ARTIFICIAL SURFACE-COMPLEMENT PROTEINS—L-ASCORBIC ACID: PLATELET INTERACTION AT THE INTERFACE—AN UNDERSTANDING*

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ABSTRACT

The contribution of platelets to thrombus formation is well known. An attempt is made in this paper to study the platelet adhesion using washed calf platelets onto polycarbonate surfaces in the presence and the absence of complement proteins and vitamin C.

The results suggest that complement proteins can modify the platelet-polymer interaction and further the C1 complement is capable of inhibiting platelet adhesion induced by an artificial surface, but the effect of C2 and C3 complement is relatively less prominent. An enhanced reduction in platelet adhesion has been demonstrated with 1.5 mg% vitamin C infusion probably *via* the encouraged cyclic nucleotide activity. C1 inhibited more efficiently fibrinogen induced platelet adhesion, which has been further reduced in presence of vitamin C. These results suggest a possible interaction between complement proteins and polymer surface but do not exclude a platelet/complement reaction. It may be suggested that the binding of C proteins to the platelets or to an artificial surface modifies or masks the platelet receptor sites for the inducer or the polymer surface itself and cause the reduction of platelet adhesion to an artificial surface. Vitamin C further reduces the adhering platelets which may be due to reduced availability of Ca^{++} ions and enhancement of C1 esterase.

INTRODUCTION

THE exposure of blood to artificial surfaces leads to adsorption of plasma proteins with subsequent deposition of platelets and leukocytes and further activates the blood coagulation, complement and fibrinolytic systems[1]. The complement system[2,3] refers to a complex group of proteins in normal blood serum which play an important role as mediators of both immune and allergic reactions together with other body systems.

Perhaps the most notable known difference between the 'artificial' and biologic situations is in the initial phase of the process where the adsorption of proteins on the artificial surface is known to occur within seconds[4-6]. Recently Uniyal and Brash[7] suggested that proteins other than those so far considered, may be substantially adsorbed to certain surface *e.g.* glass and these trace proteins may be important in blood material interactions. A number of studies[8-10] have dealt with adsorption of proteins to an artificial surface; however studies using complement proteins are not available to demonstrate its part in the thromboembolic phenomena that occur at the blood-foreign material interface.

It has been suggested that[11,12] C1 is associated with human platelets and is capable of inhibiting collagen induced platelet aggregation. Wautier *et al*[13] indicated that C1, the first component of the complement system, is made up of three sub-components C1q, C1r, and C1s which are held together by Ca^{++}. They further established the association of C1 to the platelet through its C1s subcomponent and that C1q and C1r are bound to C1s and are not themselves directly attached to the platelets.

Previous observations[14,15], have suggested *in vitro* ascorbated blood clots a little slowly than the normal blood. Vitamin C reduces the number of platelets adhered to the polymer surface and capable of inhibiting fibrinogen-induced platelet adhesion[16]. The effect of C1, C2 and C3 complement proteins was therefore studied on the interaction of platelets on an artificial surface and the possible inter-relation with vitamin C.

MATERIALS AND METHODS

Polycarbonate (Lexan), teflon (PTFE, Champion, India) and cellophane were used for our studies.

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Complement proteins used were C1 complement (human 500,000 CH_{50} units, containing 0.056 mg/ml of protein content, Cordis Laboratories, USA) C2 (human complement component, 5,000 CH_{50} units, protein content 0.011 mg/ml, Cordis Laboratories, USA) and C3 (human, 5,000 CH_{50} units protein content 0.103 mg/ml, Cordis Laboratories, USA). Fibrinogen (human fraction I, over 95% protein clottable, mol. wt. ~ 360,000, Sigma, Co. USA). L-ascorbic acid (GR. Sarabai M. Chemicals, India) and other chemicals used were of the analar grade.

Platelet Adhesion Studies

Polycarbonate solution was prepared in dichloromethane (10 g%) and cast on glass plates having ~ 0.1 mm thick films. Sizes of 2 × 2 cm were cut out, cleaned with 0.1% soap solution, distilled water and rinsed with ethanol. Teflon FEP and cellophane films were then dried in a vacuum oven at 60°C for 4 hr. The cleanliness of the surface was checked by contact angle method as described elsewhere[17].

(a) *Complement proteins adsorbed polymer surfaces*:

The dried films were divided into three batches and exposed to complement proteins and vitamin C as demonstrated below:

Batch I — 0.1 M phosphate buffer pH 7.4
Batch II — 20 μg complement protein (C1, C2, and C3 separately) in 20 ml of phosphate buffer pH 7.4, ensuring air/water interface[17]. In brief, all the films were dipped in the buffer and shaken to remove the trapped airbubbles. The protein solution was also added to make a known concentration of protein in the media to reduce the air/water interface.
Batch III — 20 μg complement protein (C1, C2 and C3 separately) in 20 ml of phosphate buffer pH 7.4, containing 1.5 mg% ascorbic acid.

All the films were kept in the respective solution for 5 hours at 4°C. Each film was taken out, rinsed with phosphate buffer and finally with distilled water. Then they were exposed to washed platelets for the adhesion studies as described below:

(b) *Complement proteins with platelet suspension to polymer surfaces*

Washed calf platelets were prepared[17] and suspended in tyrode solution[18] for the adhesion studies. Platelet adhesion to polymers was studied using platelet suspension containing varying concentrations of complement proteins (C1, C2 and C3) and 50 mg% fibrinogen as an inducing agent, in the presence of and the absence of vitamin C (1.5 mg%).

Platelet suspensions demonstrated above were exposed to the polymer surface for 15 min at room temperature (~ 30°C), rinsed with 0.1 M phosphate buffer, pH 7.4, fixed the platelets with 2.5% glutaraldehyde and stained with Coomassie Blue G. The number of platelets adhered to the polymer surfaces was counted using an optical microscope. Different vision fields were read randomly and averaged in an identical fashion for all samples. A minimum of 15 fields were counted and the data expressed as the number of platelets observed in the microscopic vision field with the standard deviation.

RESULTS

Effect of complement proteins on adhesion of platelets to polymer surface:

(i) *Effect of C1 on platelet adhesion*

The adhesion of platelets to polycarbonate was inhibited by addition of C1. As shown in table 1, 25 μg/ml C1 considerably decreased the adhesion of platelets to the polymer surface, which had been further reduced with 1.5 mg% vitamin C. Inhibition of polycarbonate surface-induced platelet adhesion by complement proteins in presence and absence of vitamin C is depicted in figure 1. C1 inhibited the platelet behaviour in a non-specific way, but is concentration-dependent and vitamin C had further enhanced the platelet inhibition. Fibrinogen-induced platelet density on the surface was also modified with C1 protein infusion to system.

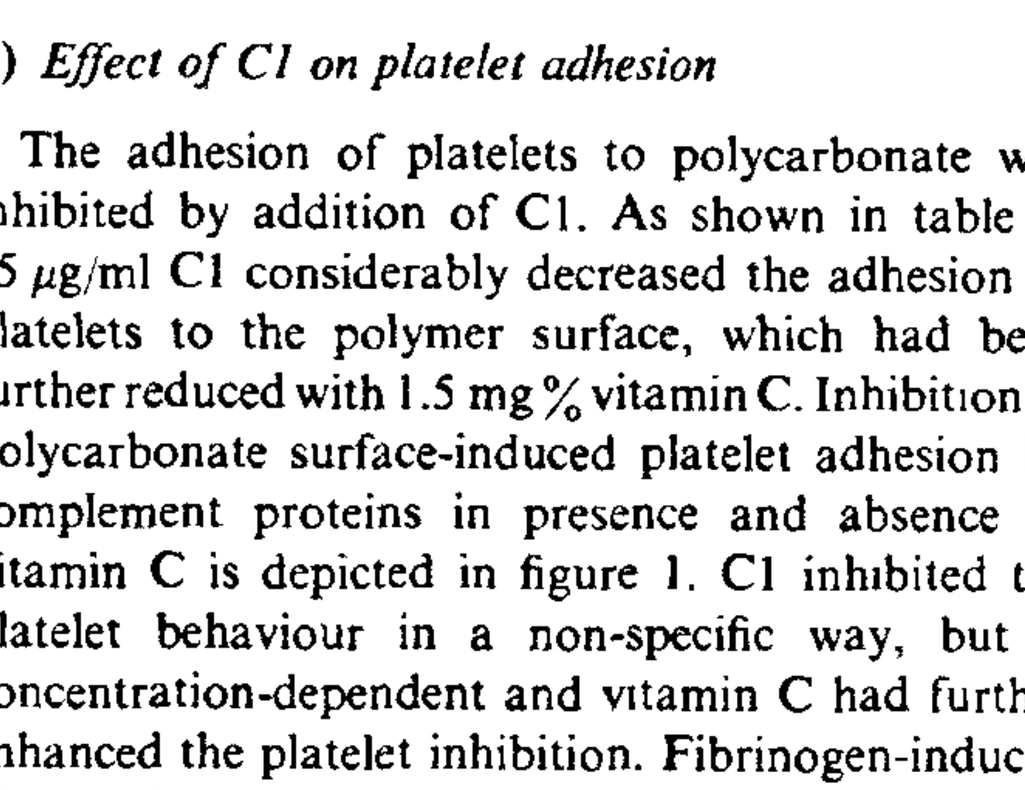

Figure 1. Concentration of C_1 protein(μgr/ml) inhibition of pc surface induced platelet adhesion by complement proteins in presence of absence of vitamin C.

Table 1 *Effect of C1 and C3 on platelet adhesion in solution form on polycarbonate*

Surfaces*	Mean[a] platelets ± S.D.
I Bare polycarbonate (PC)	6.9 ± 1.5
II PC + vitamin C (1.5 mg%)	4.8 ± 1.4
III PC + C1 protein	
5 μg/ml	3.4 ± 1.2
15 μg/ml	1.8 ± 1.0
25 μg/ml	1.0 ± 1.0
IV PC + C1 + 1.5 mg% vitamin C	
5 μg/ml + vitamin C	2.6 ± 1.2
15 μg/ml + vitamin C	1.4 ± 1.0
C1 25 μg/ml + vitamin C	0.8 ± 0.7
V PC + 50 mg% fibrinogen	16.0 ± 2.5
VI PC + Fibr. + 25 μg/ml C1	6.4 ± 1.7
VII PC + C3 protein	
5 μg/ml	4.4 ± 1.0
15 μg/ml	3.4 ± 1.5
25 μg/ml	2.8 ± 1.0
VIII PC + C3 + 1.5 mg% vitamin C	
5 μg/ml + vitamin C	3.6 ± 1.2
15 μg/ml + vitamin C	2.6 ± 0.7
C3 25 μg/ml + vitamin C	2.2 ± 0.9
IX PC + 50 mg% fibrinogen + 25 μg/ml C3	9.25 ± 1.4

* Platelet suspension containing C1, C3 and fibrinogen proteins in presence and absence of vitamin C exposed to PC (as shown in surfaces I to IX)
[a] Values expressed as the average of the number of platelets adhered to the surface in the microscopic vision field with standard deviation (at least from 15 observations of duplicate experiments).

Table 2 *Effect of C1, C2 and C3 on platelet adhesion in the adsorbed form on polymeric substrates*

Surfaces*	Mean[a] Platelets ± S.D.
I Bare polycarbonate (PC)	6.9 ± 1.5
II PC + C1 adsorbed PC	2.5 ± 1.2
III PC + C1 adsorbed PC in the presence of 1.5 mg% vitamin C	1.6 ± 0.7
IV PC + C2 adsorbed PC	3.7 ± 1.3
V PC + C2 adsorbed PC in the presence of 1.5 mg% vitamin C	2.6 ± 1.0
VI PC + C3 adsorbed PC	3.6 ± 1.4
VII PC + C3 adsorbed PC in the presence of 1.5 mg% vitamin C	2.8 ± 1.0
VIII Bare Teflon FEP	3.5 ± 2.0
IX C1 adsorbed teflon	1.4 ± 0.7
X C3 adsorbed teflon	2.1 ± 1.1
XI Bare cellophane	7.0 ± 2.0
XII C1 adsorbed cellophane	2.1 ± 1.0
XIII C3 adsorbed cellophane	3.6 ± 0.7

* Platelet suspension exposed to PC, cellophane, Teflon, C1, C2 and C3 absorbed surfaces in presence and absence of Vitamin C (as demonstrated in surfaces I to XIII)
[a] Values expressed as the average of the number of platelets adhered to the surface in the microscopic vision field with standard deviation (at least from 15 observations)

(b) *Effect of C2 and C3 on platelet adhesion*

The reduction of platelets adhered to the polymer surface by C3 is not significant as shown in table 1 in comparison with C1 protein. Figure 1 also demonstrated a similar trend though the effect of C3 protein towards platelet-polymer binding is concentration dependent. Table 2 shows the platelet adhesion effect of C1, C2 and C3 protein adsorbed polymer substrates; in presence and absence of vitamin C. The platelet-polymer binding had been modified dramatically with C1 precoated surfaces, compared to C2 and C3 precoated substrates.

DISCUSSION

The results indicate that C1 modifies platelet polymer interaction, but the effect is relatively less prominent in C2 and C3. The presence of vitamin C (1.5 mg%) with complement proteins seems to encourage further the inhibition of platelet adhesion.

Platelets possess membrane receptors for a wide variety of materials including fibrinogen[19]. The mode of assembly of complement proteins on aggregated antibody or to the cell surface and the mechanism of their subsequent activation are not yet clear[2]. However it seems that the binding of C proteins to the platelets or to an artificial surface modifies or masks the platelet receptor sites for the inducer studied[19] (fibrinogen) or the polymer surface itself and causes the reduction of platelet binding to the polymer surface.

Adenine 3',5'-cyclic monophosphate (CAMP) has been recognized as an important mediator of platelet functions secondary to that of Ca^{++} ions, but cannot alone mediate either aggregation or release. It has been generally accepted that an increase of platelet CAMP results in an inhibition of platelet responses to stimuli[20]. The reduction observed in platelets due to C1 may be due to the activation or inhibition of some of the platelet membrane enzymes and increased CAMP levels. It has been observed earlier that[16] vitamin C reduces the number of platelets adhered to the polymer surface which may be due to the increased levels of cyclic nucleotides. Inter-relation of vitamin C, complement and surface-induced platelet adhesion is important to determine the exact mechanisms possible.

It has been reported that[12], Clq and Cls (sub-components of the first component of complement) can inhibit platelet adhesion and aggregation induced by type I and type III collagens. Earlier studies have demonstrated[21] that Clq (the sub-component of the first component of complement) can inhibit platelet adhesion towards an artificial surface and the inhibition is concentration-dependent. C1 had a greater effect on platelet adhesion as compared to C2 and C3, perhaps due to the inhibitory effects of Clq and Cls. The fibrinogen-induced platelet adhesion was reduced to ~ 50% in the presence of 25 μg/ml of C1 protein as demonstrated in table 1. Also, the inhibitory action was greater with C proteins in platelet suspension rather than with adsorbed surfaces. In the suspension experiment C proteins can partly get adsorbed to the polymer substrate; however the media still contains relatively flexible, biologically less-distorted and active molecules for simultaneous interaction with platelet membranes, compared to preadsorbed substrates, where the C protein molecules are conformationally distorted. This is obvious from our platelet adhesion results indicated in tables 1 and 2. These results may suggest that C proteins modify the platelet reactivity which seems to be the main factor responsible for the platelet–polymer interaction.

Vitamin C reduces the number of platelets adhered to polymer surface due to increased levels of cyclic nucleotides and capable of inhibiting fibrinogen-induced platelet adhesion[16]. It may be stated vitamin C increases the C1 esterase activity and splits C1 to its subcomponents causing inhibition of platelets from adhering to the surface. This is further supported by the *in vivo* observation in guinea pigs that an increased intake of vitamin C significantly increases the C1 esterase activity[22].

Our *in vitro* observations with washed platelets do not explain the complex *in vivo* system. For this purpose detailed studies with artificial surfaces and their inter-relationship with complement proteins and other blood components are required.

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