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# Binding of vitronectin and clusterin by coagulase-negative staphylococci interfering with complement function

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Coagulase-negative staphylococci (CoNS) are commonly associated with infections of prosthetic devices mediated by adsorbed host factors on biomaterial surfaces. Complement activation is known to occur and induce unspecific inflammation around the biomaterials. Human vitronectin (Vn) and clusterin (Clu), two potent inhibitors of complement, can be bound by CoNS. With a hypothesis whether binding of Vn or Clu influences complement activation, two measurements were determined. For Vn, complement activation was measured with a mouse anti-activated human C9 antibody. In the presence of Vn-binding strain, *Staphylococcus hemolyticus* SM13I, complement activation on a surface pre-coated with Vn occurred as it did in the absence of Vn pre-coating. For *S. epidermidis* 3380, which does not express binding of Vn, complement activation on a Vn-presented surface was significantly decreased. For Clu, erythrocytes lysis was measured to reflect the end product of complement activation (membrane attack complex). The complement-induced hemolysis increased when human serum was pre-incubated with Clu-binding strains, *S. epidermidis* J9P. The enhancement of hemolysis by J9P decreased when serum was supplemented by exogenous Clu. The data imply that interaction between CoNS and Vn or Clu interferes with one of their physiological functions, complement inhibition.
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## 1. Introduction

Implantation of biomaterials is common in modern medicine. In addition to infections, biomaterials in contact with blood may be complicated by hemolysis, leukopenia, and disrupted coagulation hemostasis (post-perfusion syndrome) which are mediated by surface-associated complement activation [1]. Gram-positive strains, such as staphylococci, are resistant to lysis by membrane attack complex (MAC). However, MAC can stimulate cells to produce and release inflammatory mediators [2].

Soluble Vn is able to inhibit the lysis by binding to either (i) the forming of MAC or (ii) the metastable site of C5b-7 [3, 4]. We have shown that immobilized Vn also can inhibit complement activation [5]. Clu's inhibitory effect of complement assembly was derived from three sites and two modes of action. These were related to direct inhibition of C9 assembly at sites on C5b-8 and C5b-9 and to binding to C5b-7 to prevent membrane attachment. The effect on C9 assembly appeared to be the most potent [6].

According to previous reports and our findings, CoNS strains bind soluble urea-denatured Vn and immobilized Vn [7, 8], and one *S. epidermidis* strain J9P binds Clu only in fluid phase (unpublished data). The Clu-binding of J9P was expressed when bacteria were grown on ISO-sensitest (ISO) agar, but not when the bacteria were cultured on blood agar (unpublished data). In order to prove that the binding of these CoNS strains to Vn or Clu could influence complement activation and contribute to the pathogenesis of these bacteria, complement activation tests were determined in this study.

## 2. Materials and methods

### 2.1. Chemicals

Human urea-denatured Vn and native Clu was purified according to the methods established previously [9, 10]. Fresh human serum (NHS) was purchased from the Blood Bank, Lund University Hospital and stored in $-80\,^{\circ}$C. Vn deleted serum was obtained with a collagen coupled column as described by Gebb *et al.* [11]. Mouse anti-activated C9 monoclonal antibody (aE11) was a kind gift from Prof. T Lea (Oslo, Norway). All common chemicals were of analytical grade, purchased from KEBO (Spånga, Sweden). ISO-sensitest (ISO) agar was purchased from Oxoid (Hampshire, UK). Todd–Hewitt broth (THB) was purchased from LabM (Bury, UK).

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ELISA plates (MaxiSorb) were from NUNC (Roskilde, Denmark).

## 2.2. Bacteria and culture media

*Staphylococcus hemolyticus* SM131 and *S. epidermidis* 3380 were isolated from patients with osteomyelitis. SM131 expresses Vn-binding, and 3380 does not [8, 12]. They were cultured in THB. *S. epidermidis* J9P was grown on ISO agar to express Clu-binding. After growth on blood agar this strain was used as negative Clu-binding control. Bacteria were cultured 24 h at 37 °C, and then washed twice in 0.1 M phosphate-buffered saline (PBS, pH 7.2) and resuspended at a final density of $10^9$ cells/ml for various assays.

## 2.3. Buffer preparation for complement activation

Veronal-buffered saline (VBS), pH 7.5, with 0.1 mM $Ca^{2+}$ and 0.5 mM $Mg^{2+}$ was used for total complement activation. VBS with 10 mM MgEGTA and 2.5 mM $Mg^{2+}$ (MgEGTA) was used for the alternative pathway.

## 2.4. Complement activation evaluated by enzyme-linked immunosorbent assay (for immobilized Vn).

Wells of ELISA plates were coated with 100 µl Vn (5 µg/ml in PBS) overnight at 4 °C, and then rinsed three times in PBST (PBS containing 0.05% Tween 20). After the wells were blocked by 1% BSA in PBS for 1 h at 37 °C, one hundred microliters of bacterial suspension (SM131 and 3380) was added to each well at a concentration of $10^7$ cells per ml and incubated 1 h at 37 °C. Unbound bacteria were removed by PBST. One hundred microliters of Vn-depleted serum diluted 1 : 3 in MgEGTA was incubated with the wells at 37 °C for another hour under gentle agitation. After washing, the wells incubated with aE11 antibody for 1 h at 37 °C. Secondary antibody and color developing reagents were added as described previously [13]. The results were read at 492 nM in a Multiscan Plus 314 reader (Labsystems OY, Helsinki, Finland).

## 2.5. Complement-mediated hemolysis assay (for soluble clusterin)

Hemolysis assay was performed by a previously described method with minor modification [14]. Briefly, 200 µl bacterial suspension ($1 \times 10^9$ cells) was incubated with 200 µl fresh 1% NHS for 1 h at 22 °C. One hundred microliters sheep erythrocytes ($2 \times 10^8$ cells) treated by antibody against sheep erythrocyte (Dade Behring Marburg GmbH, Marburgs, Germany) was added for another hour at 37 °C. The entire procedure was carried out in VBSG (Veronal-buffered saline containing 0.1 mM $Ca^{2+}$, 0.5 mM $Mg^{2+}$ and 0.1% gelatine). After centrifugation ($2000 \times g$, 5 min) to remove unlysed cells, 200 µl of supernatant was transferred to ELISA plates and the absorbance at 550 nm was estimated. Percentage of lysis was determined by a formula: OD I-OD II/OD III − OD IV × 100 in which I refers to the absorbancy of the samples in NHS, II refers to the absorbancy of the samples in heat-inactivated (56 °C for 30 min) serum, III refers to the absorbancy of 100 µl erythrocytes ($2 \times 10^8$ cells) lysed by water, and IV refers to the absorbancy of 100 µl erythrocytes ($2 \times 10^8$ cells) incubated in VBSG.

Alternatively, 1% NHS was supplemented by two-fold diluted exogenous Clu from 2 µg in 200 µl volume, and mixed with the same volume of bacterial suspension prior to addition of erythrocytes.

## 2.6. Statistical analyses

One-way ANOVA was employed when multiple groups were compared. Mann-Whitney *U* test and unpaired student *t* test were also used when appropriate. A *P* value of $< 0.05$ was considered to represent a significant difference.

# 3. Results

## 3.1. Intrinsic complement activation of two Vn-binding CoNS strains

Since the alternative pathway is mainly responsible for surface-associated complement activation [15], MgEGTA buffer was used in these tests.

In order to evaluate whether immobilized Vn processes an inhibitory effect for complement activation. Vn depleted serum as the source of complement factors, was added into wells with or without Vn-coating. Non-Vn-coated surface (ELISA plate) showed a higher level of activation (0.38 AU) than Vn-coated surface (0.15 AU) ($P < 005$) (Fig. 1).

Two CoNS strains, SM131 and 3380 were incubated with Vn-coated or non-Vn-coated surfaces prior to the addition of Vn-depleted serum. After pre-incubation with Vn-binding strain SM131, there was no difference in

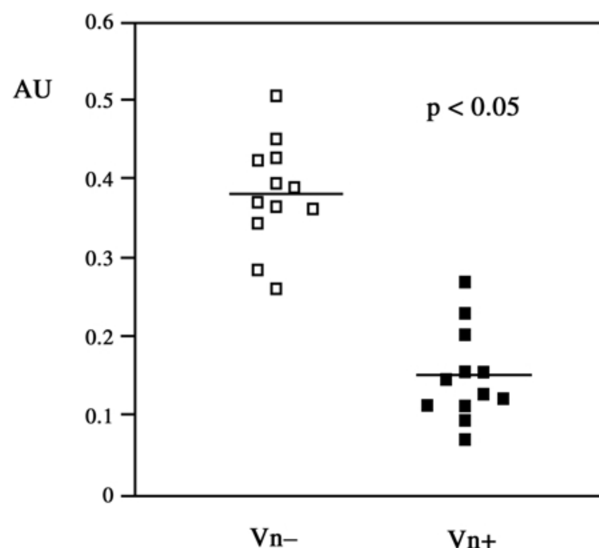


*Figure 1* Polystyrene surfaces with or without Vn-activated complement in Vn-depleted serum, which was diluted in MgEGTA buffer. The level of complement activation was measured by anti-C9 antibody. The surface without Vn showed a higher level of activation (0.38 AU) than that with Vn (0.15 AU) ($P < 0.05$, Mann–Whitney *U* test). Data are represented as median shown by horizontal lines ($n = 12$).

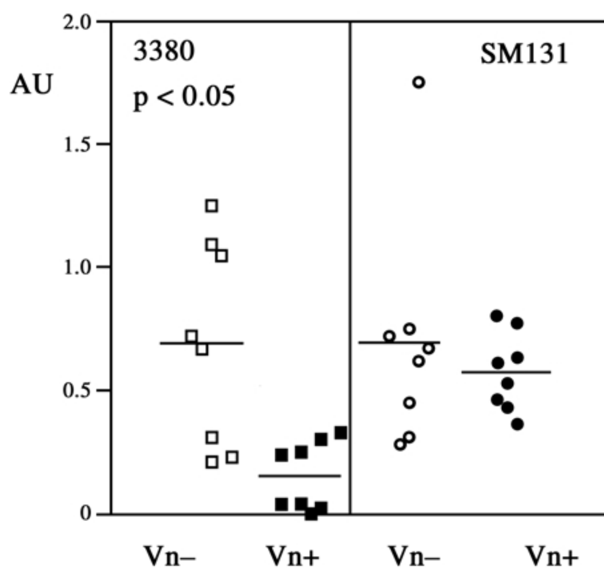


*Figure 2* Polystyrene wells with or without immobilised Vn were incubated with either Vn-binding strain SM131 or non-Vn-binding strain 3380. The surfaces were then incubated with Vn-depleted serum which was diluted 1 : 3 in MgEGTA buffer, and the level of complement activation was detected with an anti-C9 antibody. The data are represented as median values showed by horizontal lines ($n = 8$). Strain 3380 without Vn and with Vn yielded values of 0.69 and 0.15 AU, respectively ($P < 0.05$, Mann–Whitney *U* test). SM131 without Vn and with Vn yielded values of 0.69 and 0.57 AU, respectively (no significant difference).

complement activation between surfaces with Vn (0.57 AU) and without Vn (0.69 AU) (Fig. 2). *Staphyloccus epidermidis* 3380, which does not express Vn binding, was also tested in the same procedure. The complement activation on surface with Vn was significantly lower compared to that on surface without Vn (0.15 AU vs. 0.69 AU) ($P < 0.05$) (Fig. 2).

## 3.2. Complement-mediated hemolysis assays (for soluble clusterin)

Based on the fact that Clu can inhibit the hemolytic activity of complement by binding to MAC and prevent cytolysis, we predicted that Clu-binding bacteria could reduce the serum Clu level and consequently enhance MAC formation, thereby increasing hemolysis. In order to ascertain the background of hemolysis, the antibody-sensitive erythrocytes were incubated with 1% NHS. The cell lysis was $\sim 90\%$ of total erythrocytes added. After bacterial inoculation (blood agar culture), the hemolysis decreased to $\sim 70\%$. This finding indicates that bacteria consumed complement factors during the incubation with NHS. NHS pre-incubated with *S. epidermidis* J9P grown on blood agar induced 77% erythrocytes lysis (according to mean values), whereas NHS pre-incubated with J9P grown on ISO agar induced 91% cell lysis (Fig. 3). As it was not clear from above tests whether the amount of serum Clu influenced erythrocyte lysis, increasing amounts of exogenous Clu (from 0 to 2 μg) were supplemented to NHS during the pre-incubation with the clusterin-binding strain (J9P cultured on ISO agar). A corresponding decrease in the level of hemolysis was observed (Fig. 4). Hemolytic ability of NHS pre-incubated with the non-Clu-binding strain (J9P grown on

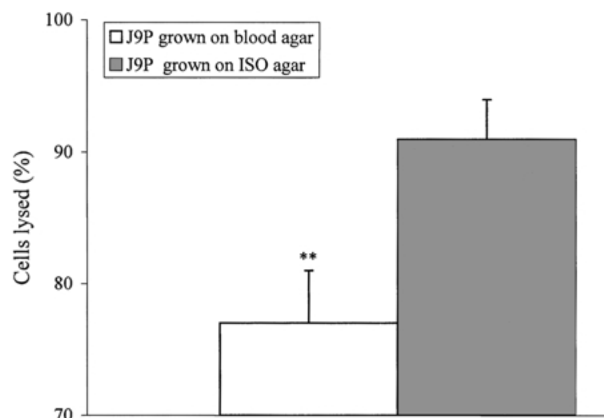


*Figure 3* Complement activation was measured as lysis of antibody sensitive sheep erythrocytes. The data are expressed as lysis percentage to total erythrocytes added (mean values with SD), and quadruple samples were repeated three times ($n = 12$). A significant difference compared to ISO agar culture is indicated (** $P < 0.01$, Student *t* test).

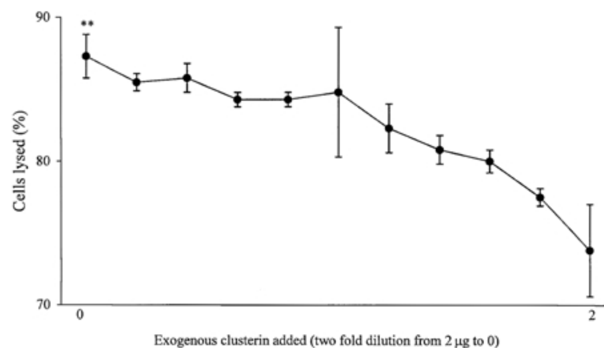


*Figure 4* The effects of exogenous clusterin on the lysis of antibody sensitive sheep erythrocytes mediated by NHS which was pre-incubated with clusterin binding bacteria, *S. epidermidis* J9P. Exogenous clusterin (2 μg) was two-fold diluted and incubated with bacteria at the same time in NHS prior to erythrocytes were added. Duplicate samples were tested twice. Data are represented as mean values with SD. ** $P < 0.01$ (One-way ANOVA test) compared to ending point (2 μg exogenous clusterin added).

blood agar) was not influenced by supplement of exogenous Clu (data not shown).

## 4. Discussion

Immobilized Vn inhibited complement activation since the complement activation was lower on a Vn-coated surface compared to a surface without Vn. On the other hand, some of CoNS stains bound immobilized Vn [8]. It is reasonable to speculate that bacterial binding could disrupt binding of immobilized Vn with complement factors. The consequence of this disruption could abate Vn's inhibitory effect for complement activation. Complement activation was increased when a Vn-binding strain, *S. hemolyticus* SM131, was introduced to a Vn-coated surface prior to serum added, whereas *S. epidermidis* 3380 which does not bind Vn did not show similar influence. This finding proves our hypothesis that bacterial binding of immobilized Vn interferes with complement activation.

With the same hypothesis the role of Clu as a regulator of complement activity was investigated. The selected bacteria, *S. epidermidis* J9P bound Clu only in fluid

phase, therefore we used another method, erythrocyte lysis test, to evaluate complement activation. Higher cell lysis was induced by Clu-binding strain (J9P grown on ISO agar) due to that the assembly of the MAC increased after Clu was removed from serum to these bacterial surfaces' binding sites. The protection against erythrocyte lysis offered by exogenous Clu confirms the crucial role played by Clu. Therefore, it could be reasoned that, the reduction observed for soluble Clu in NHS caused by Clu-binding bacteria might be compensated by the addition of exogenous Clu. Furthermore, exogenous Clu may also occupy bacterial binding sites, thereby preventing loss of endogenous Clu.

The findings described above could be argued that complement functions might be greatly influenced if both clusterin and vitronectin can be bound by certain staphylococcal strains colonizing biomaterial surfaces.

In spite of the fact that J9P did not bind immobilized Clu, it binds other adsorbed plasma proteins, such as fibrinogen or fibronectin, and the bacterial binding of soluble Clu was not influenced by binding of other proteins (unpublished data). This indicates that the bacteria can colonize a biomaterial surface mediated by other host proteins and keep its binding capacity with soluble clusterin.

In conclusion, Vn or Clu-binding bacteria may abate the protective effects of Vn or Clu against complement induced cell injury. Therefore, bacterial binding of Vn or Clu may contribute to complications to the use of biomaterials.

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