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# Potentiation of liposome-induced complement activation by surface-bound albumin

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**Abstract**

Large anionic multilamellar liposomes containing 71% membrane cholesterol (MLV) caused complement (C) activation in human serum in vitro, as reflected in significant rises in S protein-bound terminal complex (SC5b-9) and C3a-desarg levels. Increasing the albumin content in serum by 1–4 g/100 ml led to 50–100% further increase in MLV-induced C activation, while higher amounts of exogenous human serum albumin (HSA) gradually lost the capability to potentiate liposomal C activation. HSA alone had no influence on SC5b-9 formation at any level below 12%. Complement activation by liposomes and the potentiating effect of supplemental HSA were greatly reduced or eliminated in the absence of C1q or in the presence of 10 mM EGTA/2.5 mM $Mg^{2+}$, pointing to the involvement of the classical pathway. Potentiation of C activation by supplemental HSA was not unique to MLV-induced activation, as deposition of HSA on the membrane of 'Centricon' ultrafiltration units also potentiated the C-activating effect of the polycarbonate membrane. Fatty acid (FA) or non-monomeric protein contamination in HSA were unlikely to be playing a role in the described effects, as 96% pure, FA-rich (Buminate) and 99% pure, FA-free HSA had identical effects on liposomal C activation. While highlighting a new modulatory mechanism on liposomal C activation, the above data raise the possibility that deposition of extravasated HSA at sites of tissue injury may serve a hitherto unrecognized proinflammatory function. 

*Keywords:* Serum albumin; Liposome; Complement; Inflammation; Phospholipid bilayer; Polycarbonate membrane; Natural antibody

## 1. Introduction

It is known that liposomes, synthetic nanoparticles, microspheres and essentially all foreign particulate substance exposed to blood can bind serum albumin, along with a variety of other plasma proteins, including immunoglobulins and complement (C) [1–3]. The amount and spectrum of bound proteins depend on the size, charge and other physicochemical characteristics of particles, and show substantial interspecies, individual and experimental variation. In the case of anionic liposomes, the binding of albumin to the membrane was shown to be substantial, and to involve both electrostatic and hydrophobic interactions [1,4,5]. Given the abundance of albumin among plasma proteins and their prone-

Abbreviations: C, complement; Chol, cholesterol; DMPC, dimyristoyl phosphatidylcholine; DMPG, dimyristoyl phosphatidylglycerol; EGTA, ethyleneglycol tetraacetic acid; HSA, human serum albumin; MLV, multilamellar vesicles; PBS, phosphate-buffered saline; SC5b-9, S protein-bound C5b-9

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ness to bind to liposomes, relatively little is known about the influence of albumin on the association of other proteins to liposomes, in particular, C proteins. We became interested in this question following a study in which we observed that co-incorporation of human serum albumin (HSA) with hemoglobin in liposomes led to substantial acceleration of C activation by these vesicles relative to that observed with only hemoglobin-containing control liposomes [6]. This finding was not explained previously, and gave rise to the hypothesis that HSA exposed on the liposome surface might accelerate C activation by the phospholipid bilayer.

The present study was initiated to test the above hypothesis. As C activator liposomes we used negatively charged large multilamellar vesicles (MLV) that contained 71 mol% cholesterol (Chol) in the membrane, as these liposomes were shown in a previous study to be very efficient C activators in pigs [7]. The particular questions we addressed were: (1) does supplementation of serum with HSA have an influence on liposomal C activation; (2) if yes, what are the characteristics of the effect in regards to HSA concentration dependence, activation pathway involved, specificity to liposomal membranes and the influence of HSA purity?

## 2. Materials and methods

### 2.1. Materials

Dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol (DMPG) and cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, AL). HSA (Buminate 25%), prepared by Cohn–Oncley fractionation of plasma, was from Baxter Health (Glendale, CA). Fatty acid free HSA, prepared from essentially globulin-free HSA and Zymosan were from Sigma Chemical Co. (St. Louis, MO). C1q-depleted human serum and the enzyme-linked immunoassay (ELISA) kits for measuring SC5b-9 and C3a-desarg were obtained from Quidel Co. (San Diego, CA). MLV consisting of DMPC/DMPG/Chol (24:5:71 mol%) were prepared by thin-layer hydration/vortex mixing, as described previously in detail [7]. Human serum, obtained from healthy volunteers as described earlier [6], was stored at −70°C until use.

### 2.2. Complement activation by liposomes ± HSA

Liposomes were incubated with serum in Eppendorf tubes in a shaking water bath (80 cycles/min) for 30 min at 37°C. Typically, to 35–40 μl undiluted serum the HSA, other additives ($EGTA/Mg^{2+}$) and the activator (MLV, zymosan) were added to give a final volume of 50–120 μl, using PBS for volume control. The reaction was started by the addition of the activator, and stopped by supplementing the volume to 1 ml by adding 880–950 μl 'sample diluent' of the SC5b-9 ELISA kit, which contained 10 mM EDTA, 25 mg/ml bovine serum albumin, 0.05% Tween 20 and 0.01% thimerosal (pH 7.4). C activation was quantified by measuring S protein-bound terminal complex (SC5b-9) or C3a-desarg formation by the respective ELISA kits, as described earlier [6].

### 2.3. Complement activation by polycarbonate membranes ± HSA

Similar incubations as above were also performed in sealed 10 kDa cutoff Centricon centrifugal ultrafiltration units (Millipore Corp., Bedford, MA) in the absence of liposomes, with HSA deposited on the filter membrane prior to incubation. After rinsing the Centricon unit with PBS, 2 mg HSA (in 100 μl PBS) was placed on the membrane and the unit was centrifuged at 1500 rpm for 30 min to deposit the protein on the filter. This was followed by placing 100 μl serum on the membrane, incubation and measurement of SC5b-9 as described above. The controls included serum incubated in Eppendorf tubes and on filter without prior deposition of HSA on the membrane.

### 2.4. Extraction of lipids from HSA

The lipid content of HSA was reduced by a single-step extraction with chloroform/methanol (2:1 v/v). The organic phase was dried in a Rotavapor and the amount of extracted lipids was established from the weight increase of the (dried) flask relative to its pre-experiment value.

### 2.5. Statistical methods

The levels of SC5b-9 and C3a-desarg in serum were expressed as mean ± S.D. for triplicate or quadruplicate wells. Significance of differences between two groups was determined by Student's two-sample *t*-test, and that among three groups was established with analysis of variance (ANOVA) followed by the Student–Newman–Keuls test.

## 3. Results

### 3.1. Complement activation by liposomes with and without HSA

Fig. 1A shows that incubation of MLV with serum led to a significant, 4-fold rise of SC5b-9 formation relative to PBS control ($P < 0.001$), indicating C activation by liposomes with subsequent formation of the terminal complex. Addition of HSA (Buminate) to the serum at 2% caused no activation by itself, but together with MLV, 2% HSA caused a further 2-fold increase of SC5b-9 formation relative that observed with MLV alone, indicating potentiation of C activation by the liposomes. Fig. 1A also shows that SC5b-9 formation was greatly reduced in the presence of 10 mM EGTA/2.5 mM $Mg^{2+}$ both in the presence and absence of HSA, indicating a major involvement of the classical pathway in liposome-induced C activation and its potentiation by HSA.

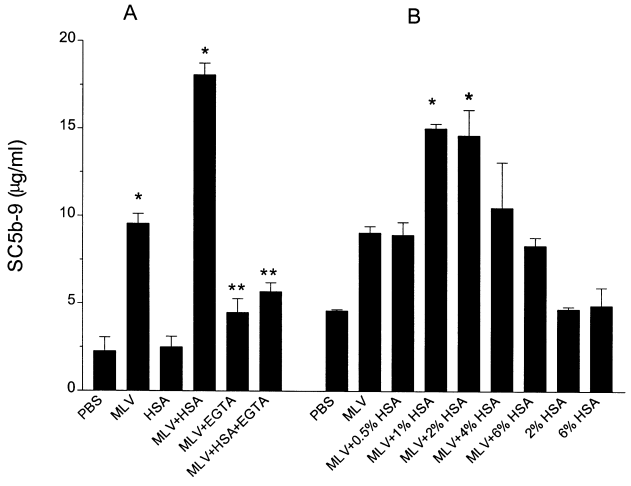


Fig. 1. The influence of HSA on C activation by multilamellar liposomes in human serum in vitro. (A) MLV was incubated with undiluted human serum in the presence and absence of 2% HSA and/or 10 mM EGTA/2.5 mM $Mg^{2+}$ as described in the text. The bars show mean ± S.D. for triplicate determinations. *Significant differences between MLV and MLV+HSA vs. all other groups ($P < 0.01$). **Significant differences between MLV+EGTA and MLV+HSA+EGTA vs. MLV and MLV+HSA, respectively ($P < 0.01$), as determined by ANOVA with subsequent pairwise comparisons using the Student–Newman–Keuls test. (B) The concentration dependence of the potentiating effect of HSA on MLV-induced C activation in another normal human serum. Similar experiment as shown in A, except that the concentration of added HSA was varied as indicated. *Significant difference vs. all other groups except MLV+4% HSA ($P < 0.01$).

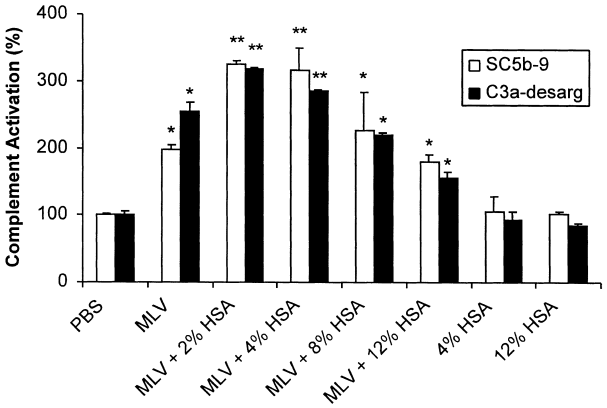


Fig. 2. The influence of HSA on C activation by multilamellar liposomes in human serum in vitro. Similar experiment as shown in Fig. 1A,B, in yet another (third) serum. Here, in addition to SC5b-9, C3a-deasarg was also determined and the levels of both markers were expressed as percentage of PBS baselines (which were 119.4 ± 7.7 and 4.8 ± 0.06 μg/ml for C3a-desarg and SC5b-9, respectively). *Significant differences relative to PBS; **significant differences relative to MLV and MLV+8% HSA (except SC5b-9 for MLV+8% HSA) at ($P < 0.05$), as determined by ANOVA with subsequent pairwise comparisons using the Student–Newman–Keuls test. Other details are the same as in Fig. 1.

Fig. 1B presents similar experiments in another normal human serum, except that the amount of added HSA was varied in the 0.5–6% range. Although the baseline was somewhat higher in this subject, the C-activating effect of MLV and its potentiation by 2% added HSA was qualitatively similar. The experiment showed that 0.5% and 6% HSA were ineffective in enhancing the C-activating effect of MLV, while 1% and 2% HSA were equipotent enhancers. HSA alone caused no C activation at 2% and 6%.

The experiment shown in Fig. 2 essentially repeated Fig. 1B using yet another (third) human se-

rum, and HSA doses 2%, 4%, 8% and 12%. In addition, we have tested the changes of C3a-desarg level, which is another marker of C activation, a measure of C3a anaphylatoxin production. In order to allow direct comparison of the two analytes' responses, we converted their absolute serum levels to percent of baseline. While corroborating the dose dependence of the C activation-potentiating effect of HSA in the 1–4% range, the data demonstrated remarkable parallelism between the changes of C3a-desarg and SC5b-9, providing evidence that (1) the described C activations were associated with anaphylatoxin production, (2) the potentiation of MLV-induced SC5b-9 production by HSA reflected increased C activation, rather than modulation of the terminal pathway only, and (3) SC5b-9 correlates with C3a production, in addition to its established property of reflecting C5a formation. Taken together, it is unlikely that the HSA-induced increase in MLV-induced SC5b-9 formation would be due to a change in the equilibrium between soluble (S-protein-bound) and membrane-bound C5b-9, or that HSA would enhance SC5b-9 formation directly, without increasing C3a anaphylatoxin production.

### 3.2. Complement activation by polycarbonate membranes with and without HSA precoating

In an attempt to establish whether or not the C activation-potentiating effect of HSA was restricted to liposome-induced C activation, we used another test system as well wherein serum was exposed to a polycarbonate membrane surface with and without prior coating of the membrane with HSA. More specifically, serum was incubated in the sample holder of polycarbonate membrane-based centrifugal ultrafiltration (Centricon) units with and without precoating their membrane with an amount of HSA that is present in the serum under conditions of potentiation of liposomal C activation (2 mg). As shown in Fig. 3, incubation of serum in Centricon units led to a minor, but statistically significant increase in SC5b-9 production relative to that observed with serum incubated in Eppendorf tubes. This polycarbonate membrane-induced C activation was significantly greater in the case of HSA-precoated membranes, providing qualitative evidence that the C activation-potentiating effect of HSA was not restricted to activation by liposomes. The HSA concentration dependence and other quantitative details of C activation in the above Centricon system were not investigated further.

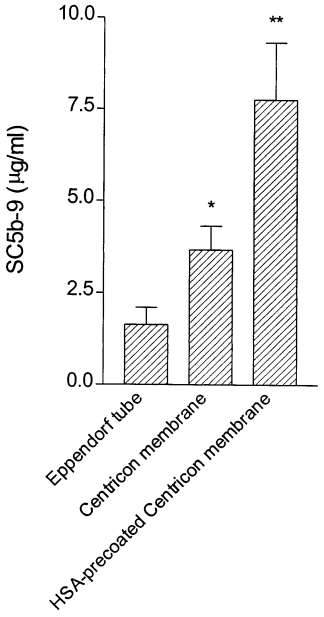


Fig. 3. Complement activation by Centricon filter membranes with and without prior deposition of HSA on the membrane. The membranes were rinsed with PBS and 2 mg HSA (100 µl from a 2% stock) was placed on the membrane followed by centrifugation for 10 min at 4000 rpm at room temperature. Human serum (100 µl) was then incubated on the HSA-precoated and non-precoated filters as described in the text, while control serum was incubated under identical conditions in Eppendorf tubes. The serum remained on top of the membrane without volume loss throughout the incubation regardless of precoating with HSA. The measurement of SC5b-9 production and other conditions of the experiments were the same as in Fig. 1. *Significant difference relative to serum incubated in Eppendorf tube; **significant difference relative to non-coated Centricon membrane as determined by ANOVA with subsequent pairwise comparisons using the Student–Newman–Keuls test.

### 3.3. Complement activation by liposomes with and without HSA in C1q-depleted serum

Fig. 4 shows that MLV with and without 2% added HSA caused no rise in SC5b-9 when C1q-depleted serum was used instead of whole serum. This lack of response was not due to reduced levels or function of C3 or the terminal cascade components, as zymosan, a known alternative pathway activator,

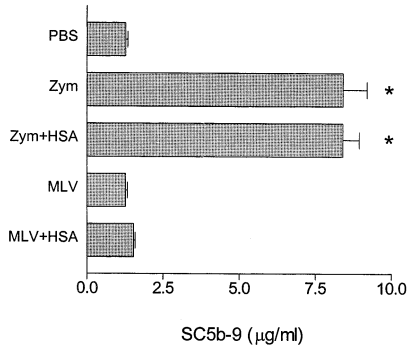


Fig. 4. The role of C1q in MLV ± HSA-induced C activation. Similar experiments as in Figs. 1 and 2, except that C1q-depleted serum was used for the incubations. Zym ± HSA denote 5 mg/ml Zymosan ± 2% HSA. The rise they caused in SC5b-9 levels were significantly greater than in all other groups (*$P < 0.001$) as determined by ANOVA with subsequent pairwise comparisons using the Student–Newman–Keuls test. HSA caused no changes in Zym-induced C activation.

caused massive SC5b-9 production. Thus, C1q played an essential role both in C activation by MLV and its potentiation by HSA.

### 3.4. The role of fatty acid and non-monomeric protein contaminations in the C activation-potentiating effect of HSA

HSA prepared by Cohn–Oncley fractionation of plasma is known to contain free fatty acids (FA) and non-monomeric protein contaminations (HSA dimers and higher molecular mass proteins, mainly globulins). The explore the possibility that the potentiation of liposomal C activation by HSA might be due to the above contaminating molecules, we compared the effects of Buminate, FA-depleted Buminate and highly pure, FA-free HSA in similar experiments as described in Figs. 1 and 2. As shown in Table 1, the C activation-potentiating effects of the three preparations were essentially similar, arguing against a major role of FA or non-monomeric components in the observed effects of HSA.

## 4. Discussion

The present study reports an unexpected potentiating influence of HSA on C activation by multilamellar anionic liposomes and by a synthetic polycarbonate membrane. The C-activating effect of certain liposomes has been analyzed previously in numerous laboratories and was established to involve a variety of mechanisms and control factors (for review see [8]). The high-cholesterol MLV we used in the present study was reported recently to be a very strong activator of pig C both in vivo and in vitro [7], which was confirmed in human and rat serum as well (Baranyi et al., submitted). Complement activation by the latter liposomes was shown to correlate with their binding of naturally occurring anti-lipid antibodies [7], which, together with the present observations on the inhibitory effects of EGTA/$Mg^{2+}$ and C1q depletion, point to a major role of classical pathway activation.

Table 1
Potentiation of liposome-induced C activation by HSA preparations of different purity and free fatty acid content

| | Purity (%) | FA content (g/100 ml) | SC5b-9 (μg/ml) | |
|---|---|---|---|---|
| | | | Serum A | Serum B |
| PBS | | | 0.82 ± 0.42 | 1.91 ± 0.26 |
| MLV | | | 11.50 ± 0.59* | 7.00 ± 0.67* |
| MLV+HSA (Buminate) | 96[a] | 0.29[b] | 18.30 ± 0.51** | 12.76 ± 1.61** |
| MLV+FA-depleted HSA | 96[a] | 0.10 | 18.39 ± 0.91** | |
| MLV+FA-free HSA | 99 | < 0.001 | | 15.20 ± 0.64** |

Entries are mean ± S.D. for $n = 3$ wells in each group. *, **, significant difference at $P < 0.01$ relative to PBS and MLV, respectively, as determined by ANOVA followed by the Student–Newman–Keuls test. Multilamellar liposomes (MLV) ± 2% of the specified HSA were incubated with two different sera (A and B), and SC5b-9 levels were measured as described in Section 2. The specified purity and FA (octanoic acid) content of the different HSA preparations were either given in their label, or was measured as described in Section 2.

[a]Buminate contained 96% HSA monomers, 2% dimers and 2% high molecular mass proteins (mainly globulins).

[b]Obtained by conversion of 0.08 mmol octanoic acid/g ($M_r$ of octanoic acid is 144.2).

In contrast to the above extensive information on liposomal C activation, the observation that HSA could modulate this activation has not been reported before. Considering the wide use of HSA in human therapy as a colloidal plasma expander with remarkable safety record [9], it was unexpected that raising the normal albumin level in serum (3.5–5%) by 1–4% with a clinically used HSA preparation (Buminate) would cause a significant increase in liposome-induced elevation of serum SC5b-9, suggesting potentiation of liposomal C activation.

In an attempt to understand the mechanism of the phenomenon we raised and ruled out several possibilities. Thus, the findings that the potentiating effect of HSA required C1q and $Ca^{2+}$, and that it was manifested not only in increased SC5b-9 formation but also in increased C3a-desarg generation, pointed to stimulation of classical pathway C3 conversion rather than the enhancement of terminal complex formation. The facts that the relatively inhomogeneous, FA-rich Buminate, lipid-depleted Buminate and the highly pure, FA-free HSA preparations had quantitatively similar effects on MLV-induced C activation essentially ruled out a major causal role of FA or non-monomeric protein components. We also provided evidence that the enhancement of C activation by HSA was not specific to phospholipid bilayer vesicle membrane-induced activation but it was present with a synthetic, flat and solid C activator surface, raising the possibility that the phenomenon may represent an universal positive feedback mechanism of C amplification. The observation that HSA alone did not cause C activation in the absence of a C activator surface, taken together with the above observations and considerations suggest that the C activation-potentiating effect of HSA may be due to physical or physicochemical changes in its structure following binding to the membrane.

Immune aggregates and many other large molecular mass polymers can activate C via antibodies and direct binding of C1q [10,11]. Of particular relevance to the present study, Milich et al. [12] have demonstrated that purified human C1q, immobilized on polystyrene beads, bound polyalbumin, but not monomeric albumin or polymers of various other plasma proteins. Our data showing a key role of C1q in both C activation and the potentiation of MLV-induced C activation by HSA raises the possibility that surface-bound HSA molecules polymerize, or associate in aggregates, and bind C1q in a fashion described in the above study [12] for polyalbumin. This would lead to increased C activation relative to that caused by C1q binding via anticholesterol and antiphospholipid antibodies [6,7,13,14]. Based on this explanation one may rationalize the gradual decline of C activation-potentiation at HSA concentrations $>4\%$ as a consequence of dissociation of membrane-bound HSA–C1q complexes. Like with circulating immune complexes, the formation of HSA-C1q complexes may critically depend on the relative concentrations of their components. After saturating liposomal HSA binding sites at about 4% exogenous HSA, addition of further protein is expected to increase the free to membrane-bound HSA ratio, potentially destabilizing surface-bound HSA–C1q complexes.

In addition to the above C1q-mechanism, a further possible explanation for our observations is that HSA increased C activation via increasing the binding of antibodies to the membranes. It is known that the binding of antiphospholipid antibodies to their phospholipid head group epitope critically depends on the presence of another epitope, provided by $\beta_2$-glycoprotein I, which strongly interacts with phospholipids via its hydrophobic lipid binding site (it is also known as apolipoprotein H) [15]. Because HSA, too, has capability for intense hydrophobic interactions with lipids [4,5], in principle, membrane-bound HSA complexes could bind excess amounts of IgM-type antibodies via associative binding, in a fashion similar to that described for phospholipid-$\beta_2$–glycoprotein I complexes. One may also speculate on the potential presence of naturally occurring antibodies with reactivity to neoepitopes on surface-bound (poly)albumin or aggregated albumin. Polyalbumin appears to be prone for complex formation with antibodies, as reflected in reports on the binding of polymerized HSA to hepatitis B surface antigen (HBsAg) only in the presence of anti-HbsAg IgM [16], or the binding of HSA-specific antibodies to erythrocyte-associated (aggregated) HSA but not to soluble HSA [17,18]. These and possible other hypotheses regarding the mechanism of the C activation-potentiating effect of a limited amount of exogenous, membrane-deposited HSA remain to be explored in future studies.

Concerning the practical implications of our study, besides its wide use as a plasma expander [9], HSA has been studied as a potential carrier system for various therapeutic agents, based on the expectation that nanoparticles and microspheres made from albumin are biodegradable and may provide long circulation time for the associated agents [19–24]. The present results highlight a potential problem with HSA-based carrier systems, inasmuch as unforeseen acceleration of C activation may not only counteract circulation longevity but may cause anaphylactoid reactions or other nonspecific 'pseudoallergic' symptoms (recently called C activation-related pseudoallergy (CARPA) [7].

Finally, our data raise the possibility that albumin may play a hitherto unrecognized proinflammatory function by promoting C activation at sites of massive extravasation of proteins and deposition in injured tissues, such as occur upon traumatic hemorrhage or exudative inflammation. The resultant increase in chemotactic anaphylatoxins would recruit granulocytes and macrophages, thereby accelerating the inflammatory process.

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