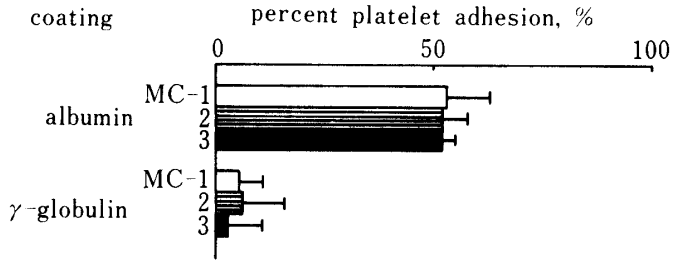

Fig. 7. Effects of Coating on Adhesion of Washed Platelets

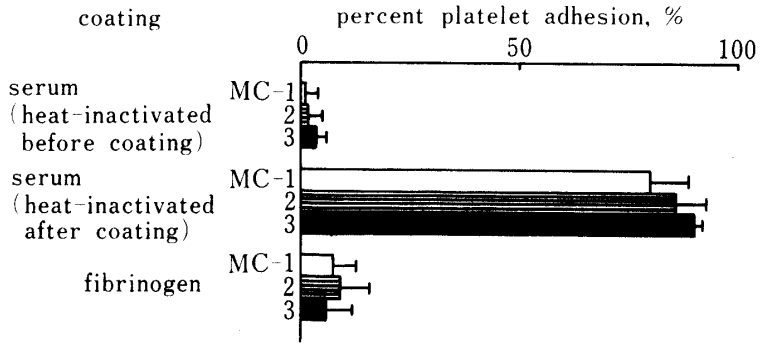

Fig. 8. Effects of Coating on Adhesion of Washed Platelets

that an albumin coating is not able to exert a full inhibitory effect. Therefore, it seems that if albumin was adsorbed irreversibly on the MC surface, the protein might inhibit platelet adhesion to the MC. Thus, albumin and γ-globulin are not the platelet adhesion-facilitating components in the serum.

In the meantime, a nearly complete inhibitory effect was found when coating was done with the serum heated at 56 °C for 30 min (Fig. 8). Comparison of this result with that obtained with fresh serum coating strongly suggests that certain complement is involved in the platelet adhesion to the MCs. It was reported[8] that the complement was activated in contact with synthetic polymer surfaces to affect the response of leucocytes or platelets on the polymer. Therefore, it is assumed that the complement is rapidly adsorbed on the MC surface, and then the platelets adhere to the MC through the adsorbed complement. Moreover, the complement adsorption was found to be strong and heat-resistant, since platelet adhesion was not inhibited even when the MC suspension was heated at 56 °C for 30 min after being coated with fresh serum (Fig. 8). This may not be surprising since there are many reports[9] showing that some enzymes become heat-resistant when they are immobilized on polymer surfaces. In addition, it seems that the adsorbed complement should be in the activated state for platelet

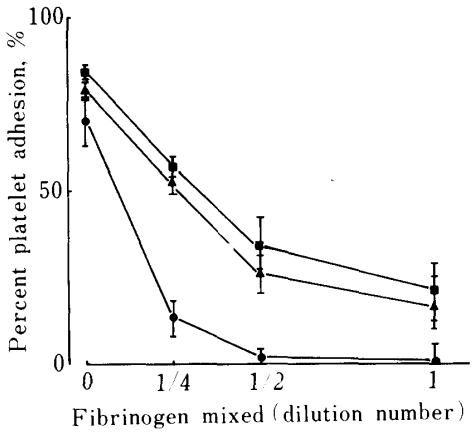

Fig. 9. Effects of Competitive Adsorption of Complement and Fibrinogen

The ordinate represents the percentage of adhering platelets 20 min after mixing coated MC and washed platelets. The abscissa shows the fibrinogen content in the coating mixture: each MC was coated with mixtures of fresh serum and an equal volume of one of a series of diluted stock solutions of fibrinogen. ●, MC-1; ▲, MC-2; ■, MC-3. Each point indicates the mean value of 3 runs and each bar, the standard deviation.

adhesion, but it is not clear at present by what mechanism the complement becomes activated.

Although the complement can be regarded as one of the facilitating components of platelet adhesion in the serum, the adsorption of the complement does not account for the observation that the platelet adhesion is dependent on the surface negative charge of the bare MCs in the presence of plasma. The major difference between serum and plasma is the presence of fibrinogen in the latter. Hence, the effects of fibrinogen coating were examined, and the results are given in Fig. 8. Fibrinogen was also found to decrease platelet adhesion to the MCs. Considering the finding that complement facilitated but fibrinogen inhibited platelet adhesion to the MCs, it is assumed that the difference in platelet adhesion which is dependent on the surface negative charge of the bare MCs, as was found in PRP, can be ascribed to a counter-operative effect of the complement and fibrinogen. In order to verify the above assumption, mixtures of fresh serum and fibrinogen were used to coat each of the MCs and platelet adhesiveness to the coated MCs was examined. The results are shown in Fig. 9. In the absence of fibrinogen, the washed platelets adhered well to the MCs through the adsorbed complement, but the adhesion decreased as the amount of fibrinogen increased in the mixture. Moreover, the inhibitory effect of fibrinogen on platelet adhesion was more prominent for MC-1 than for MC-2 and MC-3. It was shown in the previous paper[2b] that the rate and amount of adsorption of fibrinogen were greater for the microcapsules having lower negative charges than for those having higher ones. Therefore, fibrinogen would be adsorbed more readily on MC-1 than on MC-2 and MC-3, preventing the adsorption of the complement, and this would presumably be the main cause of the observed difference in platelet adhesion in PRP among the microcapsules with different surface negative charges.

Thus, it is suggested that competitive adsorption of the complement and fibrinogen is of great importance in controlling platelet adhesion to the microcapsules. However, it is not clear which one or ones of the complement are effective in controlling platelet adhesiveness, although C3b seems the most probable candidate since rabbit platelets are known to have C3b receptors on their surface. Further work is necessary to resolve these questions.

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