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Platelet Adhesion to Artificial Red Blood Cells having Different Membrane Compositions

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Sheep hemolysate-loaded artificial red blood cells with different membrane compositions were prepared by making use of the interfacial polycondensation reaction between diamines and terephthaloyl dichloride.

When these artificial red blood cells were added to rabbit platelet-rich plasma, the platelets rapidly adhered to the artificial red blood cells and obvious differences were seen in platelet adhesion between cells of different membrane compositions. On the other hand, platelet adhesion was not affected by the membrane composition in the absence of the plasma, suggesting that platelet adhesion is strongly affected by plasma components.

Among various substances in plasma, the major components, proteins, could be expected to play an important role in platelet adhesion. Therefore, three kinds of proteins, fibrinogen, γ-globulin and albumin, were chosen as typical plasma components and differences in their adsorption behavior on artificial red blood cells were examined. As a result, it was found that fibrinogen and γ-globulin were adsorbed very rapidly and in large amount on the artificial red blood cells while albumin was very little adsorbed under physiological conditions. It was further found that the rate and amount of protein adsorption were dependent on the membrane composition of the artificial red blood cells. The platelet adhesion was found to be affected by coating of the artificial red blood cells with these proteins, but the results were not consistent with the glycosyl transferase hypothesis.

Accordingly, it was considered that the dependence of platelet adhesion on the membrane composition of artificial red blood cells is caused by differences in the complex layers of plasma proteins formed rapidly on the artificial red blood cells.

Keywords——microcapsule; artificial red blood cell; blood compatibility; platelet adhesion; protein adsorption

Introduction

As a result of the progress in surgical techniques, the demand for bolod for transfusion has increased consistently. However, as the supply of blood is dependent on blood donations from volunteers at present, chronic shortage of blood for transfusion has become a serious problem. In addition, it is difficult to store blood for a long time, it is necessary to match blood groups at the time of transfusion, and care is necessary to avoid infection of patients with viral diseases such as serum hepatitis caused by the blood used for transfusion. All these problems have contributed to the shortage of blood suitable for transfusion.

In view of this, the development of blood substitutes, especially of erythrocyte substitutes, is very desirable.[1–3] In our laboratory, attempts have been made to prepare artificial red blood cells by making use of microencapsulation techniques. These artificial red blood cells enclose mammalian hemolysate within an ultra thin synthetic polymer film which has a high permeability towards smaller solutes. Their oxygen dissociation curves, enzyme activities and rheological properties have so far been found to be comparable to those of native erythrocytes.[4,5] However, it was also found that platelets adhere rapidly to these artificial red blood cells consisting of poly(N^{α},N^{ϵ}-terephthaloyl L-lysine) film when they come into contact with blood.[6] This may present a serious problem when these artificial red blood cells are introduced into a living body because the cells may cause thrombosis or angiostenosis.

In the present paper, sheep hemolysate-loaded artificial red blood cells with different membrane compositions were prepared by making use of the interfacial polycondensation reaction between diamines and terephthaloyl dichloride, platelet adhesion to the artificial red blood cells thus prepared was investigated, and the possible causes of platelet adhesion to the artificial red blood cells were studied.

Experimental

Preparation of Artificial Red Blood Cells——Preparation of hemolysate-loaded microcapsules was carried out by making use of the interfacial polycondensation reaction between diamines and terephthaloyl dichloride according to the method described earlier.[5,6] Ten ml of a mixture of a hemolysate and 0.4 M diamine (hemolysate: diamine=1: 1 v/v) was mechanically dispersed in 50 ml of a mixed organic solvent (chloroform–cyclohexane, 1: 3 v/v, containing 15 %v/v sorbitan trioleate as an emulsifier) with a Chemistirrer (Tokyo Rika Kikai Co., Model B-100) at 1360 rpm for 10 min to yield a water-in-oil emulsion. Then, without stopping the stirring, 50 ml of 0.04 M terephthaloyl dichloride solution dissolved in the mixed organic solvent was quickly added to the emulsion and the stirring was continued for another 10 min. At the end of this period, 75 ml of cyclohexane was added to the dispersion to stop the polycondensation reaction. The dispersion was centrifuged to separate the newly formed hemolysate-loaded microcapsules and they were transferred into water with the aid of sorbitan monooleate. Then, the aqueous dispersion was centrifuged to remove fine capsules and excess surfactant, and the separated microcapsules were washed repeatedly with deionized water. The microcapsules thus obtained were treated with glutaraldehyde to reinforce the membrane by crosslinking, and then washed repeatedly with an isotonic phosphate buffer solution (pH 7.4) and dispersed finally in the same medium.

The diamines used were piperazine and mixtures of piperazine and each of L-lysine, L-histidine, L-arginine, and L-ornithine, in which piperazine: L-amino acid=1: 1 M/M. The hemolysate-loaded microcapsules thus prepared were used as artificial red blood cells and are designated hereafter as P-ARBC, L-ARBC, H-ARBC, A-ARBC, and O-ARBC, respectively.

The hemolysate used was prepared as follows: sheep whole blood was centrifuged to remove plasma and particulates other than red blood cells. The collected red blood cells were washed twice with physiological saline. Then, two volumes of distilled water were added to one volume of the packed red blood cells, and the mixture was incubated at room temperature for 30 min to achieve complete hemolysis of the cells. At the end of this period, the mixture was centrifuged to remove the debris and the hemolysate thus obtained was concentrated by dialyzing it against a 12.5 % w/v PEG 4000 solution until the volume reached about a half of the original volume.

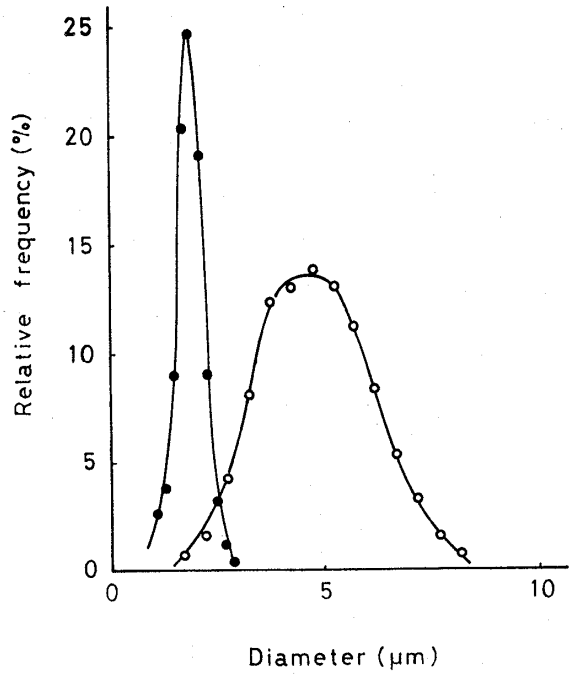

Fig. 1. Size Distribution Curves of Rabbit Platelets (●) and Artificial Red Blood Cells (H-ARBC) (○)

Preparation of Platelet Samples——Nine ml of blood was withdrawn from the auricular artery of a rabbit into a polypropylene syringe containing 1 ml of 3.8%v/v sodium citrate solution. This anticoagulant-treated blood was centrifuged at 1000 rpm for 15 min. The upper layer in the centrifuge tube was carefully pipetted out and used as platelet-rich plasma (PRP). After removal of PRP, the remaining liquid in the centrifuge tube was subjected to further centrifugation at 3000 rpm for 30 min and the upper clear layer was obtained as platelet-free plasma.

In order to separate platelets from plasma, PRP was gel-filtered according to the method described by Tangen *et al.*[7] A slurry of Sepharose 2B (Pharmacia Fine Chemicals) was slowly poured into an acrylic resin column (1.5 cm diameter, 20 cm length) having a gel-supporting nylon net (40 μm, pore size) until the height of the gel bed reached about 15 cm. The eluant was passed through the column overnight. The eluant consisted of 0.1 %w/v glucose, 5×10^{-3} M $CaCl_2$, 9.8×10^{-4} M $MgCl_2$, and 5.4×10^{-3} M KCl dissolved in a mixture of 1 volume of 0.145 M Tris-HCl (pH 7.4) and 9 volumes of 0.140 M NaCl. About 2.5 ml of PRP

was gently layered on top of the gel and fractions of about 2 ml were collected. The fractions containing platelets could be detected with the naked eye and were used as gel-filtered platelets.

Measurement of Platelet Adhesion to Artificial Red Blood Cells——The principle of the measurement is as follows: the size distributions of rabbit platelets and artificial red blood cells measured with a Coulter Counter, Model ZB-1 (Coulter Electronics Inc.), are shown in Fig. 1. Here, only the size distribution curve of H-ARBC is illustrated since the other ARBCs showed similar curves. Rabbit platelets show a narrow distribution curve with a mean diameter of 2.0 μm, while the ARBC exhibits a broader curve with a mean diameter of around 4.5 μm. Therefore, if rabbit platelets adhere to the ARBC, the number of single platelets, which can be counted by setting the threshold values of the Coulter Counter between 1.6 and 2.4 μm, will decrease.

The practical operation was carried out as follows: 0.2 ml of an ARBC suspension was added to 0.8 ml of a rabbit platelet suspension in a 2 ml polystyrene cuvette under constant stirring (800 rpm). Samples of 20 μl each were pipetted out of the mixture at given time intervals and the number of single platelets remaining not adhered to ARBC in each sample was counted with the Coulter Counter by setting the threshold values between 1.6 and 2.4 μm. The measurement was carried out at 25±1°C.

Determination of Protein Adsorption onto ARBC——A protein solution made up with a Ca^{2+}, Mg^{2+}-free Tyrode buffer (pH 7.4) was mixed with an equal volume of ARBC suspension at 25±1°C. After a given period of time, the mixture was centrifuged and the amount of protein remaining in the supernatant was colorimetrically determined by using the method of Lowry.[8] The amount of protein adsorbed onto ARBC was calculated from the difference between the concentrations before and after adsorption. The proteins used were bovine fibrinogen (95% clottable, Pentex, Miles Laboratory Inc.), bovine γ-globulin (Sigma), and bovine serum albumin (Povite Producten N.V.).

Results and Discussion

An optical micrograph of H-ARBC is shown in Fig. 2. These artificial red blood cells are spherical in shape with a mean diameter of about 11 μm. The other kinds of ARBC were also spherical though their mean diameters differed slightly. Here, the value of 11 μm is considerably larger than that of 4.5 μm obtained with the Coulter Counter. This is presumably

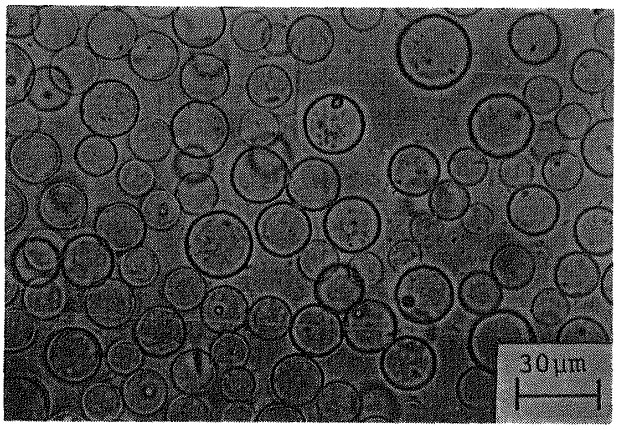

Fig. 2. Optical Micrograph of H-ARBC prepared by Making Use of the Interfacial Polycondensation Reaction

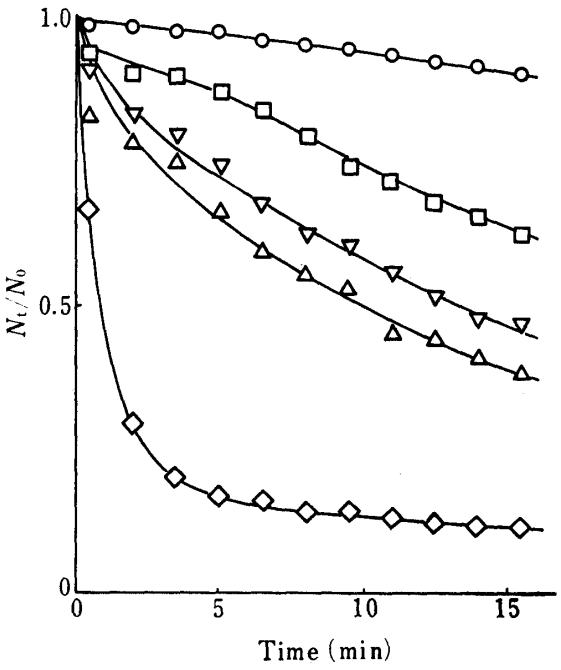

Fig. 3. Decrease with Time in the Number of Single Platelets in PRP caused by the Addition of Artificial Red Blood Cells

P-ARBC, ○; A-ARBC, □; O-ARBC, ▽; L-ARBC, △; H-ARBC, ◇.
The ordinate represents the ratio of the number of single platelets at a time t, N_t, to the initial value, N_0, before mixing PRP and ARBC.

due to the semipermeability of the membrane of ARBC, since the principle of the Coulter Counter is based on measuring the difference in electrical resistance generated when a particle passes through a given aperture. Therefore, the mean volumes and the mean surface areas of all kinds of ARBC were determined from the size distribution curves obtained from optical micrographs, and these values were used in the following experiments.

In order to examine platelet adhesion to ARBC, each kind of ARBC was added to PRP and the decrease in the number of single platelets remaining not adhered was determined. The results are given in Fig. 3. Here, the total surface area of ARBC added was adjusted to be approximately constant. The ordinate represents the ratio of the number of single platelets at a given time t, N_t, to the initial value, N_0, before mixing PRP and ARBC. In this and the following figures, each plot gives mean values for at least six experiments and the standard deviation was at most 20%. The number of single platelets did not decrease markedly in the case of P-ARBC, while it decreased most in the case of H-ARBC, followed by L-ARBC, O-ARBC, and A-ARBC in that order. The decreases caused by O-ARBC and L-ARBC were not very different from each other. However, in the case of H-ARBC, the number of single platelets showed a sudden decrease and the decrease tended to level off after 5 min. This might indicate that the rate and the degree of platelet adhesion to ARBC depend on the kinds of ARBC, that is, on the membrane composition. It may still be possible that a similar curve could be obtained as a result of platelet aggregation in view of the principle of the measurement, since it is reported[9] that platelets change their shape, release substances which induce platelet aggregation, mainly ADP, and thereby cause self-aggregation when they adhere to foreign surfaces. Therefore, adenosine, which is a competitive inhibitor of platelet aggregation caused by ADP, was added in advance to PRP, and then ARBC were mixed with it. The decrease with time in the number of single platelets then showed the same tendency as that seen in Fig. 3. In addition, it was found that ARBC form large aggregates in PRP while they are well dispersed in platelet-free plasma. Figures 4 and 5 show these findings. Hence, it seems likely that the platelets adhere to ARBC and the ARBC then aggregate with each other through the adhering platelets. Consequently, it may be safe to conclude that the results obtained in Fig. 3 do not reflect platelet aggregation, but their adhesion to ARBC.

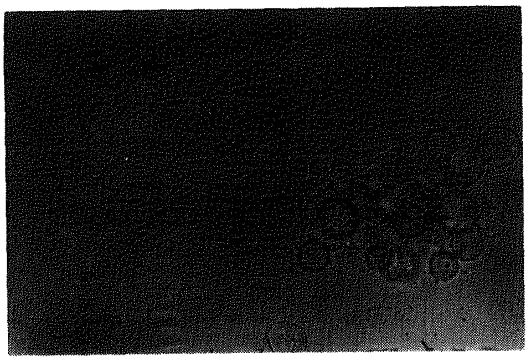

Fig. 4. Optical Micrograph of H-ARBC in PRP

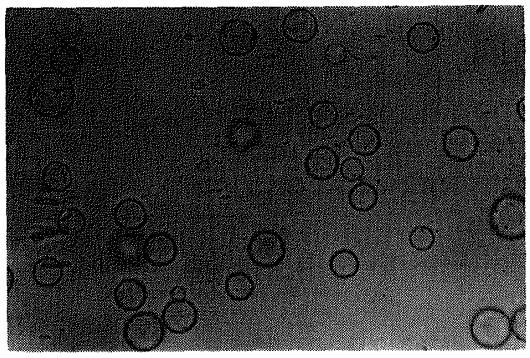

Fig. 5. Optical Micrograph of H-ARBC in Plasma

Since it is widely accepted that plasma components play an important role in platelet adhesion to a foreign surface, PRP was gel-filtered in order to eliminate the effect of plasma components. ARBC were then added to the gel-filtered platelet suspension and the decrease with time in the number of single platelets was examined. The results are shown in Fig. 6. Here, the results are shown only for three kinds of ARBC; P-ARBC, L-ARBC, and H-ARBC. They were chosen since it was found that P-ARBC adhered very little, H-ARBC most strongly and L-ARBC to an intermediate degree, and these three kinds of ARBC were used hereafter

in the experiments. As can be seen from Fig. 6, gel-filtered platelets adhered relatively well to the ARBC. In this case, however, platelet adhesion was not affected by the membrane composition of ARBC, and almost identical decrease curves were obtained. As gel-filtration itself might cause some modification in platelet adhesiveness, autologous plasma was added in advance to the suspension of gel-filtered platelets and platelet adhesion was examined on these ARBC again. The results are shown in Fig. 7, where the changes dependent on the membrane composition clearly appear once again in the number of adhering platelets. In addition, these ARBC were coated with plasma by mixing them with the plasma and incubating for 1 h prior to addition to PRP and the decreases with time in the number of single platelets were examined (Fig. 8). As can be seen from a comparison of Fig. 8 with Fig. 3, platelet adhesion was accelerated in the presence of P-ARBC and L-ARBC while it remained almost unaffected by the treatment in the case of H-ARBC. Therefore, it seems quite likely that the platelets adhere to ARBC not directly but through some kinds of plasma components preadsorbed onto ARBC, and the adsorbed plasma components affect the subsequent platelet adhesion. Moreover, the rate of formation, molecular conformation, *etc.*, of the adsorbed layer would be dependent on the membrane composition of ARBC, which affects the adhesiveness of the platelets.

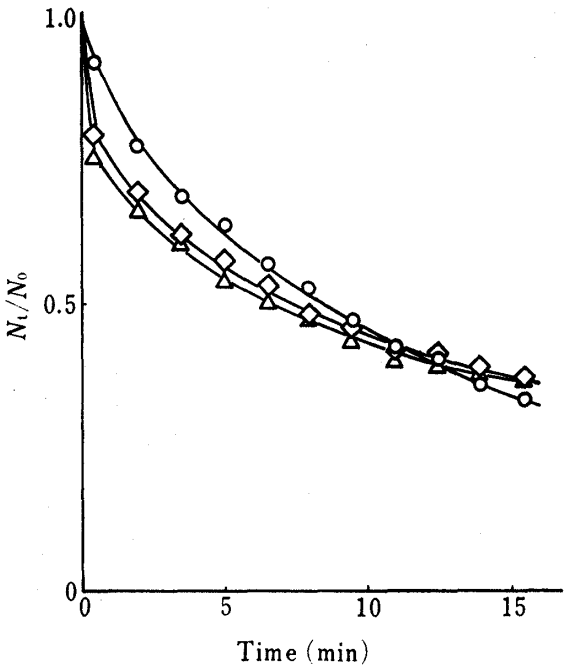

Fig. 6. Decrease with Time in the Number of Single Platelets in the Buffer caused by the Addition of Artificial Red Blood Cells

P-ARBC, ○ ; L-ARBC, △; H-ARBC, ◇.
The ordinate represents the ratio of the number of single platelets at a given time t, N_t, to the initial value, N_0, before mixing platelet suspension and ARBC.

It is believed in general that a foreign surface, when it comes into contact with blood, readily adsorbs plasma components, especially plasma proteins. Brash[10] suggested that proteins are irreversibly adsorbed physically on hydrophobic polymers to form a monolayer, and the adsorbed protein molecules are closely packed in the end-on state, with their morphology remaining unchanged. Moreover, it is reported[11] that platelets adhere more strongly to the surface with a protein layer than to those without it. Accordingly, we investigated whether the rate and amount of protein adsorption are affected by the membrane composition of ARBC using bovine serum albumin, bovine γ-globulin, and bovine fibrinogen as test proteins. Figures 9 and 10 show the rates of adsorption of fibrinogen and γ-globulin on ARBC, respectively. For both proteins, adsorption reached equilibrium within a few minutes independently of the membrane composition. Figures 11 and 12 illustrate the adsorption isotherms of fibrinogen and γ-globulin on ARBC, respectively. P-ARBC showed the highest adsorption among the three kinds of ARBC, and no appreciable difference in the amount adsorbed between L-ARBC and H-ARBC could be detected. The saturated amounts of fibrinogen adsorption onto P-ARBC, H-ARBC, and L-ARBC were approximately 3.09, 0.62, and 0.56 $\mu g/cm^2$, respectively. In the case of γ-globulin, the saturated amounts of adsorption onto P-ARBC, H-ARBC, and L-ARBC were about 3.24, 1.47, and 1.25 $\mu g/cm^2$, respectively. Baszkin and Lyman[12] calculated the saturation amounts of proteins adsorbed monomolecularly onto the surface based on the geometrical dimensions of the proteins. For fibrinogen, the values are 0.18 (side-on adsorption) and 1.70 $\mu g/cm^2$ (end-on adsorption).

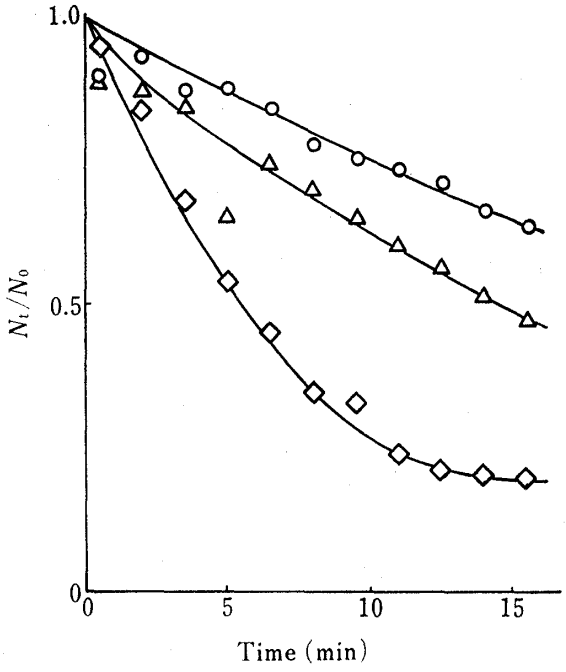

Fig. 7. Decrease with Time in the Number of Single Platelets in the Mixture of Buffer Solution and Plasma caused by the Addition of Artificial Red Blood Cells

P-ARBC, ○; L-ARBC, △; H-ARBC, ◇.
The ordinate represents the ratio of the number of single platelets at a given time t, N_t, to the initial one, value, N_0, before mixing platelet suspension and ARBC.

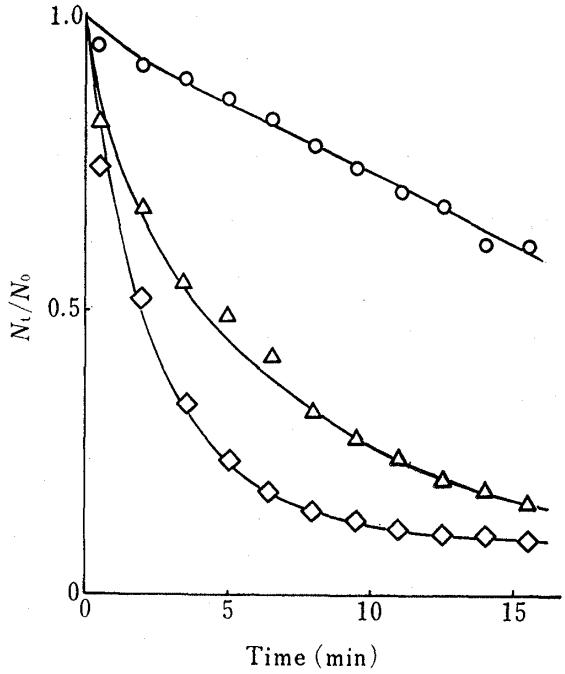

Fig. 8. Decrease with Time in the Number of Single Platelets in PRP caused by the Addition of Plasma-precoated Artificial Red Blood Cells

P-ARBC, ○; L-ARBC, △; H-ARBC, ◇.
The ordinate represents the ratio of the number of single platelets at a given time t, N_t, to the initial value, N_0, before mixing PRP and ARBC.

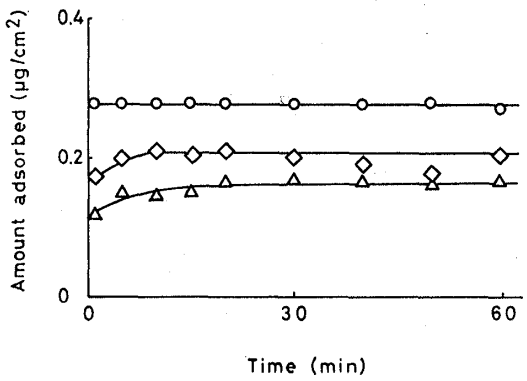

Fig. 9. Adsorption Rates of Fibrinogen on Artificial Red Blood Cells

P-ARBC, ○; L-ARBC, △; H-ARBC, ◇.
Fibrinogen concentration added was 0.1 mg/ml.

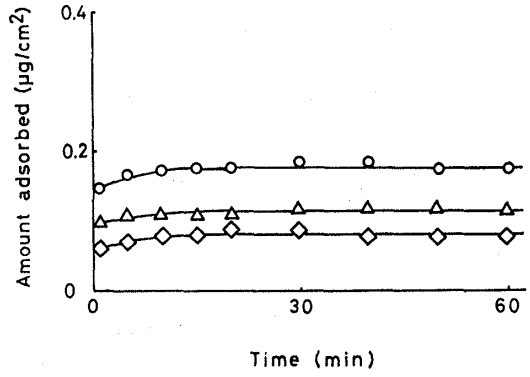

Fig. 10. Adsorption Rates of γ-Globulin on Artificial Red Blood Cells

P-ARBC, ○; L-ARBC, △; H-ARBC, ◇.
γ-Globulin concentration added was 0.2 mg/ml.

Similarly, for γ-globulin the values are 1.27 (side-on adsorption) and 1.85 $\mu g/cm^2$ (end-on adsorption). As these values are obtained on the assumption that no conformational change of the protein molecules takes place on adsorption, they may not be directly applicable. Nevertheless, comparison of these values with the amounts adsorbed of fibrinogen and γ-globulin on ARBC suggests that the adsorption onto P-ARBC corresponds to more than a monomolecular layer. The amounts of adsorption onto L-ARBC and H-ARBC are numerically intermediate between those for side-on adsorption and end-on adsorption, indicating that the adsorption may be monomolecular. Hence, the mode of adsorption and structure of the adsorbed layer of fibrinogen or γ-globulin are likely to depend on the membrane composi-

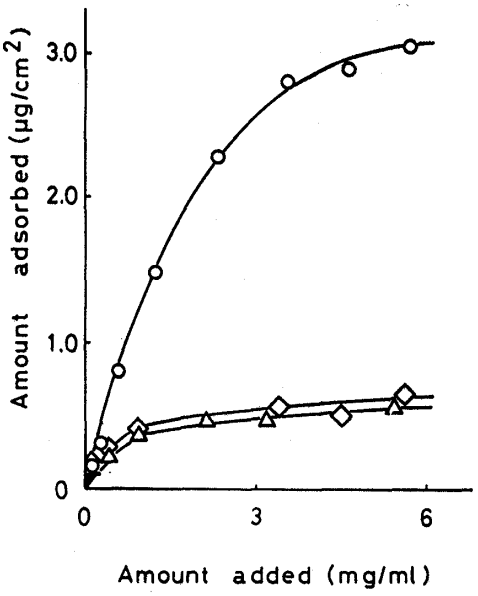

Fig. 11. Adsorption Isotherms of Fibrinogen on Artificial Red Blood Cells

P-ARBC, ○; L-ARBC, △; H-ARBC, ◇.
ARBC were incubated with each fibrinogen solution for 2 h at 25±1°C.

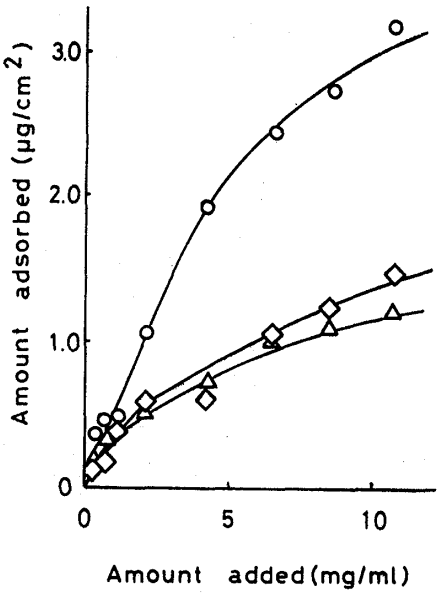

Fig. 12. Adsorption Isotherms of γ-Globulin on Artificial Red Blood Cells

P-ARBC, ○; L-ARBC, △; H-ARBC, ◇.
ARBC were incubated with each γ-globulin solution for 2 h at 25±1°C.

tion. In regard to albumin, which is the protein found in greatest abundance in plasma, no data for the rate of adsorption or adsorption isotherm could be obtained because the amount adsorbed of the protein was too small to be measured by the method used in this study. However, the possibility of albumin adsorption cannot be ruled out, since it was suggested that a small quantity of albumin is adsorbed onto microcapsules.[13]

Papers[14,15] have appeared describing that fibrinogen or γ-globulin, when adsorbed onto hydrophobic polymer surfaces, accelerates platelet adhesion, while albumin inhibits it. This is called the glycosyl transferase hypothesis. Therefore, in order to check the effect of proteins, ARBC were mixed with each of the proteins, the coated ARBC were added to PRP, and the number of platelets adhering to the ARBC 15 min later was calculated. In addition, these three kinds of plasma proteins were mixed at the same proportion as in blood and ARBC were coated with the mixture, because it was expected that their adsorption pattern and effect on platelet adhesion might differ from those exhibited by the individual proteins. The results are summarized in Fig. 13. This figure shows that both fibrinogen and γ-globulin accelerate platelet adhesion to P-ARBC and L-ARBC, while they inhibit it onto H-ARBC. In contrast, albumin has no effect on P-ARBC, while it inhibits platelet adhesion to L-ARBC and H-ARBC. Some of the results obtained here are consistent with the glycosyl transferase hypothesis whereas others are not. Hence, the glycosyl transferase hypothesis may not necessarily apply to all cases, suggesting that the adsorbed proteins may undergo considerable changes in their conformation and orientation depending on the nature of the surface of ARBC. When the treatment was done with such a low concentration of the protein mixture that monolayer adsorption on the cells could be assumed, the number of platelets adhering to L-ARBC and H-ARBC decreased more than in the cases where the individual plasma proteins were used, while that to P-ARBC was large as compared with the value obtained with albumin coating. Nevertheless, the same trend as for the bare ARBC in the number of adhering platelets was still seen. In contrast, at high protein concentration close to that in native plasma, no appreciable difference in platelet adhesion was observed among the different kinds of ARBC. It seems, therefore, that the protein layer formed on the ARBC surface consists of a complex

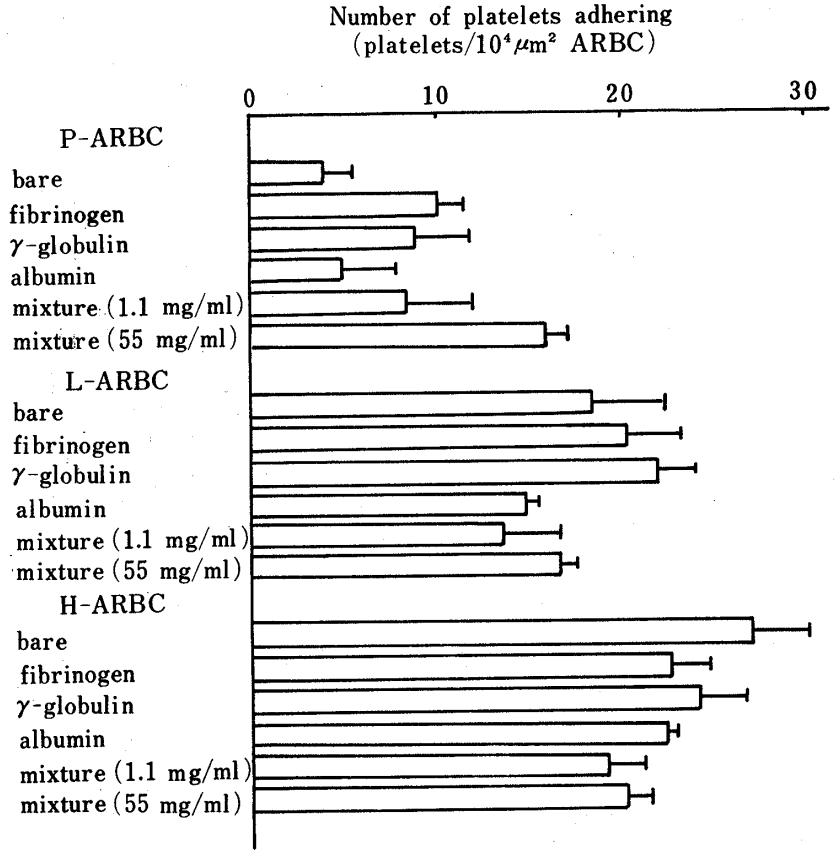

Fig. 13. Number of Platelets adhering to Bare and Protein-coated ARBC

ARBC were mixed with each protein solution for 2 h, the coated ARBC were added to PRP, and the number of platelets adhering to ARBC 15 min later was calculated.

Concentrations of the proteins used were 0.1 mg/ml for fibrinogen, 0.2 mg/ml for γ-globulin, 0.8 mg/ml for albumin, 1.1 mg/ml for mixture (1.1 mg/ml) (fibrinogen: γ-globulin: albumin=1 : 2: 8), and 55 mg/ml for mixture (55 mg/ml) (fibrinogen: γ-globulin: albumin =1: 2: 8). The bar represents the S.D. for 6 experiments.

mixture of the three proteins, and its rate of formation, molecular composition, orientation, and conformation may depend on the membrane composition. Moreover, the number of adhering platelets seems to lose its dependency on the membrane composition of ARBC with increase in the amount of the proteins adsorbed.

From the results obtained in this work it can be concluded that when ARBC come in contact with blood, an adsorbed layer of plasma components, especially of proteins, is formed very rapidly on the ARBC surface. The molecular composition, orientation, and conformation of the adsorbed layer formed in the early stage depend on the membrane composition of ARBC, which may in turn cause the differences in platelet adhesion illustrated in Fig. 3. However, at present, it is not obvious how the adsorbed layer of proteins changes with membrane composition and what sort of protein layer affects platelet adhesion. In addition, we have chosen in this work three kinds of proteins, albumin, γ-globulin and fibrinogen, as typical plasma proteins, but it seems unlikely that these three kinds of proteins fully reflect the results obtained, even though they exerted an appreciable influence on platelet adhesion, and the effects of other plasma proteins cannot be neglected. These questions remain to be answered.

Finally, the method of assessing platelet adhesion adopted in this work has the advantage of allowing a rather simple and numerical determination of platelet adhesion in the early stage. Hence, it may be useful for kinetic analysis and elucidation of the detailed mechanism of platelet adhesion under certain conditions.

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