Inhibition of serum complement haemolytic activity by lipid vesicles containing phosphatidylserine

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The effect of artificial model membranes on the complement system was investigated. Incubation of the model membranes with human serum resulted in consumption of complement haemolytic activity when phosphatidylserine-containing vesicles were used. The activation of the complement system appeared to proceed through the alternative pathway. This conclusion was supported by the failure of [^{125}I]Clq to bind to the membranes suggesting that the classical pathway was not involved. Although always obtained when phosphatidylserine was present in the model membranes, the activation of complement was enhanced by the contemporaneous presence of phosphatidylethanolamine. Liposomes prepared from lipid extracts of red blood cells were also able to stimulate a concentration-dependent activation of complement. Fresh, intact erythrocytes, however, could not initiate the same effects unless opsonized by antibodies. When artificially aged in vitro, red blood cells were lysed if incubated with normal human serum or with Clq-depleted serum. However, no lysis was obtained if the 'aged' erythrocytes were incubated with serum pretreated with ammonia to destroy the C3 component of complement. It is suggested that one of the mechanisms of macrophage recognition of senescent erythrocytes might be provided by the activation of the alternative pathway of complement if phosphatidylserine becomes exposed on the surface of the aging cells.

Complement activation *Phospholipid* *Erythrocyte* *Membrane asymmetry*

1. INTRODUCTION

Macrophages routinely phagocytose erythrocytes at the end of their 120-day lifespan [1]. The ability of macrophages to selectively recognize, and remove from the blood stream, cells that are no longer functional, while leaving the mature viable cells unharmed, has been attributed to binding of antibodies, opsonizing erythrocytes, by the Fc receptors on the surface of macrophages [2].

Very little is known about the mechanism employed but probably the recognition of aged erythrocytes by the macrophages involves the removal of sialic acid from their cell surface. Evidence for this includes the demonstration that neuraminidase treatment of erythrocytes led to a decreased survival time of the cells when they were injected into an animal's circulation [3]. However, other investigations have also shown significant age-related alterations in the structure and function of membrane components of human erythrocytes, including decrease in membrane deformability [4] and phospholipid concentrations [5]. Stuart and Cummings [6] have suggested that the elimination of senescent erythrocytes from the peripheral circulation is related to such changes and is mediated by the deposition of normal human serum components. This has been supported by results presented by Alderman et al. [2] indicating that upon removal of sialic acid, IgG binds to the newly exposed antigens on the surface of senescent cells, but not younger erythrocytes. Macrophages would then recognize the opsonized

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RBC via the Fc receptors and phagocytose them.

Recently, the asymmetric distribution of phospholipids in RBC membranes has been implicated in the recognition system [7]. Of the four major phospholipids of the erythrocyte membrane, phosphatidylserine (PS) is found nearly exclusively on the cytoplasmic surface [8] while the outer monolayer of the membrane is rich in neutral phospholipids, particularly sphingomyelin (SM) with phosphatidylcholine (PC) and phosphatidylethanolamine (PE) present on both membrane sides though not to the same extent. Abnormalities in the lipid distribution in membranes have already been associated with biological phenomena, such as platelet aggregation [9], cell transformation [10] and sickle cell anaemia [11]. The outer surface of sickle cells, for example, has been found to be enriched in PE and PS, compared with the normal RBC [12]. These changes were balanced by the decrease in PC in that layer.

Recently, negatively charged vesicles composed of PS and PC were found to bind to macrophage and be phagocytosed at significantly higher rates than neutral vesicles composed exclusively of PC [13]. In view of this, PS has been suggested to provide a signal that triggers macrophage recognition [14]. Although the nature of the signal has not been established, direct interaction of the macrophages with PS or indirect action via protein-independent PS-Ca^{2+} interactions [15] have been proposed. However, in view of the reported damage to Forssman antigen-bearing liposomes by complement, or complement-mediated lysis of antigen-free liposomes [16,17] we decided to explore a possible role of antibody-independent serum complement activation in the macrophage recognition of senescent cells.

2. MATERIALS AND METHODS

2.1. *Materials*

Ovalbumin, egg phosphatidylcholine (in $CHCl_3$), egg phosphatidylethanolamine (in $CHCl_3$), bovine brain phosphatidyl-L-serine, and bovine brain sphingomyelin were purchased from Sigma, St. Louis, MO. Sheep erythrocyte antiserum (haemolysin) was purchased from the Commonwealth Serum Laboratory, Melbourne, Australia. Normal human serum was supplied by the Red Cross Blood Bank, Sydney, Australia. Human C1q was isolated from serum, and labelled with ^{125}I using lactoperoxidase [18]. Rabbit IgG was prepared according to Wilkinson [19]. Equivalence ovalbumin-antiovalbumin rabbit IgG immune complexes were prepared as described by Sutton et al. [20].

2.2. *Human sera*

Human serum depleted in complement component C3 and C4 was prepared as described by Lachmann et al. [20a]. Normal human serum was depleted of C1q by adsorption with an equal volume of antiovalbumin immune complexes (1 mg/ml) in the presence of 0.01 M EDTA according to Barbaro [21]. Control serum was diluted 1:2 with 10 mM phosphate, 150 mM NaCl, pH 7.2 (PBS), containing 0.02 M EDTA and, except for adsorption, was similarly treated. After the treatment, 0.01 M $CaCl_2$ was added to the serum to neutralize the effect of EDTA.

2.3. *Extraction of lipid from erythrocyte membranes*

Human erythrocyte ghosts were prepared by hypotonic lysis of erythrocytes at 4°C followed by 4–5 washes with lysis buffer (5 mM sodium phosphate, pH 8) according to standard methods. The membrane lipids were extracted by shaking the erythrocyte ghost membranes with a mixture of methanol:chloroform (2:1) for 15 min. 1 ml chloroform and 1 ml water were then added per ml of ghosts, and shaken again to separate the two liquid phases. After centrifugation (3000 × *g*, 10 min), the bottom chloroform layer was collected and the solvent evaporated in vacuo at 37°C. The lipids were finally dissolved in chloroform:methanol (2:1, v/v).

2.4. *Lipid vesicle preparations*

The lipid vesicles were prepared from total lipid extract of erythrocyte membranes or from phospholipids mixed in different proportions. Chloroform was evaporated under a nitrogen stream leaving the lipids as a thin film in a flask. In order to ensure complete removal of the solvent, the film was left under vacuum at least 1 h. The lipids were suspended by shaking on a vortex mixer in PBS to obtain a lipid concentration of 14 mg/ml and the suspension left at 37°C for 10 min. The

milky suspension was sonicated at 4°C under nitrogen for 2 h with a Branson B15 sonifier, equipped with a micro-tip probe and tuned for maximum power output. After sonication, titanium fragments released from the sonication probe and any undispersed phospholipid were removed by centrifugation (30000 × *g* for 60 min). The preparations were then chromatographed on a Sepharose 4B column. Since Sepharose adsorbs lipids, the column was pre-equilibrated with PC vesicles prepared by the same method to avoid large losses. An upward flow rate of 0.3 ml/min was used and the eluate was monitored by the turbidity measured at 300 nm.

2.5. *In vitro aging of erythrocytes*

Erythrocytes were aged in vitro by washing them 3 times with 0.1 M phosphate buffered saline, pH 7.4, and storing them for 2 weeks at 4°C.

2.6. *Complement haemolytic activity*

Normal human serum was desensitized by 3 cycles of adsorption against sheep erythrocytes. Desensitized serum (0.4 ml, 1:10 dilution) was incubated at 37°C for 30 min in the presence of the appropriate phospholipid preparation (0.2 mg) in barbitone buffered saline (BBS) containing $MgCl_2$ and $CaCl_2$ [22] in a final volume of 0.5 ml.

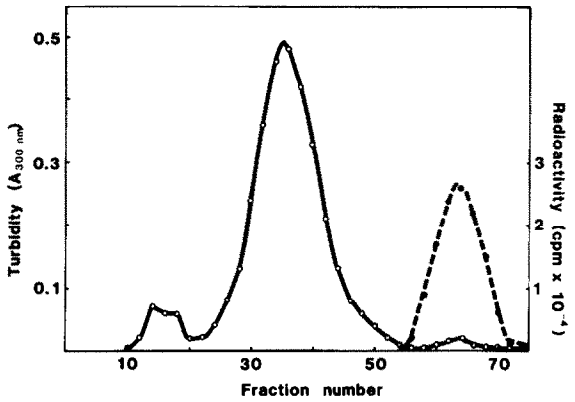

Fig.1. Gel filtration of a typical sonicated phospholipid vesicle preparation on a Sepharose 4B column (1 × 90 cm). The column was pre-equilibrated with a phosphatidylcholine preparation and eluted with PBS. The turbidity of the eluate was monitored at 300 nm (○—○). When [^{125}I]C1q was preincubated with the vesicle suspensions, its elution was followed by the radioactivity associated with the protein (●---●).

Haemolysin-activated sheep erythrocytes (2.5 × 10^7 cells, 0.1 ml) were then added and after 1 h incubation at 37°C, the non-damaged cells were spun down at 3000 × *g* for 5 min at 4°C. The residual complement activity of the serum was determined from the extent of lysis of the cells measured by the absorbance at 541 nm. Spontaneous haemolysis control (sensitized RBC + BBS) and complement activity control (sensitized RBC + normal serum + BBS) were included in each set of experiments. When the haemolytic activity of serum was tested against fresh or artificially aged RBC, the appropriate erythrocyte suspension (2.5 × 10^7 cells, 0.1 ml) was incubated with 0.5 ml of normal human serum or with serum depleted of the C1q or the C3 components of complement (1:10 dilution). The extent of cell lysis after 1 h incubation at 37°C was monitored as above.

3. RESULTS

The basic procedure used in preparing the phospholipid vesicles was essentially identical to the method described by Huang and Thompson [23] for the preparation of egg phosphatidylcholine vesicles. Experimental evidence obtained using a variety of techniques has shown that phospholipid liposomes obtained by prolonged ultrasonic irradiation in dilute aqueous buffers are almost exclusively composed of vesicles, each comprised of a single continuous bimolecular lipid layer [24,25].

Fig.1 shows the elution profile of freshly sonicated lipid vesicle suspensions chromatographed on a Sepharose 4B column. Two peaks were obtained: a small void volume peak containing all larger liposomes that did not penetrate into the Sepharose gel and a single large, but symmetrical, peak containing the smaller more homogeneously sized liposomes [23]. Binding of [^{125}I]C1q to the vesicle preparations was attempted by incubating the protein with the freshly sonicated vesicles for 1 h at 25°C before the chromatographic step. As shown by the gel filtration elution profile (fig.1), however, no radioactivity was found to be associated with either of the two lipid fractions. [^{125}I]C1q eluted later in a clear fraction indicating no association with the phospholipid fraction used (peak II) in our experiments.

The ability of the vesicles to trigger the complement system was tested by measuring the residual complement activity of serum samples preincubated with the preparations at 37°C for 30 min. The vesicles depleted serum haemolytic activity according to the relative ratio of the three individual phospholipids used in the preparations: PC, PE and PS (fig.2). The haemolytic activity of complement was found to be inhibited when PS was used in the preparation of the vesicles. This effect was found to be enhanced by the simultaneous presence of PE.

The effect of temperature on the preincubation of the vesicles with serum was also investigated by incubating the vesicles with serum on ice or in a water bath at 37°C. About 25% of the haemolytic activity was consumed by a 3 min incubation at 37°C, and almost 90% depletion was achieved by 30 min, whereas no consumption of complement was observed in the sample kept cold (fig.3).

Lipid vesicles obtained from an extract of erythrocyte ghost membrane lipids were also able to inhibit the complement haemolytic activity of

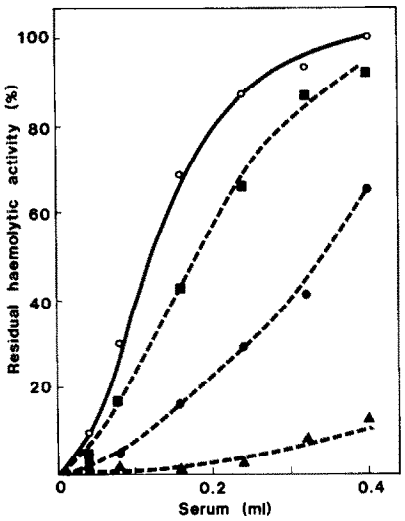

Fig.2. Residual haemolytic activity of human serum preincubated with lipid vesicles. The vesicles were prepared with PC:PE, 9:1, w/w (■---■); PC:PS, 9:1, w/w (●---●); or PC:PE:PS, 8:1:1, w/w (▲---▲) and incubated with various amounts of normal human serum. The residual complement activity of serum was then determined as described in section 2. Serum preincubated with barbitone buffer was used as a control (○—○).

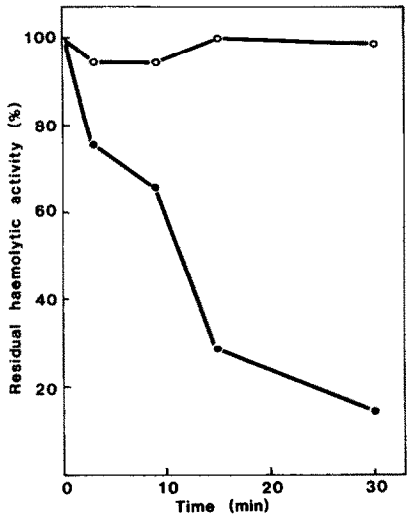

Fig.3. Temperature dependence of complement haemolytic activity inhibition of phospholipid vesicles. The vesicles were prepared with PC:PE:PS (8:1:1, w/w) and incubated with serum at 4°C (○—○) or at 37°C (●—●). The residual complement activity of serum was determined as described in section 2.

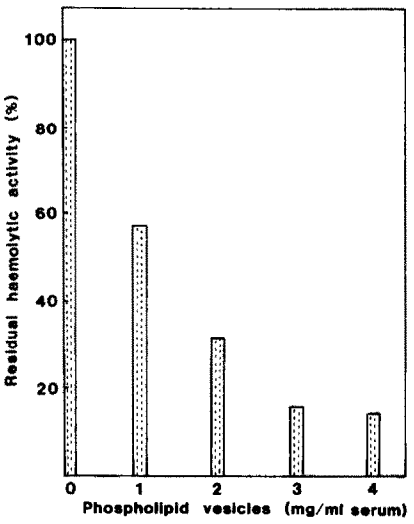

Fig.4. Residual haemolytic activity of serum preincubated with lipid vesicles prepared from phospholipids extracted from erythrocyte membranes. The residual haemolytic activity of 0.4 ml normal human serum was determined after preincubation with various amounts of lipid vesicles as described in section 2.

serum as shown by the concentration-dependent inhibition of such a preparation (fig.4).

We also prepared lipid vesicles with a phospholipid composition similar to that of the outer layer of RBC membrane (44% PC, 12% PE, 44% SM and no PS) and that of the inner layer (15% PC, 47% PE, 10% SM and 28% PS) and determined the effect of such vesicles on serum complement haemolytic activity. The results of these experiments are shown in table 1. Vesicles with a phospholipid composition similar to the outer layer of the erythrocyte membrane did not significantly decrease the haemolytic activity of serum after 1 h incubation. In contrast, vesicles with phospholipid composition similar to the inner surface of erythrocytes, considerably inhibited the serum haemolytic activity.

When freshly isolated, intact, unsensitized erythrocytes were exposed to normal human serum, no significant lysis was observed. On the other hand, when artificially aged cells were exposed to the same serum, up to 28% lysis was recorded at optimal concentrations (fig.5). The data obtained suggest that a structural change took place in about 28% of the cells after incubation for 2 weeks, and that this change made the cells vulnerable to serum-mediated damage. C1q-adsorbed serum was equally effective in lysing the erythrocytes indicating that C1q was not required, for recognition of surface antigens, by the complement system. This excludes the involvement of the classical pathway. In contrast, C3-depleted serum was unable to display the same lytic activity, implying that the alternative pathway of complement might have been responsible for the observed cell damage. This suggests that the recognition of senescent cells by macrophages in vivo may be initiated by binding of C3b fragments, formed during activation of the alternative pathway, to their receptors on the surface of macrophages.

Table 1

Residual haemolytic activity of lipid vesicles incubated with normal human serum

Phospholipid preparation	Residual haemolytic activity (%)
PC	96
PC:PE (90:10)	93
PC:PS (90:10)	64
PC:PE:PS (80:10:10)	7
PC:PE:SM (44:12:44)	88
PC:PE:SM:PS (15:47:10:28)	2
RBC lipid extract	19

The weight ratio of phospholipids used in the preparations is shown in parentheses. The residual haemolytic activity of 0.4 ml serum (1:10 dilution) was determined after preincubation with 0.1 mg phospholipid preparation as described in section 2

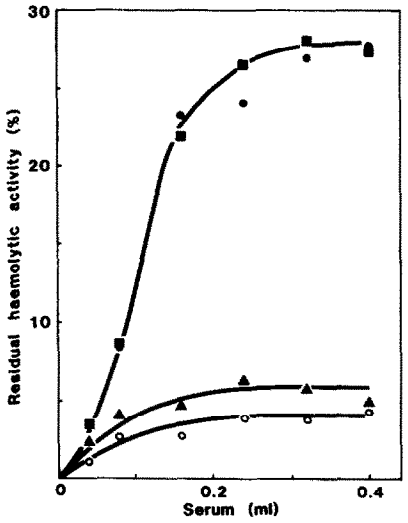

Fig.5. Lytic activity of serum against erythrocytes. Fresh (○—○) or artificially aged (●—●) erythrocytes were incubated with normal human serum as described in section 2. Artificially aged cells were also incubated with C1q-depleted (■—■) or with C3-depleted serum (▲—▲).

4. DISCUSSION

The results of our study indicate that phospholipid vesicles displaying PS and PE on their surface are able to activate the alternative pathway of complement in human serum.

Recently, Tanaka and Schroit [7] have reported interaction of isolated macrophages with RBC displaying PS in their surface membrane. Although phagocytosis was implied, the method employed was unsatisfactory because it only measured attachment to the cells and phagocytosis did not necessarily follow. A more recent paper by Schroit et al. [14], however, has confirmed that addition of PS to RBC enhances their recognition by

macrophages resulting in phagocytosis. The experiments, in this case, were performed in vivo and hence, in the presence of serum proteins.

Significant differences in the distribution of PS in RBC has been suggested to play a physiological role in the maintenance of homeostasis [7]. Although no such differences have been found in a normal population of erythrocytes, the proposal is still valid because, as suggested by Schroit et al. [14], one would not expect to find transformed old cells in peripheral blood because they would be rapidly removed from circulation by phagocytic cells.

However, abnormal organization of aminophospholipids has been demonstrated within the lipid bilayer of sickled erythrocytes [12]. The most notable change that occurred during sickling was the translocation of PE and PS from the inner to the outer lipid bilayer. Lipid asymmetry caused by hypoxia-induced sickling in erythrocytes, causes the sickle cells to bind avidly to human monocytes. In contrast, oxygenated sickled cells, which are morphologically normal in appearance and do not express PS on their outer surface, do not bind to the monocytes [7].

The factors controlling the rearrangements of phospholipids within the lipid bilayer during sickling are unknown although it has been suggested that spectrin may play an important role in stabilizing membrane phospholipid distribution in human erythrocytes [26]. Spectrin has been shown to be irreversibly deformed in irreversible sickle cells [27].

The results of our experiments using artificial phospholipid vesicles support the possibility that PS in exposed membrane surfaces may promote serum complement activation, which would then probably mediate biological effects, such as macrophage recruitment. The results of experiments on the effect of preincubation temperature of the vesicles with serum demonstrated that activation was not caused by non-specific adsorption of complement components, as shown by the lack of lytic inhibition when the preincubation was performed at 4°C. Although other explanations are possible, it is feasible that the observed complement activation by PS/PE in artificial vesicles may also be responsible for the observed damage to the artificially aged RBC if PS and/or PE become exposed on the cell surface after losing enough sialic acid residues.

The alternative pathway of complement seems to be involved in the activation process because C1q was shown not to bind to the vesicle preparations. These results are in agreement with the observation that sheep erythrocytes are able to activate the alternative pathway of complement when sialic acids are removed from their surface [28]. In addition, artificially aged RBC were damaged by C1q-adsorbed serum but not by C3-depleted serum.

The presence of complement-activating phospholipids only at cytoplasmic surfaces of RBC is not only consistent with phospholipid asymmetry in membranes, but might also serve an important biological purpose. It could be judged as potentially dangerous to have complement-activating phospholipids at the outer surface of blood cells, which would bring about immediate lysis. This fact itself could provide one of the physiological reasons for an asymmetric phospholipid distribution in RBC membranes.

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