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Both Plasma- and Leukocyte-Associated C5a Are Essential for Assessment of C5a Generation In Vivo

Geir Hetland, MD, PhD, Oddvar Moen, MD, Kåre Bergh, MD, PhD, Kolbjørn Högåsen, MD, PhD, C. Erik Hack, MD, PhD, Tom Eirik Mollnes, MD, PhD, and Erik Fosse, MD, PhD

Institute for Immunology and Rheumatology, The National Hospital, Oslo, Norway

Background. Measurement of C5a in plasma is hampered by the rapid clearance of C5a as a result of cell binding. Therefore, an assessment of whether cell-bound C5a might better reflect C5a generation in vivo is essential.

Methods. We quantified plasma and leukocyte-bound C5a in samples from patients undergoing cardiopulmonary bypass, which is known to be associated with complement activation. C3 activation products and the terminal complement complex were measured as well.

Results. Plasma levels of C3 activation products and the terminal complement complex increased rapidly and significantly after the onset of cardiopulmonary bypass until they reached a plateau after 30 minutes. The concentration of plasma C5a increased steadily to twice

baseline at the end of bypass. The concentration of leukocyte-associated C5a increased threefold after 10 minutes of cardiopulmonary bypass, when a plateau was reached. A positive correlation was found between levels of plasma C3 activation products or terminal complement complex and plasma C5a plus cell-associated C5a but not between C3 activation products or terminal complement complex and either one of the C5a variables.

Conclusions. We conclude that both plasma C5a and leukocyte-associated C5a are needed for monitoring in vivo C5a generation.

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C5a, a potent proinflammatory mediator generated during complement activation, is formed when C5 is converted into C5a and C5b. C5b binds to C6-C9 to form the terminal complement complex (TCC), which is either inserted into cell membranes as cytolytic C5b-9 or solubilized by vitronectin/S protein and clusterin (SC5b-9). C5a disappears from the circulation within minutes after its production or administration [1, 2]. It binds to specific C5a receptors (C5aRs) on myeloid cells [1, 3]. C5aR (CD88) has recently also been discovered on human epithelial cells [4] and nonmyeloid cells in the lung, including vascular smooth muscle and endothelial cells, the liver, and other organs [5]. This distribution of C5aR may explain some biologic properties of C5a, such as induction of spasmogenic activity, enhancement of vascular permeability, and trapping of C5a in vascularized tissue [2]. Possibly it also contributes to the various organ dysfunctions occasionally seen after cardiopulmonary bypass (CPB) [6, 7], which promotes marked activation of complement and generation of C5a [8, 9]. Consistent with this, elevated levels of TCC have been found to precede development of the adult respiratory distress syndrome [10].

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Address reprint requests to Dr Hetland, Institute of Immunology and Rheumatology, The National Hospital, Fr. Qvamsgate 1, 0172 Oslo, Norway.

C5a is difficult to detect in plasma by immunoassay, even under conditions with abundant terminal complement activation such as CPB [8, 11]. Only with a very sensitive enzyme immunoassay is C5a detected, despite its rapid disappearance from the fluid phase, in activated as well as normal plasma [9, 12]. Recently, it was observed that a large amount of C5a internalized by polymorphonuclear leukocytes (PMNs) in vitro remains antigenically intact [13].

The purpose of the present study was to examine whether C5a could be measured intracellularly in cells taken from patients with complement-activating conditions and whether cell-associated C5a might better reflect C5a generation in vivo than plasma C5a. In samples from patients undergoing CPB, C5a both in plasma and in association with peripheral blood leukocytes (PBLs) was examined using an enzyme immunoassay based on a monoclonal antibody to a neoepitope in C5a/C5a desArg. The results were related to plasma levels of C3 activation products (C3bc) and TCC.

Material and Methods

Experimental Design

This study was carried out as an addition to a European multicenter study on heparin-coated CPB with a membrane oxygenator (Univox; Baxter, Irvine, CA), described in detail elsewhere [14]. The PMN counts and the C3bc and TCC measurements were obtained from that study and included here to examine the correlation with C5a.

For this study, blood samples from 6 patients randomized to heparin-coated CPB and 9 patients randomized to uncoated CPB were available. The patients were included in the study after giving informed consent. The aim of the present study was not to evaluate the effect of heparin-coated circuits on complement activation during CPB but rather to evaluate the merit of assessing cell-bound C5a as a variable for complement activation. Therefore, differences in complement activation between the two groups are not discussed.

Sampling and Biochemical Analyses

Test samples were drawn from the arterial line at the start of CPB, after 10 and 30 minutes, and at the end of CPB. Samples for routine hematologic analysis were obtained in EDTA (ethylenediaminetetraacetic acid) vacutainers and kept at room temperature until analyzed in a Technicon analyzer (Technicon Instrument Corp, Tarrytown, NY). Samples (4 mL) for analysis of C3bc (which refers to the activation products C3b, iC3b, and C3c), TCC, and C5a were drawn into EDTA tubes, kept in melting ice, and centrifuged within 3 hours at 1,300 g for 10 minutes. The plasma samples were stored at -70°C before analysis in batches. Erythrocytes in the cell pellets (1 volume) were lysed by 10-minute treatment with 4 volumes of cold (4°C), isotonic (0.14 mol/L) NH_4Cl containing 10% NaHCO_3 w/w and then centrifuged, and the hemolysate was removed. The leukocyte pellets were lysed by sonication (sonicator from MSE Ltd, London, UK) for 1 minute in 100 μL of a buffer containing 50 mmol/L hydroxy-ethyl-piperazine-ethane-sulfonic acid (HEPES), 10 nmol/L EDTA, 2 mmol/L phenyl-methyl-sulfonyl-fluoride (PMSF), 0.1 mol/L iodoacetamide, and 15- μg of soybean trypsin inhibitor. Cell lysates were stored at -20°C until assayed for C5a. When necessary for the C5a determination, dilutions of cell lysates were made in phosphate-buffered saline solution. Cell association (which means uptake or binding and internalization) of C5a is reported as nanograms in cells per milliliter of whole blood and calculated on the basis of the blood sample volume (4 mL) and dilution factor.

C3bc was quantified in a double antibody enzyme immunoassay specific for a C3 neopeptide expressed on C3b, iC3b, and C3c but not on native C3 [15]. The TCC was quantified in a double enzyme immunoassay using the monoclonal antibody aE11 specific for a C9 neopeptide expressed in TCC, but not in the native C9, as capture antibody [16]. Standard was a zymosan-activated human serum pool ($n = 80$) defined to contain 1,000 arbitrary units/mL of TCC.

Quantification of C5a was performed in a sandwich enzyme immunoassay based on a monoclonal antibody to a neopeptide on C5a/C5a desArg that is concealed in native C5 and exposed on the activation fragment only [17]. The applicability of the assay has been shown in patients undergoing hemodialysis [17]. The concentrations of C3bc, TCC, C5a, and PMNs were corrected for hemodilution by multiplying the measurement with the initial hemoglobin value divided by the sample hemoglobin.

Fig 1. Kinetics for plasma C3 activation products (C3bc) and terminal complement complex (TCC) during cardiopulmonary bypass ($n = 15$) were monitored by enzyme immunoassays. Results are shown as medians and 25th and 75th percentiles. The levels were significantly increased compared with baseline at all data points ($p = 0.0001$). (AU = arbitrary units.)

Statistical Analysis

Nonparametric analysis was used because of the relatively small number of patients. Friedman's test was used to perform a nonparametric repeated-measures analysis for within-group comparisons [18]. Hotelling-Pabst test, a variant of Spearman's rank correlation analysis, was used for correlation analysis after summing the changes in values of the variables compared with baseline (eg, sum of observations minus baseline at all time points). Values of p less than 0.05 were considered statistically significant. The data are presented as medians with 25th and 75th percentiles, and in scatter plots.

Results

Generation of C3bc and TCC During CPB

From the start of CPB (baseline) to 10 minutes of CPB, there was a 13-fold and 7-fold increase in the concentrations of C3bc and TCC, respectively (Fig 1). This was followed by a further, but lesser increase in the concentration of C3bc until the end of CPB, when the level was 18 times the baseline value. The formation of TCC reached a maximum and a plateau after 30 minutes at 10-fold the baseline value. There was a positive correlation between the sum of values minus baseline for C3bc and TCC ($r = +0.75$, $p < 0.01$).

Generation of C5a During CPB

During the initial 10 minutes of CPB, the level of C5a in plasma increased slightly from a mean initial concentration of 9.7 ng/mL and then rose steadily to twice baseline by the end of the procedure ($p = 0.002$) (Fig 2).

The concentration of C5a was also measured in PBLs obtained from the same blood samples analyzed for plasma values. Significantly elevated (threefold) values ($p = 0.0001$) were observed for the PBL-associated

Fig 2. Kinetics for C5a during cardiopulmonary bypass ($n = 15$) were estimated by enzyme immunoassay analysis of plasma or peripheral blood leukocytes (PBLs) from each sample. Cellular uptake of C5a was examined after lysis of PBLs and expressed as nanograms of C5a in PBLs per milliliter of whole blood. The results are given as medians and percentiles. (See text for statistical evaluations.)

(bound and internalized) C5a after 10 minutes of CPB (see Fig 2). The PBL uptake of C5a reached a slightly higher maximum after 30 minutes (5.6 ng/mL of blood) and then leveled off to the 10 minutes' value. The mean concentration of PBL-associated C5a was as high as one third of the mean total C5a.

Because the kinetics for the PBL-associated C5a were very similar to those of plasma C3bc and TCC (see Fig 1), a correlation was anticipated between the generation of C3bc or TCC and the uptake of C5a. However, when the increase in the C3bc or TCC values, corrected for baseline, was compared with the increases either for the uptake of C5a in PBLs or for plasma C5a, no correlation was found. On the other hand, the change in either C3bc values (Fig 3A) or TCC values (Fig 3B) showed a significant positive correlation ($r = +0.70$, $p < 0.005$; and $r = +0.57$, $p < 0.025$, respectively) with the sum of the change in cell-associated C5a and plasma C5a.

Polymorphonuclear Leukocyte Counts

The number of PMNs was unchanged during the first 10 minutes of CPB (Fig 4) but doubled at 30 minutes and tripled at the end of CPB compared with baseline ($p = 0.0001$). The change in the PMN count correlated positively with the change in PBL uptake of C5a ($r = +0.55$, $p < 0.025$) but not with plasma C5a or TCC.

Comment

Our results show that quantification of both plasma C5a and PBL-associated C5a is required for an accurate assessment of the generation of C5a in vivo. Quantification of plasma C5a alone may be unreliable for this purpose, as C5a disappears rapidly from plasma presumably because of uptake by leukocytes and clearance by

A

B

Fig 3. Scatter diagrams of change (sum of observations minus baseline at all time points) during cardiopulmonary bypass ($n = 15$) in levels of whole-blood C5a [ie, peripheral blood leukocyte (PBL)-associated and plasma C5a] plotted against change in levels of (A) complement activation products (C3bc) ($r = +0.70$, $p < 0.005$) and (B) terminal complement complex (TCC) ($r = +0.57$, $p < 0.025$). The regression lines are rank based.

the liver [1, 2]. Recently, C5aR has also been identified in many nonmyeloid cells and organs [4, 5]. It is therefore likely that cells other than PBLs also contribute to the removal of C5a from plasma. This may explain in part why the values for PBL-associated C5a alone did not correlate with those for plasma TCC. Another reason may be the differences in clearance between C5a and TCC. In principle, C5a and TCC are formed in equimolar quantities, but fluid-phase TCC (SC5b-9) has a longer half-life (50 minutes) [19] than C5a (1 to 2 minutes) [1, 2]. The positive correlation between whole-blood C5a (ie, cell-associated and plasma C5a) and TCC also shows that TCC is an indirect indicator of total C5a generation.

The first samples for this study were obtained from the patients immediately before the start of CPB and not before the start of the operation. The concentration of plasma C5a at the start of CPB was close to the mean C5a concentration (11.2 ng/mL) found in healthy individuals

Fig 4. Number of polymorphonuclear leukocytes (PMN) counted during cardiopulmonary bypass (CPB). The results are plotted as medians and 25th and 75th percentiles of 15 CPB procedures. The number of PMN was significantly increased compared with baseline after 30 minutes ($p = 0.0001$).

using the current C5a enzyme immunoassay [17]. Therefore, anesthesia and the traumatizing effect of the operation per se probably had minimal effect, if any, on complement activation and C5a generation, a finding noted previously [9]. Cardiopulmonary bypass-induced complement activation and generation of C5a occur very early in the procedure. The 10-minute delay in the PMN increase after the start of CPB agrees well with the pattern observed during injection of C5a in rabbits [20], showing an initial neutropenia lasting less than 15 minutes, followed by a marked neutrophilia. The neutropenia probably results from a transient adherence to the endothelium, whereas the neutrophilia is due to demargination and release from bone marrow stores of leukocytes [20]. The positive correlation found between change in PMN number and PBL-associated C5a, but not plasma C5a, probably indicates that the C5a-induced response in PMNs is exerted after PMN binding of the molecule. Because monocytes, but not lymphocytes, also express C5aR [1], a stronger correlation than this ($r = +0.55$) would have been anticipated between the change in PBL-associated C5a and the number of PMNs and monocytes.

The kinetics of plasma and cell-associated C5a agreed well. The initial increase in both during the first 10 minutes of CPB reflected the generation of C5a in the fluid phase that was taken up by the cells at an increasing rate. Thereafter, the PBL uptake of C5a leveled off, probably because the generation of plasma C5a reached a plateau, as was suggested by the course of TCC levels (see Fig 1). Because the C5a concentration in the samples was at half maximal concentration (ie, 2 to 7 nmol/L) and lower than saturation (≥ 30 nmol/L) for ligand binding to C5aR [3], it is unlikely that the plateau in the cell-associated C5a was due to "desensitization" for the uptake mechanism. One reason for the observed plateau in C5a uptake paralleling the increased plasma C5a

levels could be increased degradation of plasma C5a to C5a desArg, which has 50-fold less affinity for C5aR than the parent molecule [21]. However, another more probable reason is that the observed postoperative increase in plasma C5a could represent degradation fragments of C5a/C5a desArg still bearing the C5a neopeptide that were released from the leukocyte C5aR [9].

The background for the present study was the recent finding that a large portion of C5a internalized by PMNs in vitro stays antigenically intact in the cells for a considerable time [13]. New evidence also suggests that C5a taken up by PMNs in vitro is recycled in complex with its receptor back to the cell surface, from where it may be released and degraded (Hetland, Pfeifer, Hugli: unpublished results). Thus, if there is a time-dependent release from leukocytes of mostly antigenically intact C5a/C5a desArg, it would cause a rebound effect and explain both the increase in plasma C5a at the end of CPB despite the constant levels of TCC and the observed postoperative increase in C5a [9]. Moreover, a release of C5a from PBLs together with degradation of plasma C5a to C5a desArg [21] would account for the constant level of cell-associated C5a despite the increase in PBLs during CPB.

In conclusion, because leukocytes constitute a major depot for C5a in vivo, the quantification of whole-blood C5a (ie, cell-associated and plasma C5a) appears to be a more exact and reliable marker for the generation and metabolism of C5a during CPB than measurement of plasma C5a alone. C5a bound to the recently discovered C5aR on nonmyeloid cells in various organs may be processed similarly and should also be taken into account.

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