

# Effect of Complement Inhibition and Heparin Coating on Artificial Surface-Induced Leukocyte and Platelet Activation

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**Background.** Exposure of blood to artificial surfaces, as in cardiopulmonary bypass, induces an inflammatory response involving complement, leukocyte and platelet activation. To elucidate the specific role of complement in this process, studies were performed on blood circulated in polyvinyl chloride tubing in the absence and presence of complement inhibitors. Parallel experiments were performed with heparin-coated polyvinyl chloride tubing, which is known to prevent complement and cell activation.

**Methods.** A novel experimental model was used, based on human whole blood anticoagulated with lepirudin. Complement activation products, myeloperoxidase, lactoferrin, and thrombospondin were quantified in enzyme immunoassays. Leukocyte CD11b expression and leukocyte-platelet conjugates were detected by flow cytometry.

**Results.** Increased levels of C3 activation products, alternative pathway convertase, and the terminal SC5b-9 complex, combined with unchanged levels of C1rs-C1-inhibitor complexes and marginal changes in C4 activation demonstrated that complement was activated

through the alternative pathway. Granulocyte and monocyte CD11b expression and granulocyte-platelet conjugate formation were efficiently attenuated by blocking either factor D, C3, C5, or C5a receptor. In contrast, monocyte-platelet conjugate formation and release of myeloperoxidase, lactoferrin, and thrombospondin were not reduced by complement inhibition. Heparin-coated polyvinyl chloride tubing efficiently reduced all inflammatory markers studied, except for C1rs-C1-inhibitor complexes, which increased, consistent with the enhancing effect of heparin on C1-inhibitor function. This effect did not, however, reduce fluid-phase classic pathway activation induced by heat-aggregated immunoglobulin G.

**Conclusions.** Leukocyte and platelet activation in response to artificial materials occur by mechanisms that vary in their dependence on complement. Heparin coating precludes both the complement-dependent and complement-independent reactions.

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Patients undergoing extracorporeal circulation, as during cardiopulmonary bypass (CPB), suffer a systemic inflammatory reaction, which is partly caused by exposure of blood to the artificial surfaces of the extracorporeal circuit, but also by surface-independent factors like surgical trauma and ischemia-reperfusion injury [1-3]. This adverse response to a foreign material, however, has been shown to be substantially reduced by modifying the artificial surface with heparin [4], although as yet the mechanisms of this improved biocompatibility are incompletely understood.

Exposure of blood to polyvinyl chloride (PVC), a syn-

thetic polymer frequently used in extracorporeal devices, leads to an inflammatory reaction by activation of both plasma cascade systems and cells [5-7]. Several in vitro models have been developed for comparative studies of the biocompatibility of unmodified and surface-heparinized PVC tubing [8, 9]. It has previously been demonstrated in our laboratory that end point-attached, covalently linked heparin (CBAS) completely prevents PVC-induced activation of complement [8], leukocytes [10], and platelets [11]. However, it is not clear to what extent the activation of cells in blood exposed to unmod-

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ified PVC is linked to the activation of complement, which is a first-line defense system and as such a candidate for the triggering of secondary inflammatory responses. Furthermore, in *in vitro* models, the choice of anticoagulant is crucial as the complement system is readily affected by both calcium-binding anticoagulants and heparin. We have recently described an *in vitro* full blood model anticoagulated with lepirudin and shown that in concentrations sufficient for anticoagulation, the complement system is unaffected [12]. Thus, the model allows selective studies on the role of the complement system in artificial surface-induced inflammation.

The aim of the present study was to elucidate the role of complement in PVC-induced activation of leukocytes and platelets by use of specific complement inhibitors. Heparin-coated PVC was used for comparison. Additionally, we investigated whether surface-immobilized heparin may counteract classic pathway activation induced in the fluid phase, in view of the well-known enhancing effect of heparin on C1-inhibitor function [13].

## Material and Methods

### Reagents

Heparin-coated (CBAS, Carmeda BioActive Surface) and uncoated PVC tubing were provided by Carmeda AB (Stockholm, Sweden). Sterile phosphate-buffered saline was from Life Technologies (Paisley, UK), and lepirudin (Refludan) from Hoechst (Frankfurt am Main, Germany). Human immunoglobulin G (IgG) was purchased from Kabi (Uppsala, Sweden). Paraformaldehyde and ethylene diamine tetraacetic acid (EDTA) were from Sigma-Aldrich (St. Louis, MO), and LDS-751 from Molecular Probes (Leiden, The Netherlands). The flow cytometer (FACScalibur) and the antibodies used for flow cytometry (anti-CD11b PE, anti-CD14 PE, anti-CD14 FITC, and anti-CD61 FITC) were all from Becton Dickinson (San Jose, CA).

**COMPLEMENT INHIBITORS.** Compstatin is a 13-amino acid cyclic peptide that binds to and inhibits cleavage of C3 [14]. The acetylated form of compstatin (Ac-ICVVQDWGHRCT-NH<sub>2</sub>) and the control peptide (IA-VVQDWGHRAT-NH<sub>2</sub>) have been described previously [15]. The cyclic hexapeptide AcF[OPdChaWR], a C5a receptor (C5aR) antagonist [16], was synthesized as previously described [17]. The monoclonal antibodies 166-32 (anti-factor D; IgG1; blocks factor D function) and 137-30 (anti-C5; IgG1; blocks cleavage of C5) have been described earlier [12, 18]. The antibodies, as well as an isotype control antibody, were produced in one of the authors' laboratory (M.F.).

**PREPARATION OF HEAT-AGGREGATED IMMUNOGLOBULIN.** A 10 mg/mL solution of human IgG in phosphate-buffered saline, pH 7.2, was incubated in a water bath at 63°C for 15 minutes. The resulting heat-aggregated IgG preparation was cooled immediately, and stored at -20°C until use.

### Experimental Model

The model has previously been described in detail [8] but was modified on the critical point of anticoagulation. Blood was drawn from healthy laboratory volunteers using lepirudin, a recombinant form of hirudin, instead of heparin as anticoagulant. Hirudin is a highly specific thrombin inhibitor, shown to have no effect on the complement system. This is in contrast to heparin, which can either potentiate or attenuate complement activation, depending on the concentration used [12]. Samples of blood were supplied with specific complement inhibitors or equal volumes of saline and incubated at 37°C for 4 minutes. A volume of 750  $\mu$ L of blood was then transferred to segments of PVC or CBAS-coated tubing (length, 30 cm; internal diameter, 3 mm). Each segment was closed end-to-end and incubated by rotating slowly at 37°C for 15 minutes, if not otherwise stated. After incubation, 100  $\mu$ L of the blood was used for flow cytometric studies as described below, while 500  $\mu$ L was mixed with 10  $\mu$ L of 0.51 mol/L EDTA (final concentration 10 mmol/L) and centrifuged for 15 minutes, 3,220 *g* at 4°C. The plasma was frozen in aliquots at -70°C for later analysis of complement activation and neutrophil degranulation products. Studies of the release of thrombospondin were performed in separate experiments with blood circulated in loops of tubing for 5 minutes only, owing to the rapid activation of platelets. After incubation, 450  $\mu$ L of blood was mixed with 50  $\mu$ L of the platelet-stabilizing anticoagulant CTAD (citrate, theophylline, adenosine, and dipyridamole) and treated as previously described [19]. All experiments were run in triplicate. The median value was compared with the baseline value, which was defined as 100%. Thus, "n" indicates the number of donors for each type of tubing or inhibitor, and as PVC tubing was included as the control in every experimental setup, this number exceeds the number for each complement inhibitor.

### Flow Cytometric Studies

**CD11B.** Immediately after incubation, blood samples were fixed with an equal volume of 0.5% (vol/vol) paraformaldehyde for 4 minutes at 37°C in polypropylene tubes. Fixed blood cells were stained for 15 minutes at room temperature with anti-CD11b PE, anti-CD14 FITC, and the nuclear dye LDS-751 (FL-3) in polystyrene tubes. The cells were resuspended in 2 mL of phosphate-buffered saline and incubated for another 15 minutes before analysis in the flow cytometer with a threshold in FL-3 to exclude red blood cells and debris. Both granulocytes and monocytes were gated in a (side scatter) SSC/FL-1 dot plot, and CD11b expression was measured as median fluorescence intensity.

**LEUKOCYTE-PLATELET CONJUGATES.** Fixed blood cells were stained with anti-CD14 PE, anti-CD61 FITC, and LDS-751 and resuspended as described above. A threshold was set in FL-3, and the conjugates were gated in an SSC/FL-2 dot plot. Formation of conjugates was measured as median fluorescence intensity in FL-1 (green fluorescence).

### Enzyme Immunoassays

**COMPLEMENT ACTIVATION PRODUCTS.** Activation of the classic complement pathway was determined in two previously described enzyme immunoassays, one measuring C1rs-C1-inhibitor complexes and the other C4bc, the latter also being indicative of the lectin pathway [20, 21]. Both antibodies were a kind gift from Professor C. E. Hack, Amsterdam, The Netherlands. Activation of the alternative pathway was detected by quantifying the alternative convertase C3bBbP as previously described [12]. Activation of C3, the common link of all three initial pathways, was quantified in an enzyme immunoassay using the monoclonal antibody bH6 specific for a neopeptide exposed in C3b, iC3b, and C3c [22], and activation of the terminal C5-C9 pathway in an enzyme immunoassay using the monoclonal antibody aE11 specific for a C9 neopeptide in the SC5b-9 complex (TCC) using a modification of an assay described in detail previously [23]. A schematic presentation of the complement system with the three initial activation pathways and the activation products measured in the present study is given in Figure 1.

**THROMBOSPONDIN.** Release of thrombospondin was quantified using an assay previously developed in our laboratory [19].

**NEUTROPHIL DEGRANULATION.** The neutrophil granula proteins myeloperoxidase and lactoferrin were quantified by previously described enzyme immunoassays [24, 25].

### Statistics

If not otherwise stated, all complement inhibitors were tested in at least six separate experiments with blood from different donors. Wilcoxon's test for paired observations was used, with a two-tailed *p* value less than 0.05 considered statistically significant.

## Results

### Complement Activation in Blood Exposed to Polyvinyl Chloride and Heparin-Coated Polyvinyl Chloride Tubing

Circulation of lepirudin-anticoagulated human whole blood for 15 minutes in segments of tubing rotating as closed loops resulted in a significant increase in the alternative pathway convertase C3bBbP and in the soluble TCC for PVC tubing, but not for H-PVC tubing (Fig 2, left and middle). Conversely, the level of C1rs-C1-inhibitor complexes, a classic pathway indicator, was markedly elevated in the H-PVC tubing (Fig 2, right), but not in plain PVC, consistent with the well-known effect of heparin on C1-inhibitor function. C3 activation (C3bc) induced by PVC paralleled the increase in C3bBbP, whereas PVC hardly activated C4 (C4bc; data not shown).

### Effect of Specific Inhibitors on Polyvinyl Chloride-Induced Complement Activation

The formation of C3bBbP in blood circulated in PVC tubing was efficiently suppressed by monoclonal anti-

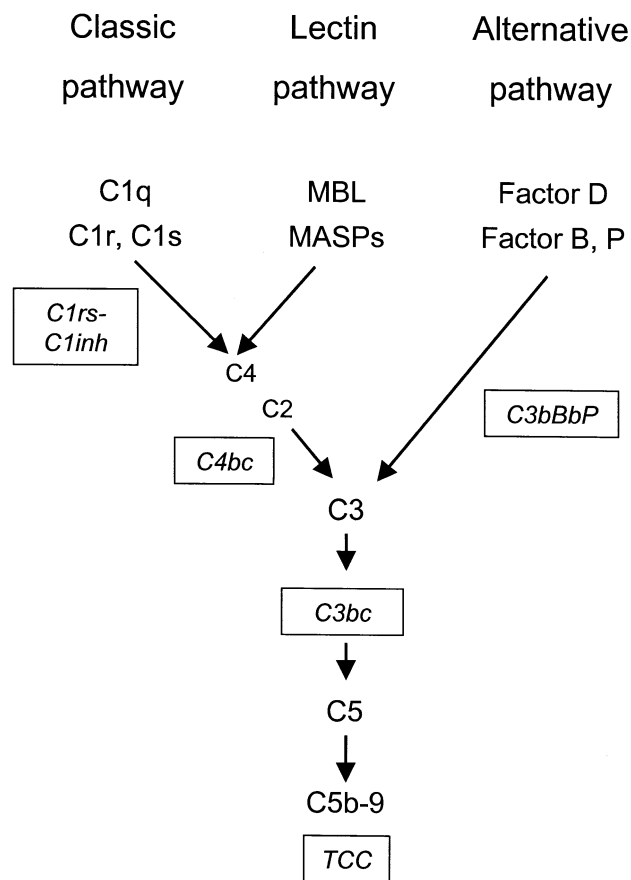


Fig 1. The complement system. The three initial and the terminal activation pathways of complement are schematically illustrated with the activation products measured in the present study in boxes. C1rs-C1-inhibitor (C1rs-C1inh) complexes are specific for the classic pathway, C4bc is generated both from the classic and the lectin pathways, C3bBbP is specific for the alternative pathway, C3bc is generated from any of the three initial pathways, and the terminal SC5b-9 complement complex (TCC) is the final activation product of the terminal pathway. (MASP = MBL-associated serine protease; MBL = mannose-binding lectin.)

factor D antibody (10  $\mu\text{g}/\text{mL}$ ) and by the C3 inhibitor compstatin (100  $\mu\text{mol}/\text{L}$ ), but not by monoclonal anti-C5 antibody (50  $\mu\text{g}/\text{mL}$ ; Fig 2, left). The lack of an inhibitory effect of the anti-C5 antibody was expected as this inhibitor interferes with the complement system at a stage after C3 activation. Formation of TCC was efficiently reduced by all three inhibitors and most pronounced for anti-C5 (Fig 2, middle). No C1rs-C1-inhibitor complexes were found in blood exposed to PVC, consistent with the inability of PVC to activate the classic pathway (Fig 2, right). Neither of the negative controls, an isotype-matched monoclonal antibody and a control peptide, had any effect on the generation of either of the activation products measured (data not shown).

### Leukocyte CD11b Expression

The expression of CD11b on granulocytes and monocytes increased after circulation of blood in PVC tubing, but

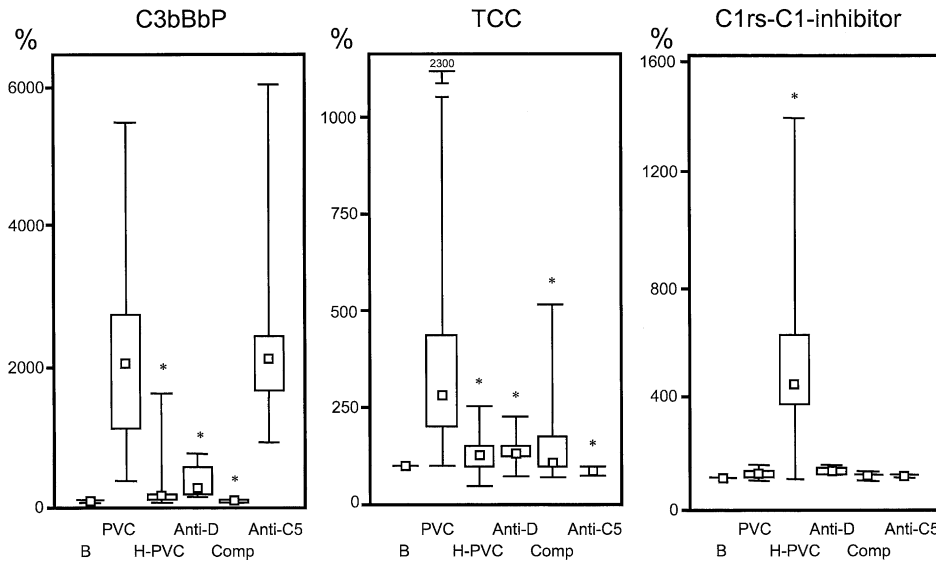


Fig 2. Activation of complement by uncoated (PVC) and heparin-coated polyvinyl chloride (H-PVC), and the effect of specific complement inhibitors. Human whole blood with or without complement inhibitors was circulated for 15 minutes in segments of tubing rotated as closed loops, whereupon complement activation was determined as the plasma concentrations of C3bBbP (left), terminal SC5b-9 complement complex (TCC; middle), and C1rs-C1-inhibitor complexes (right). Inhibitors were added to blood circulated in uncoated PVC tubing only. Data are expressed as percent of baseline (preincubation value) set as 100%. Median, 25th to 75th percentiles, and total range are shown;  $n = 6$  for each inhibitor,  $n = 18$  for PVC and H-PVC. \* $p < 0.05$  versus PVC. (Anti-C5 = anti-C5 [50  $\mu\text{g}/\text{mL}$ ]; Anti-D = anti-factor D [10  $\mu\text{g}/\text{mL}$ ]; B = baseline; Comp = compstatin [100  $\mu\text{mol}/\text{L}$ ]; TCC = terminal complement complex.)

not at all after incubation in H-PVC tubing (Fig 3). In the presence of anti-factor D (10  $\mu\text{g}/\text{mL}$ ), compstatin (100  $\mu\text{mol}/\text{L}$ ), or anti-C5 (50  $\mu\text{g}/\text{mL}$ ), the PVC-induced up-regulation of CD11b on granulocytes was substantially reduced (Fig 3, left). Blocking of the C5a receptor with a synthetic receptor antagonist counteracted CD11b expression and thus revealed that this activation mechanism was mainly C5a mediated (Fig 3, left). Similarly, monocyte CD11b expression was significantly attenuated by inhibition of complement, although not quite to the same extent as for granulocytes. No expression of CD11b

on monocytes could be demonstrated after exposure to H-PVC (Fig 3, right). Neither of the negative controls, an isotype-matched monoclonal antibody and a peptide, had any effect on CD11b expression (data not shown).

#### Leukocyte-Platelet Conjugate Formation

Exposure of blood to PVC, but not to H-PVC, induced a substantial increase in the formation of granulocyte-platelet and monocyte-platelet conjugates (Fig 4). The granulocyte-platelet conjugate formation was attenuated by compstatin, and substantially inhibited by anti-factor

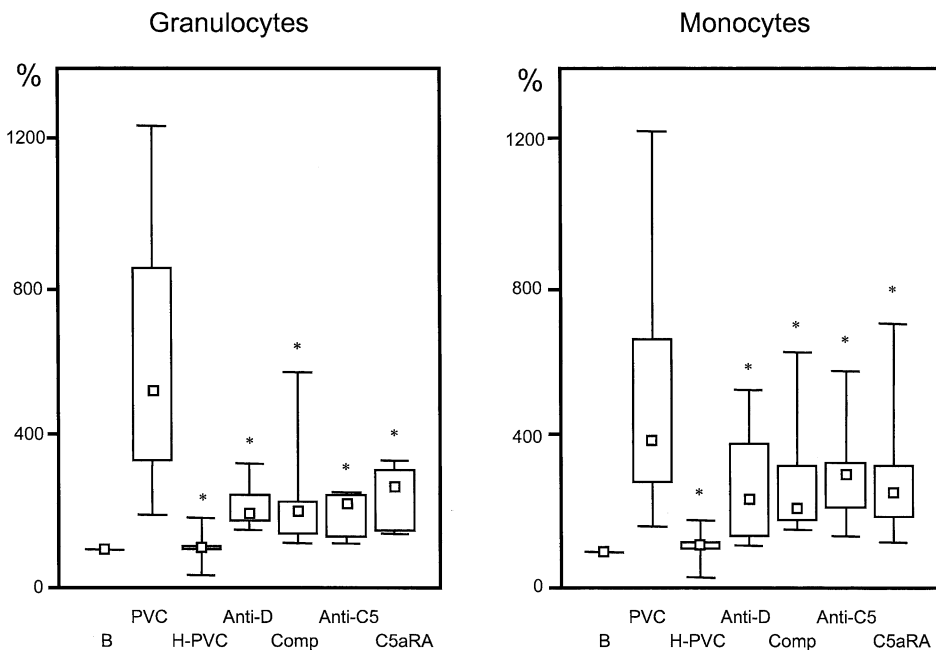
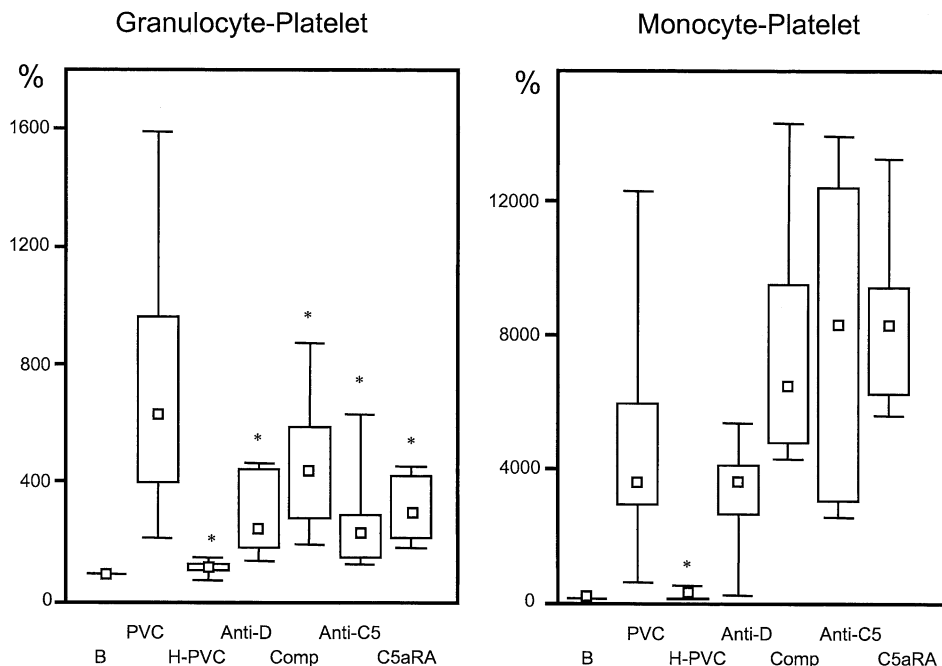


Fig 3. Expression of CD11b on leukocytes in blood exposed to uncoated (PVC) and heparin-coated polyvinyl chloride (H-PVC), and the effect of complement inhibitors. Human whole blood with or without complement inhibitors was circulated for 15 minutes in tubing loops, followed by measurement of CD11b expression on granulocytes (left) and monocytes (right) by flow cytometry. Inhibitors were added to blood circulated in uncoated polyvinyl chloride tubing only. Data are given as percent of baseline (B) median fluorescent intensity, which is set as 100%.  $n = 6$  for each inhibitor;  $n = 24$  for PVC and H-PVC. \* $p < 0.05$  versus PVC. (C5aRA = C5a receptor antagonist [5  $\mu\text{mol}/\text{L}$ ]; Comp = compstatin [100  $\mu\text{mol}/\text{L}$ ].)

Fig 4. Formation of leukocyte-platelet conjugates in blood exposed to uncoated (PVC) and heparin-coated polyvinyl chloride (H-PVC), and the effect of complement inhibitors. Human whole blood with or without complement inhibitors was circulated for 15 minutes in tubing loops, after which granulocyte-platelet (left) and monocyte-platelet (right) conjugates were quantified by flow cytometry. Inhibitors were added to blood circulated in uncoated polyvinyl chloride tubing only. Data are given as percent of baseline (B) median fluorescence intensity, which is set as 100%.  $n = 6$  for each inhibitor,  $n = 24$  for PVC and H-PVC. \* $p < 0.05$  versus PVC. (Comp = compstatin [100  $\mu\text{mol/L}$ ].)



D and anti-C5 (Fig 4, left). Blocking the C5aR revealed that granulocyte-platelet conjugate formation was largely C5a mediated (Fig 4, left). Contrary to granulocyte-platelet conjugates, the PVC-induced formation of monocyte-platelet conjugates was not reduced by complement inhibition. Interestingly, the finding that no monocyte-platelet conjugates were formed in blood circulated in H-PVC (Fig 4, right) shows that a yet unknown mechanism triggering this conjugate formation is apparently complement-independent and not activated by H-PVC. Neither of the negative controls, an isotype-matched control antibody and a control peptide, had any effect on the formation of either type of conjugate (data not shown).

#### Platelet Release Reaction

Exposure of blood to PVC induced a rapid increase in plasma thrombospondin concentrations, reaching a maximum after 5 minutes. Addition of complement inhibitors (anti-factor D, compstatin, or anti-C5) had no effect on this reaction (Fig 5). The thrombospondin release was much less pronounced in response to H-PVC tubing (Fig 5).

#### Neutrophil Degranulation

Circulation of blood in PVC tubing led to a substantial increase in plasma concentrations of the neutrophil proteins myeloperoxidase and lactoferrin. This release reaction was less rapid than platelet release of thrombospondin, with a gradual increase during the 4-hour incubation period. Only minor amounts of these proteins were detected in plasma after incubation in H-PVC (Fig 6). Complement inhibition had no effect on the PVC-induced release of myeloperoxidase and lactoferrin (data not shown).

#### Effect of Heparin Coating on Induced Fluid-Phase Complement Activation

Surface modification with heparin completely prevented the complement activation caused by PVC. However, the marked increase in C1rs-C1-inhibitor complexes seen for H-PVC, but not for PVC, led us to investigate whether the stimulatory effect of the surface-immobilized heparin on C1-inhibitor could possibly have an impact on fluid-phase classic pathway complement activation initiated by heat-aggregated IgG. Addition of increasing doses of heat-aggregated IgG to blood circulating in H-PVC and in PVC resulted in a corresponding rise in plasma C1rs-C1-inhibitor complexes, reaching essentially the same level at the highest heat-aggregated IgG concentrations for both materials (Fig 7, left). Inasmuch as increased levels of C1rs-C1-inhibitor complexes per se are not necessarily indicative of further propagation of complement activation, measurement of TCC was included as a marker of terminal pathway activation. As the TCC generation was similar for both materials, it can be concluded that a heparin surface is insufficient to counteract systemically induced complement activation (Fig 7, right).

#### Comment

##### Complement, Leukocytes, and Platelets in Cardiopulmonary Bypass Models

The activation of complement, leukocytes, and platelets caused by exposure of blood to the artificial surfaces of CPB circuits has been extensively studied in vitro, in animal models, and in clinical trials. Despite a vast body of literature in this field, the data in general are not conclusive and even to a large extent conflicting, both

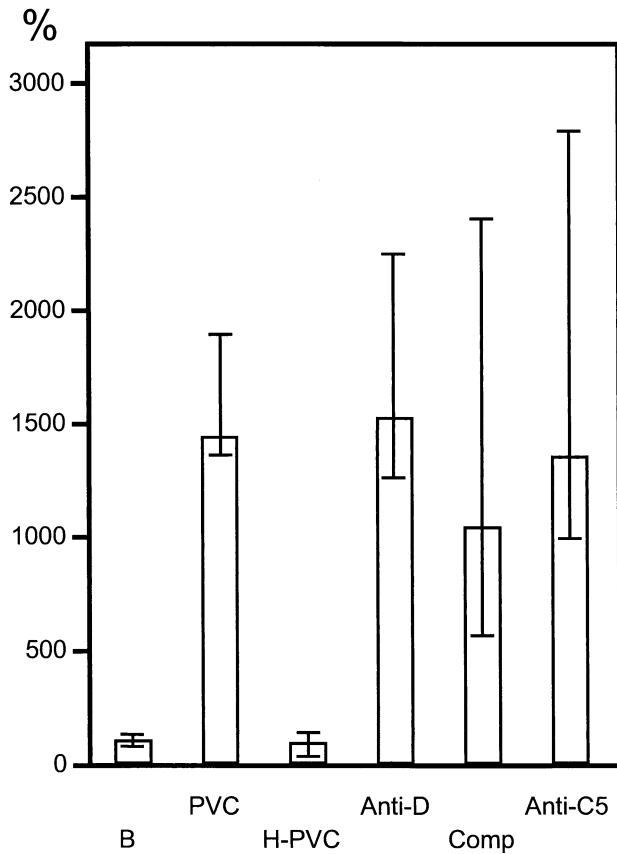


Fig 5. Platelet granula release in blood exposed to uncoated (PVC) and heparin-coated polyvinyl chloride (H-PVC), and the effect of complement inhibitors. Human whole blood with or without complement inhibitors was circulated for 5 minutes in tubing loops, followed by measurement of thrombospondin in plasma. Inhibitors were added to blood circulated in uncoated polyvinyl chloride tubing only. Median and range of three experiments, expressed as percent of baseline (B) set as 100%, are shown. (Comp = compstatin [100  $\mu$ mol/L].)

with regard to the mechanisms of the inflammatory response during CPB and the clinical benefit of attenuating the reaction. Thus, it was recently stated that “the scientific data concerning the effectiveness and safety of key principles of CPB are insufficient in both amount and quality of scientific evidence to serve as a basis for practical, evidence-based guidelines” [26]. A major reason why there is no common understanding of the coupling between activation of complement and inflammatory reactions is the lack of widely used, standardized analytical procedures for the detection and quantification of activation of these defense mechanisms. Moreover, measurement of inflammatory mediators in patients undergoing CPB only in part reflect the reactions related to the exposure of blood to artificial materials, as surface-independent factors like surgical trauma, ischemia-reperfusion injury, and, in particular, reinfusion of unprocessed blood from cardiotomy suction contribute to the inflammatory load [27].

In vitro, factors unrelated to the artificial surface can largely be eliminated, enabling selective studies of specific materials and the effects of surface modification. Nevertheless, the need for anticoagulation may interfere with the activation phenomena of interest, which can limit the suitability of experimental models, especially in the case of whole blood. Accordingly, the complement system cannot be studied in models using calcium-binding anticoagulants such as citrate or EDTA, and soluble heparin may interfere with complement by being inhibitory at high and stimulatory at low concentrations.

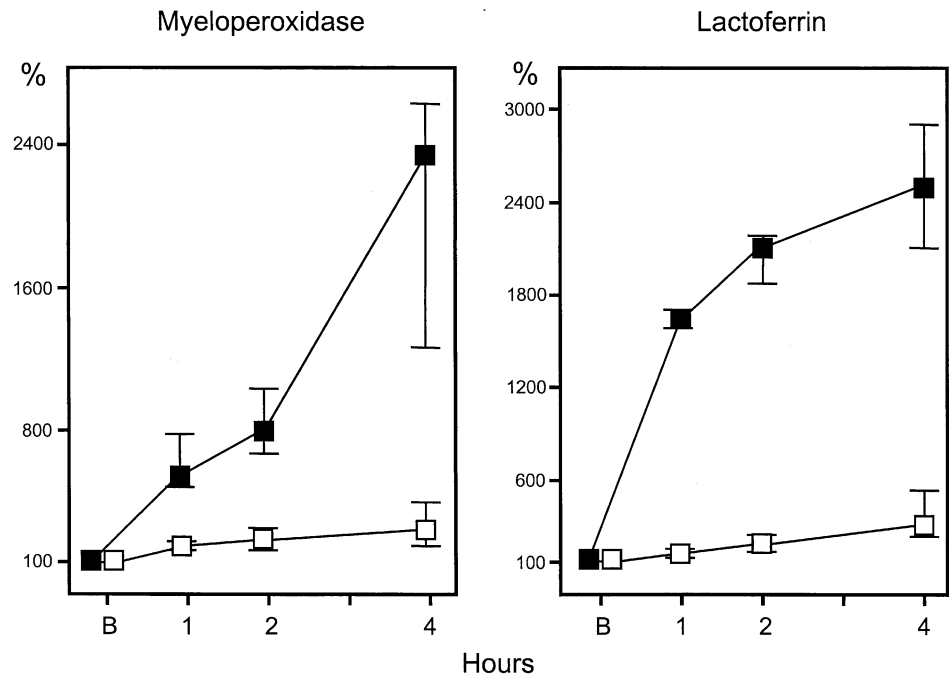
We recently described a novel model for studies of the role of complement in inflammation in human whole blood, based on the specific thrombin inhibitor lepirudin, a recombinant form of hirudin. This anticoagulant, in contrast to heparin, was found not to interfere with the complement system [12]. In this model all inflammation-related systems in blood, except for the thrombin-dependent steps of the coagulation mechanism, may undergo activation and mutually interact, without interference from an external inhibitor. In our view, this method for studies of inflammatory reactions, in combination with specific blocking of the complement system at different sites, is currently the most relevant in vitro technique for investigation of the coupling between complement and inflammation in whole blood. In the present work, the role of complement in inflammatory reactions induced by the exposure of blood to artificial surfaces is studied for the first time using this new procedure.

The model is designed to study the mechanism of the artificial surface-induced inflammatory reaction in a reductionistic manner. Thus, the limitations with respect to translating the data to a clinical CPB setting are obvious. However, our results from the novel whole blood model add important information to the understanding of the interaction between the complement system and cells in artificial surface-induced inflammation.

#### Mode of Complement Activation

Activation of complement by biomaterials is considered to occur largely by the alternative pathway, the mechanism of which has been described recently [28]. This presumption is in line with the present findings showing that exposure of blood to PVC led to formation of the alternative pathway convertase C3bBbP, whereas the effect on classic and lectin pathway markers C1rs-C1-inhibitor complexes and C4bc was insignificant. Further evidence supporting activation by means of the alternative pathway was the observation that activation of C3 and generation of TCC were both abolished by a monoclonal antibody against factor D. This antibody has previously been demonstrated to be an efficient inhibitor of the alternative complement pathway, as well as of leukocyte and platelet activation both in vitro [18] and in an animal model [29]. Moreover, the suppression of complement and leukocyte activation by compstatin in an in vitro model [30] is in agreement with this mode of activation.

Fig 6. Neutrophil degranulation in blood exposed to uncoated and heparin-coated polyvinyl chloride. Human whole blood was circulated for 1, 2, or 4 hours in loops of uncoated (closed squares) and heparin-coated polyvinyl chloride (open squares), followed by quantification of plasma myeloperoxidase (left) and lactoferrin (right). Data are given as percent of baseline (B) value set as 100%. Median and range of three experiments are shown.

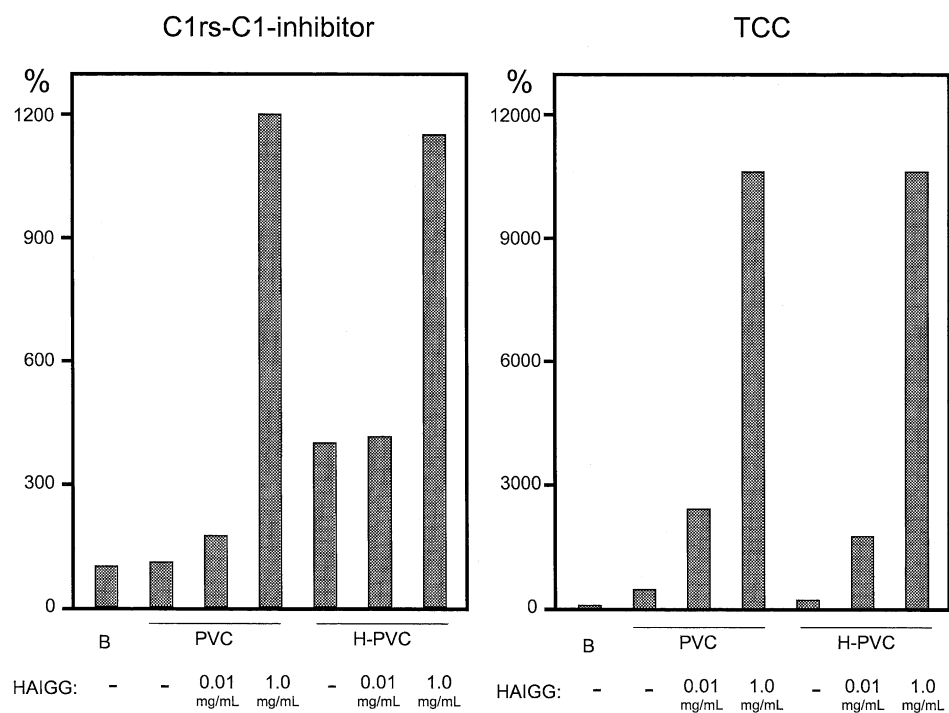


**Complement-Dependent and Complement-Independent Mechanisms of Polyvinyl Chloride-Induced Leukocyte Activation**

Inhibition of complement had diverse effects on the activation of leukocytes. The presence of complement inhibitors markedly attenuated the expression of CD11b on granulocytes, whereas the degranulation of the same cells, measured as release of myeloperoxidase and lacto-

ferrin, was unaffected. The latter observation is in accordance with complement-independent granula release, previously observed in patients undergoing dialysis [31]. The pathophysiologic role of neutrophil activation, observed as complement-independent degranulation, may differ from that of complement-dependent upregulation of adhesion molecules like CD11b. It is tempting to speculate that the exposure of adhesion molecules more

Fig 7. Effect of the heparin surface on complement activation in the fluid phase. Human whole blood with or without addition of complement activating heat-aggregated immunoglobulin (HAIGG) was circulated for 1 hour in loops of uncoated (PVC) or heparin-coated polyvinyl chloride (H-PVC) tubing. The resulting plasma concentrations of C1rs-C1-inhibitor complexes (left) and terminal SC5b-9 complement complex (TCC; right) were quantified. One representative result from three experiments, expressed as percent of baseline (B) value set as 100%, is shown.



readily will lead to granulocyte trapping in vital organs and, as a consequence, worsening of organ inflammation. Furthermore, the formation of granulocyte-platelet conjugates was a complement-dependent process, probably related to the increased expression of adhesion molecules on the granulocyte surface. In this respect, granulocytes and monocytes clearly differed in their dependence on complement, as inhibitors had a more pronounced effect on the upregulation of CD11b on granulocytes than on monocytes. In addition, the formation of monocyte-platelet conjugates was independent of complement. It could be argued that complement inhibition was not complete, and because platelet conjugate formation of granulocytes and monocytes differ in their sensitivity to complement, the effect of complement inhibition was seen for granulocytes only. However, our data, in particular for the anti-C5 antibody (Fig 2), clearly show that complement inhibition was complete. Hence, it appears unlikely that the striking difference observed between monocytes and granulocytes in terms of conjugate formation would be a result of inefficient complement inhibition. The mechanisms by which monocytes and granulocytes form conjugates with platelets under these conditions need to be further investigated.

#### *Mechanism of Complement-Mediated Leukocyte Activation by Polyvinyl Chloride*

In the present study four different complement inhibitors were used. Anti-factor D blocks the alternative pathway, compstatin blocks C3 and thereby all initial pathways, and anti-C5 blocks cleavage of C5, whereas the C5aR antagonist specifically blocks the interaction between C5a and its receptor. Interestingly, in all cases where an effect was seen on leukocyte activation, it was essentially the same for all inhibitors. Apparently, exposure of blood to PVC triggers activation of the complement system by the alternative pathway by means of factor D, leading to activation of leukocytes largely through C5a. These findings are in accordance with previously reported results showing that a monoclonal antibody blocking C5 cleavage prevented upregulation of granulocyte CD11b and formation of leukocyte-platelet conjugates [32], whereas an antibody blocking C8, subsequent to C5a formation, lacked this capacity [33]. The same group demonstrated a selective effect of C3 cleavage on monocyte activation [34] in contrast to our present results showing no difference between inhibition at the C3 or C5 level. This discrepancy is most likely a result of different experimental conditions; in the present study the surface exposure was limited to PVC tubing, whereas the experimental setup used by the other group included a complete CPB circuit with a membrane oxygenator, representing a vast surface area and presumably a much greater challenge to the complement system. Similar experimental differences may also explain why the anti-factor D antibody inhibited neutrophil degranulation (myeloperoxidase and elastase) and platelet thrombospondin release in a complete CPB circuit *in vitro* [18], in contrast to the observations in the present study.

#### *Complement and Platelets*

Platelets were activated by PVC, as revealed by the release of thrombospondin. In a previous report we showed that exposure of blood to PVC caused platelet granula release, although the upregulation of P-selectin was minor [11]. Others have demonstrated that platelets, but not leukocytes, are activated by the C5b-9 complex in an *in vitro* CPB circuit [33]. However, as mentioned above for leukocytes, it should be emphasized that this platelet activation may well be related to factors of the CPB system other than PVC. Interestingly, the CPB model, in contrast to our loop model, induced platelet expression of P-selectin [11]. Thus, similar to our present findings for neutrophils, there may be a complement-independent mechanism responsible for platelet granula release and a complement-dependent mechanism for the expression of platelet surface molecules. Different *in vitro* and *in vivo* models with different areas of artificial surface, absence or presence of membrane oxygenators, and variable degrees of surgical trauma may very well trigger these mechanisms differently. It may be speculated that complement-independent granula release and complement-dependent membrane conversion to an "adhesive" phenotype represent modest and more extensive degrees, respectively, of cellular activation induced by the artificial surface.

#### *Effect of Heparin Coating*

Surface modification by end point immobilization of heparin (Carmeda BioActive Surface, CBAS) improves the biocompatibility of artificial materials, reviewed by Olsson and coworkers [35], including the capacity to inhibit complement activation as confirmed in this report. Other recent reports have presented similar findings; eg, that heparinization of PVC reduces the expression of CD11b on granulocytes [36]. Coating with heparin makes a usually hydrophobic polymeric surface hydrophilic and changes the properties of protein binding from unspecific adsorption to a more selective uptake of plasma proteins [37]. The biocompatible, "endothelial-like" properties of end point-attached heparin may to some degree be related to the structural and functional similarity between heparin and the natural endothelial surface heparan sulfate lining the vascular wall, and possibly also to the capacity of the immobilized heparin to bind phospholipids. The present findings show that the heparin surface, unlike plain PVC, caused a marked increase in C1rs-C1-inhibitor complex formation, in accordance with a previous study using the same heparin-coated surface [38], and consistent with the known accelerating effect of heparin on C1-inhibitor function [39]. Similar observations have been made before in a microtiter plate model, where end point attachment of heparin led to an increased uptake of C1q with concomitant classic pathway activation, measured as activated C3 [40]. Although the experimental conditions used by these authors differed from ours, the net effect on the complement system was the same, ie, inhibition as a result of the blocking of the alternative pathway.

As no indication of activation of C4 or later components of the complement system could be detected in blood exposed to H-PVC, the increase of the classic pathway marker C1rs-C1-inhibitor complexes appears contradictory, indicating enhanced inhibition rather than activation at the C1 level. Similar studies of the plasma contact activation system, however, showed that coagulation factor XII, activated by exposure to the negatively charged heparin surface, is instantaneously neutralized by the coagulation inhibitor antithrombin, bound at high local concentration to and functionally accelerated by the surface-immobilized heparin molecules [41]. By this mechanism, any factor XII activation occurring on this particular heparin coating is effectively neutralized, thus blocking feedback activation of further factor XII and the subsequent onset of the coagulation system. A similar inhibitory mechanism of the classic pathway activation of the complement system by the heparin surface can be envisioned, as both C1q and C1-inhibitor interact with heparin.

Despite the fact that the heparin coating was as effective as complement inhibitors in attenuating PVC-induced complement activation, it had no effect on systemic classic pathway activation of the complement system induced by addition of heat-aggregated IgG to the circulating blood. This finding is not surprising as the neutralization of circulating activation products by the surface is likely to be diffusion-controlled, meaning that any activation product generated in the fluid phase must be transported to the surface to undergo heparin-catalyzed inhibition. This limitation of the heparin surface may have bearing on the potential clinical use of fluid-phase complement inhibitors in cardiothoracic surgery involving CPB, as obviously the inhibitory effect of the surface is not sufficient to compensate for activation caused by mechanisms unrelated to the artificial surface, such as surgical trauma, ischemia-reperfusion, and blood-gas interaction in the oxygenator circuit. Both in vivo animal models [29, 42] and clinical trials [43] have shown that specific anti-complement antibodies can attenuate the inflammatory response seen during CPB procedures and possibly reduce clinical complications and improve outcome. In a porcine model of CPB using a heparin-coated circuit, inhibition of complement with soluble complement receptor 1 (sCR1) was found to improve the clinical outcome compared with heparin coating only [44]. This observation is in agreement with our present findings that the heparin surface is unable to prevent complement activation induced in the fluid phase, although the experimental models differ considerably. Thus, combining complement inhibition with heparin-coated systems may be a rational approach to improved control of the inflammatory response during extracorporeal circulation.

The present study demonstrates that the biocompatibility of end point-attached heparin with respect to leukocyte and platelet activation cannot be fully explained by the well-documented complement inhibitory properties of this surface. When comparing these results with previous reports, it should be emphasized that we

have studied the effect of the artificial surface only, and that we have used an anticoagulant not previously used in similar studies. The fact that lepirudin does not interfere with complement activation makes it an ideal anticoagulant for this kind of investigation. Our results show that the upregulation of CD11b on both granulocytes and monocytes and the granulocyte-platelet conjugate formation were dependent on complement activation, and, as expected, these phenomena were suppressed in blood exposed to the heparin surface. In contrast, neutrophil degranulation (myeloperoxidase, lactoferrin), the platelet release of thrombospondin, and the monocyte-platelet conjugate formation were unaffected by complement inhibition, yet all these reactions were counteracted by the heparin surface. Thus, pathways other than complement exist for activation of leukocytes and platelets, the triggering of which can be prevented by modification of artificial surfaces with functionally active heparin.

In conclusion, complement is an important trigger of artificial surface (PVC)-induced cell activation, but complement-independent mechanisms are also involved. Because surface-independent complement activation is known to be involved in clinical CPB, surface heparinization and specific systemic complement inhibition may be supplemental approaches in minimizing the inflammatory load during such procedures, in particular in patients at high risk owing to preoperative organ dysfunction. However, both further basic and experimental studies, as well as controlled clinical trials, are required to establish such treatment as evidence-based medicine.

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