The artificial surface-induced whole blood inflammatory reaction revealed by increases in a series of chemokines and growth factors is largely complement dependent

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Abstract: Exposing blood to an artificial surface results in a systemic inflammatory response, including cytokine release and complement activation. We studied the artificial surface-induced inflammation in human whole blood using an extensive panel of inflammatory mediators including proinflammatory cytokines, chemokines and growth-factors and investigated the role of the complement system in the induction of this response. Using multiplex technology, 27 different inflammatory mediators were measured after circulating blood for 4 hours in polyvinyl chloride tubing. The C3 inhibitor compstatin was used to block complement activation. A significant ($p < 0.05$) increase in 14 of the 27 mediators was induced by the surface, of which 7 were chemokines (IL-8, MCP-1, MIP-1α, MIP-1β, RANTES, eotaxin and IP-10) and 5 were growth-factors (G-CSF, GM-CSF, VEGF, PDGF and FGF). The traditional proinflammatory cytokines like IL-1β, TNFα and IL-6 were not induced, although IL-6, as well as IL-15 and IL-17 increased if the surface was coated with highly bioincompatible laminaran. Inhibition of complement activation with compstatin significantly ($p < 0.05$) reduced the formation of 12 of the 14 mediators. For 10 of the 12 mediators, the inhibition was by 2/3 or more, for the remaining two the inhibition was more moderate. A highly biocompatible heparin-coated PVC surface was used as negative control and completely abolished the whole inflammatory response. The artificial surface PVC markedly induced a broad spectrum of chemokines and growth-factors, which was largely dependent on activation of complement. © 2007 Wiley Periodicals, Inc. J Biomed Mater Res 87A: 129–135, 2008

Key words: artificial surface; biocompatibility; complement; cytokine; growth factors

INTRODUCTION

Blood exposed to an artificial surface, as in cardiopulmonary bypass (CPB), results in a systemic inflammatory response involving activation of leukocytes, platelets and plasma cascade systems, including the complement system. It has previously been shown that complement activation is necessary for a number of the subsequent inflammatory reactions, since blocking complement activation with specific monoclonal antibodies or peptides attenuates or totally inhibits several secondary responses.[1–5] When studying interactions between inflammatory systems in whole blood, it is crucially important that the experimental conditions enable mutual interactions between the systems. In particular, the choice of anticoagulant in studies of complement involvement in inflammatory processes is highly important, as several anticoagulants (both calcium binding agents and heparin) interfere with complement activation and thus are unsuitable for this purpose. We have

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developed a model using human whole blood anticoagulated with the recombinant hirudin analogue lepirudin, a highly specific thrombin inhibitor, which does not interfere with complement activation and therefore enables complement to interact in the inflammatory network.[6]

This model has been documented to be highly suitable for studying the inflammatory reaction induced by artificial surfaces,[7] although it cannot directly be compared to CPB as it lacks a number of factors that are present in an *in vivo* situation. Using this model, we have previously shown that the artificial surface-induced synthesis of the chemokines interleukin 8 (IL-8) and monocyte chemoattractant protein 1 (MCP-1) is totally and moderately dependent, respectively, on complement activation, whereas the cytokines IL-1β, tumor necrosis factor alpha (TNF-α) and IL-6 were not induced by the artificial surface in this model.[4]

The aim of the present study was twofold: first, to investigate the potential of a polyvinyl chloride (PCV) artificial surface to induce a broad inflammatory response in the human whole blood model taking advantage of the novel multiplex technology enabling assay of a long range of mediators in one single sample and second, to evaluate the role of complement in the induction of this reaction, by specific inhibition with the C3 inhibitor compstatin. For this purpose we used an assay of 27 different cytokines, including chemokines and growth factors.

MATERIALS AND METHODS

The model for artificial surface-induced inflammation has been described in detail previously.[8] Lepirudin was used as anticoagulant as this does not affect complement activation.[6] The main artificial surface used in the study was PVC, a surface regularly encountered in various clinical settings, such as CPB. A heparin-coated tubing served as a negative control, as several studies have shown this surface to be virtually inert and "endothelial-like."[3,4,9,10] PVC coated with laminaran was used as a positive control. This surface is highly bioincompatible, is a potent inducer of inflammation, and was included to determine whether cytokines in the novel assay that were not induced by the PVC surface could be induced by a more powerful stimulus. Heparin-coated (CBAS®, Carmeda BioActive Surface), laminaran-coated and uncoated PVC tubing was provided by Carmeda AB, Stockholm, Sweden.

Reagents

Sterile phosphate-buffered saline (PBS) was from Life Technologies™ (Paisley UK) and lepirudin (Refludan®) from Hoechst, Frankfurt am Main, Germany.

Complement inhibitor

Compstatin is a 13 amino acid cyclic peptide which binds to and inhibits cleavage of C3. We used the compstatin analogue Ac-I[CV(1MeW)QDWGAHRC]T-NH_2, which is 264 times more active than the parent peptide I[CVVQDWGH HRC]T-NH_2. Both compstatin and a control peptide IAVVQ DWGHHRAT-NH_2 were synthesised as previously described.[11] Assessment of complement activation was made by measuring the terminal complement complex (TCC) as previously described.[12] Compstatin was used at a concentration of 25 μ*M*, as pilot experiments had shown efficient inhibition of complement activation at this dose.

Experimental set-up

Samples of blood were supplied with compstatin or equal volumes of saline and incubated at 37°C for 4 min. A volume of 750 μL blood was then transferred to segments of PVC, heparin-coated or laminaran-coated tubing (length 30 cm, internal diameter 3 mm). In a few selected experiments a control peptide was included without effect on release of inflammatory mediators (data not shown). Each segment was closed end to end and incubated by rotating slowly at 37°C for 4 h if not otherwise stated. After incubation, the blood was centrifuged for 15 min, 3220*g* at 4°C. The plasma was frozen in aliquots at −70°C for later analysis of cytokines.

Cytokine assay

Plasma samples were analyzed using a multiplex cytokine assay (Bio-Plex Human Cytokine 27-Plex Panel, Bio-Rad Laboratories, Hercules, CA) containing the following analytes: Interleukin (IL) 1 beta (IL-1β), IL-1 receptor antagonist (IL-1Ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin (CCL11), basic fibroblast growth factor (FGF), granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), interferon gamma (IFN-γ), chemokine (C-X-C motif) ligand 10 (IP-10 or CXCL10), monocyte chemoattractant protein 1 (MCP-1 or CCL2), macrophage inflammatory protein-1-alpha (MIP-1α or CCL3), macrophage inflammatory protein-1-beta (MIP-1β or CCL4), platelet-derived growth factor-BB (PDGF), regulated upon activation T cell expressed and secreted (RANTES or CCL5), tumor necrosis factor alpha (TNF-α) and vascular endothelial growth factor (VEGF). The analysis was performed according to the instructions from the manufacturer.

Statistics

Wilcoxon's test for paired observations was used, with a two-tailed p value < 0.05 considered statistically significant.

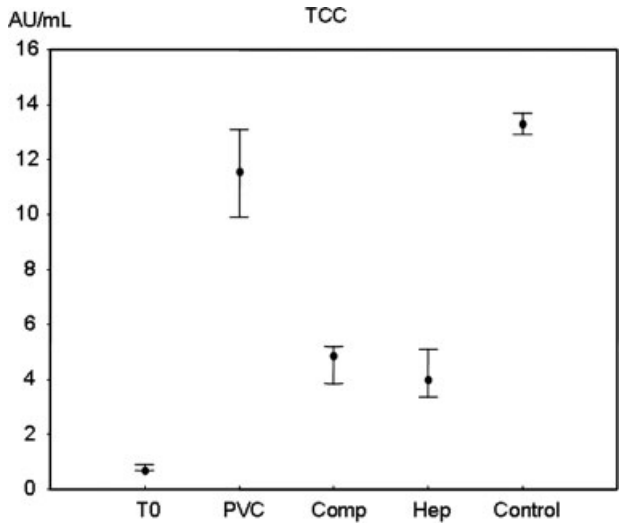

Figure 1. Median concentration in arbitrary units/mL (with 25–75 percentiles) for the terminal complement complex (TCC). T_0, baseline values; PVC, polyvinyl chloride loops; Comp, PVC loops with compstatin; Hep, heparin-coated loops; Control, control peptide. Loops incubated at 37°C for 4 h. $p < 0.05$ for PVC versus T_0, and $p < 0.05$ for compstatin versus PVC.

RESULTS

Effect of compstatin on complement activation

Complement activation was determined by measuring the terminal complement complex (TCC). Generation of TCC after incubation of blood in PVC loops increased significantly compared to baseline. This increase was attenuated by the addition of compstatin during incubation, and complement activation was of the same low magnitude as in the biocompatible heparin coated loops. As expected, the control peptide did not influence complement activation (Fig. 1).

Mediators induced by the PVC surface and the corresponding inhibition by compstatin

Fourteen of the 27 mediators increased significantly after exposure to PVC. Heparin-coated tubing (negative control) abolished all these responses (illustrated in Figures 1–5). For 12 of the 14 mediators, complement inhibition with compstatin significantly reduced the PVC-induced increase, for 10 out of 12 by 2/3 or more (Table I).

Chemokines

IL-8 increased from 8 pg/mL (8-8) (median and 25-75 percentiles) at baseline to 532 pg/mL (224-1295) after 4 h incubation ($p < 0.05$) and was significantly inhibited ($p < 0.05$) by compstatin (25 pg/mL (17-28)) (Fig. 2, left panel). MCP-1 increased from 10 pg/mL (7-12) at baseline to 120 pg/mL (59-173) after 4 h incubation ($p < 0.05$) and was significantly inhibited ($p < 0.05$) by compstatin (17 pg/mL (15-21)) (Fig. 2, right panel). MIP-1α increased from 4 pg/mL (4-8) at baseline to 46 pg/mL (4-53) after 4 h incubation ($p < 0.05$) and was significantly inhibited ($p < 0.05$) by compstatin (9 pg/mL (11-17)) (Fig. 3, left panel). MIP-1β increased from 53 pg/mL (44-67) at baseline to 940 pg/mL (502-1220) after 4 h incubation ($p < 0.05$) and was significantly inhibited ($p < 0.05$) by compstatin (298 pg/mL (204-464)) (Fig. 3, right panel). RANTES increased from 1206 pg/mL (915-1408) at baseline to 13185 pg/mL (11,120-28,491) after 4 h incubation ($p < 0.05$) and was significantly inhibited ($p < 0.05$) by compstatin (6790 pg/mL (5897-13243) (Fig. 4, left

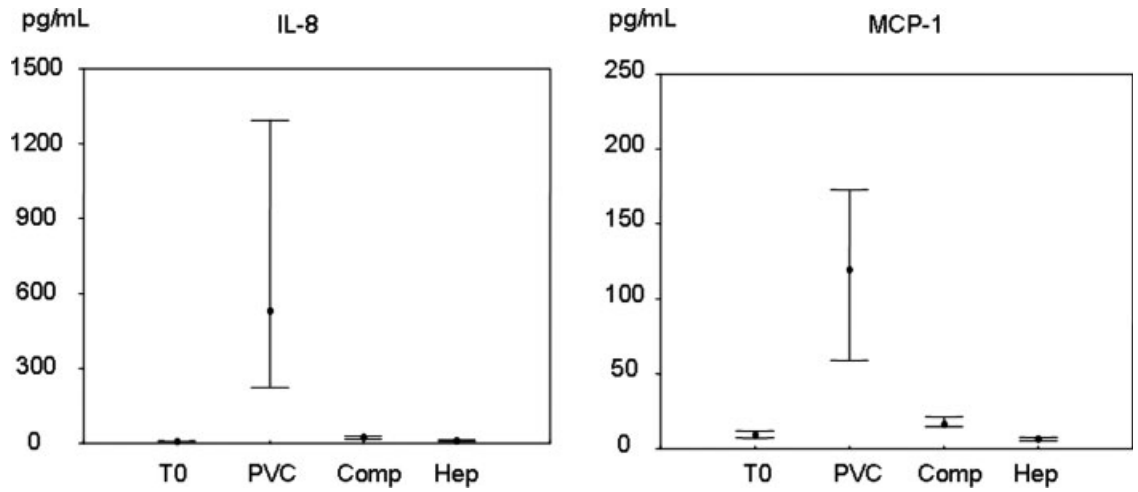

Figure 2. Median concentration in pg/mL (with 25–75 percentiles) for interleukin 8 (IL-8, left panel) and monocyte chemoattractant protein 1 (MCP-1, right panel). Loops incubated at 37°C for 4 h. $p < 0.05$ for PVC versus T0, and $p < 0.05$ for compstatin versus PVC. $N = 8$–10.

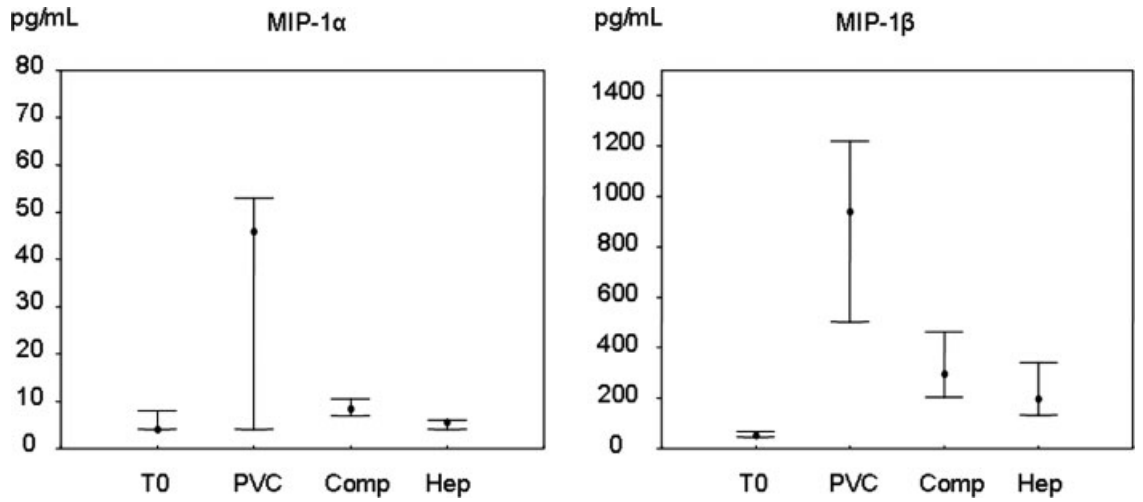

Figure 3. Median concentration in pg/mL (with 25–75 percentiles) for macrophage inflammatory protein-1-alpha (MIP-1α, left panel) and macrophage inflammatory protein-1-beta (MIP-1β, right panel). Loops incubated at 37°C for 4 h. $p < 0.05$ for PVC versus T0, and $p < 0.05$ for compstatin versus PVC. $N = 8–10$.

panel). Eotaxin increased from 40 pg/mL (27-50) at baseline to 156 pg/mL (126-192) after 4 h incubation ($p < 0.05$) and was significantly inhibited ($p < 0.05$) by compstatin (79 pg/mL (66-85)) (Fig. 4, right panel). IP-10 increased from 709 pg/mL (637-1030) at baseline to 971 pg/mL (906-1729) after 4 h incubation ($p < 0.05$) and was significantly inhibited ($p < 0.05$) by compstatin pg/mL (612 (565-1169)) (data not shown).

Growth factors

G-CSF increased from 11 pg/mL (6-25) (median and 25-75 percentiles) at baseline to 146 pg/mL (111-210) after 4 h incubation ($p < 0.05$) and was significantly inhibited ($p < 0.05$) by compstatin (29 pg/mL (23-39) (Fig. 5, left panel). GM-CSF increased from 30 pg/mL (30-30) at baseline to 483 pg/mL (352-645) after 4 h incubation ($p < 0.05$) and was significantly inhibited ($p < 0.05$) by compstatin (48 pg/mL (30-61)) (Fig. 5, right panel). VEGF increased from 12 pg/mL (11-23) at baseline to 314 pg/mL (225-377) after 4 h incubation ($p < 0.05$) and was significantly inhibited ($p < 0.05$) by compstatin (41 pg/mL (32-52)) (Fig. 6, left panel). PDGF increased from 213 pg/mL (92-357) at baseline to 7663 pg/mL (6258-13891) after 4 h incubation ($p < 0.05$) and was significantly inhibited ($p < 0.05$) by compstatin (4399 pg/mL (2366-4880)) (Fig. 6, right panel). FGF increased from 50 pg/mL (50-50) at baseline to 631 pg/mL

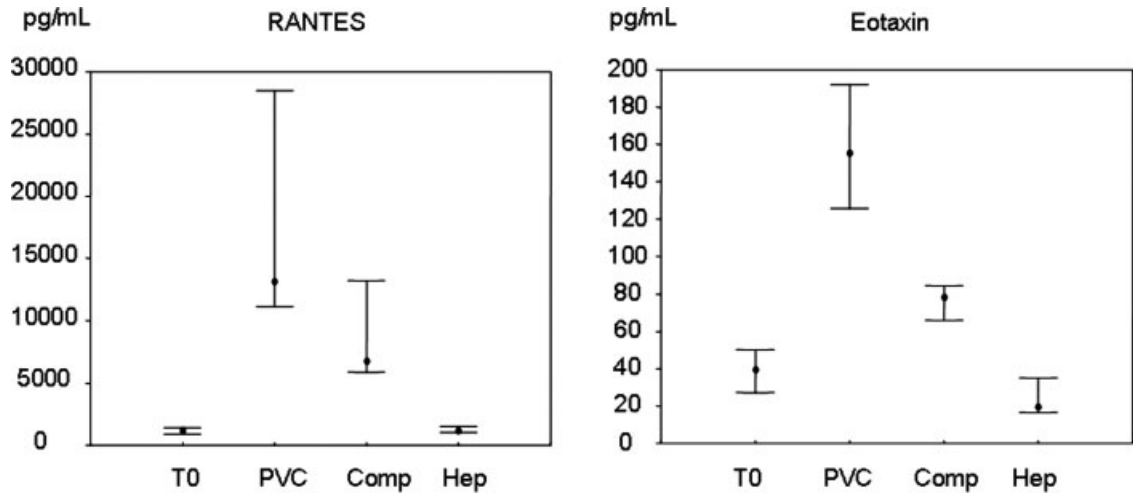

Figure 4. Median concentration in pg/mL (with 25–75 percentiles) for regulated upon activation T cell expressed and secreted (RANTES, left panel) and eotaxin (right panel). Loops incubated at 37°C for 4 h. $p < 0.05$ for PVC versus T0, and $p < 0.05$ for compstatin versus PVC. $N = 8–10$.

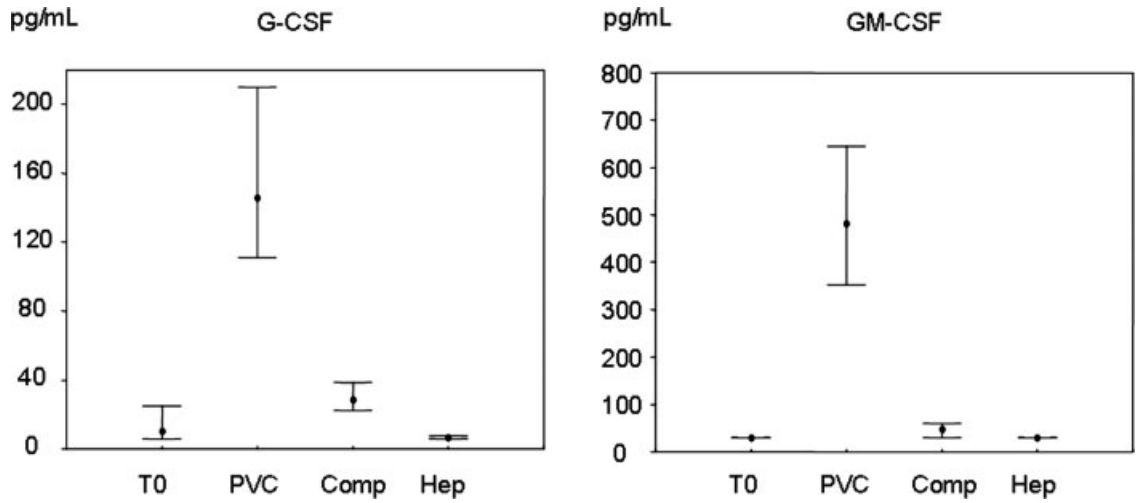

Figure 5. Median concentration in pg/mL (with 25–75 percentiles) for granulocyte colony stimulating factor (G-CSF, left panel) and granulocyte-macrophage colony stimulating factor (GM-CSF, right panel). Loops incubated at 37°C for 4 h. $p < 0.05$ for PVC versus T0, and $p < 0.05$ for compstatin versus PVC. $N = 8$–10.

(50-765) after 4 h incubation ($p < 0.05$) and was significantly inhibited ($p < 0.05$) by compstatin (50 pg/mL (50-57)) (data not shown).

The inhibitory effect of compstatin is summarized in Table I.

Other mediators induced by the PVC surface

IFN gamma increased from 43 (34-51) (median and 25-75 percentiles) at baseline to 417 pg/mL (34-721) after 4 h incubation ($p < 0.05$) and was to some extent inhibited by compstatin (128 pg/mL (103-164)), although this did not reach statistical significance (data not shown). IL-9 increased from 8 pg/mL (6-57) at baseline to 121 pg/mL (121-236) after 4 h incubation ($p < 0.05$) and was to some extent inhibited by compstatin (38 pg/mL (7-49)), although this did not reach statistical significance (data not shown).

Mediators not induced by the PVC surface

The following 13 of the 27 mediators were not increased after exposure to PVC, of which many are typical proinflammatory mediators not belonging to the chemokine- and growth factors groups: TNF-α, IL-1β, IL-1rα, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-15 and IL-17. IL-6, IL-15, and IL-17 could, however, be induced after exposure to the PVC surface coated by the bioincompatible laminaran; IL-6 (Fig. 7) and IL-17 after 4 h, whereas IL-15 was detectable only after 8 h (data not shown).

DISCUSSION

It has previously been demonstrated that contact between blood and an artificial surface, such as PVC, induces an inflammatory response, including synthesis and release of cytokines.[13–16] The results presented here extend previous findings in the way that a long range of hitherto not studied inflammatory mediators have now been included. Previous reports have been limited to a small number of cytokines analyzed in separate EIAs,[4] whereas we have analyzed a large number of inflammatory mediators simultaneously in one single plasma sample. It should be noted, however, that the present model is restricted to studying the interaction between blood and the artificial surface itself and that the results cannot directly be extrapolated to an *in vivo* situation as, for example, cardiopulmonary bypass, which in addition to the artificial surface introduces several other inflammatory stimuli including a surgical trauma, an oxygenator and a pump. It is, nevertheless, important to reveal the mechanisms by which the artificial surface itself induces an inflammatory

TABLE I
Complement Inhibition of PVC-Induced Increase in Selected Inflammatory Mediators

FGF	IP-10	IL-8	GM-CSF	MIP-1α	MCP-1	VEGF	G-CSF	MIP-1β	Eotaxin	PDGF	RANTES
100 (98-100)	100 (100-100)	99 (94-99)	96 (94-100)	94 (83-100)	93 (77-97)	88 (85-94)	85 (57-93)	74 (62-79)	69 (17-84)	55 (40-66)	42 (13-57)

Median percentage reduction with 25-75 percentiles.

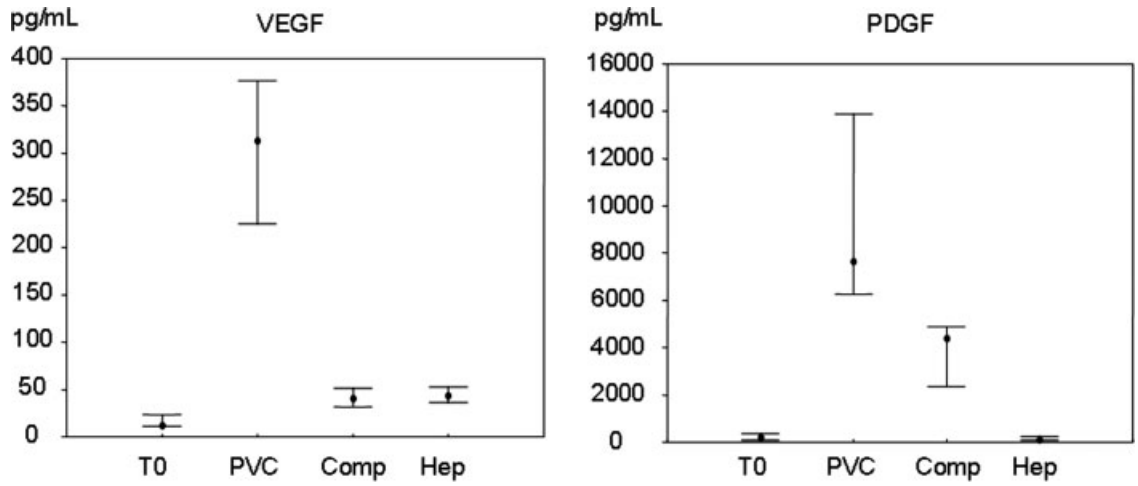

Figure 6. Median concentration in pg/mL (with 25–75 percentiles) for vascular endothelial growth factor (VEGF, left panel) and platelet-derived growth factor-BB (PDGF, right panel). Loops incubated at 37°C for 4 h. $p < 0.05$ for PVC versus T_0, and $p < 0.05$ for compstatin versus PVC. $N = 8$–10.

reaction, and for this purpose we claim that the present model currently is the most reliable to investigate the interaction between inflammatory systems in human whole blood *in vitro*.

The present data show that induction of synthesis and release of inflammatory markers in human whole blood by the PVC surface is selective in the way that some markers show a considerable increase upon stimulation, some a more modest response and some markers do not increase at all. This is in line with our previous findings where IL-8 and MCP-1 increased whereas IL-1 β, IL-6, IL-10, and TNF-α were virtually unaffected after incubation in PVC tubing.[4] We now show, using the broad panel of mediators, that this differential increase is not random, but follows a pattern where in particular the chemokines (IL-8, MCP-1, MIP-1α, MIP-1β, RANTES, eotaxin, and IP-10) and the growth factors (G-CSF, GM-CSF, VEGF, PDGF, and FGF basic) were induced, while most of the other cytokines were not (TNF-α, IL-1β, IL-1rα, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-15, and IL-17). It is reasonable to speculate that the pattern recognition receptors responding to microorganisms through toll-like receptors (TLR), in particular the CD14/MD2/TLR4 complex, is not stimulated by this artificial surface. In contrast, complement is directly activated by the surface and generates anaphylatoxins, in particular C5a, which is most likely responsible for a major fraction of the inflammatory mediators generated in this system.[2,4,9] The finding that none of the inflammatory markers increased in the heparin-coated loops confirms that the activation phenomena studied indeed reflect properties of the artificial material and that coating with heparin dramatically improves the biocompatibility, presumably due to the endothelial cell like properties of this surface modification.[17–21] In previous clinical trials with CPB, investigation of inflammatory mediators has to a large extent focused on cytokines. Based on our results, we suggest that a broader range of mediators, including chemokines and growth factors, should be evaluated in future trials.

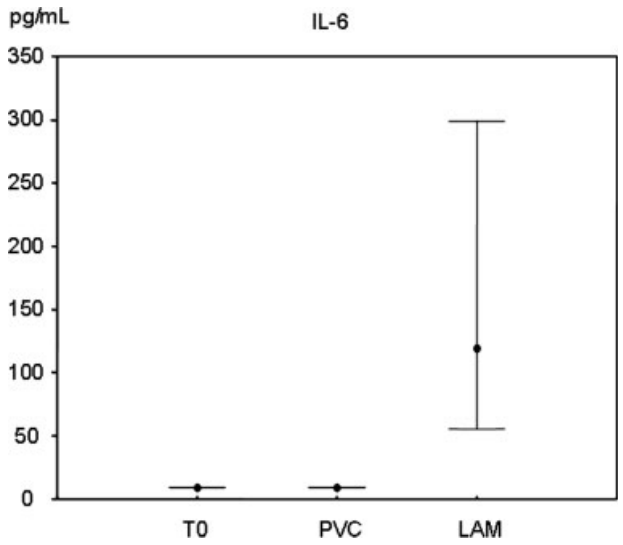

Figure 7. Median concentration in pg/mL (with 25–75 percentiles) for interleukin 6 (IL-6). LAM, laminaran-coated loops. $N = 4$, unsufficient for statistical calculations. Loops incubated at 37°C for 4 h.

In addition to variations in inducibility, the inflammatory markers also differed with respect to the degree of complement dependence. Notably, however, there was a marked and statistically significant effect of complement inhibition on the vast majority of the mediators induced by the PVC surface. We have previously shown that compstatin effectively blocks complement activation in our model,[3,9] and the present results confirm this. Furthermore,

through this efficient inhibition of complement activation, compstatin blocked the formation of IP-10 and FGF by 100% and IL-8 and GM-CSF by more than 95%.

CONCLUSION

The data presented reveal a broad inflammatory reaction in human whole blood exposed to a PVC artificial surface, mainly reflected by chemokines and growth factors and not by traditional inflammatory cytokines. This reaction is to a great extent, but not exclusively, dependent on complement activation.

Johan Riesenfeld is an employee of Carmeda AB.

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