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Blood Phagocyte Activation During Open Heart Surgery With Cardiopulmonary Bypass

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Summary

Open heart surgery with a cardiopulmonary bypass (CPB) is associated with a systemic inflammatory response which significantly contributes to adverse postoperative complications. The purpose of this study was to characterize the activation of blood phagocytes during open heart surgery with CPB. Blood samples were collected during and up to 24 h after surgery. The production of reactive oxygen species (ROS) in whole blood, the expression of surface molecules by blood phagocytes and complement activity in the plasma were determined. A cDNA microarray analysis of leukocyte RNA profile of genes was performed related to the inflammatory response. Activation of the complement was already observed at the beginning of CPB. This was followed by an increase in the neutrophil number and in both spontaneous and opsonized zymosan-activated ROS production after the onset of reperfusion. The activation of blood phagocytes was affirmed by changes in surface receptors involved in the adhesion and migration of leukocytes (CD11b, CD62L and CD31). Gene arrays also confirmed the activation of leukocytes 4 h after reperfusion. In conclusion, open heart surgery with a cardiopulmonary bypass was found to be associated with a rapid and pronounced activation of blood phagocytes and complement activation which was partly independent at the onset of CPB.

Key words

Phagocytes • Complement • Surface receptors • Reactive oxygen species • Gene expression

Introduction

Open heart surgery with a cardiopulmonary bypass (CPB) is associated with systemic inflammation, which significantly contributes to adverse postoperative complications (Dernek *et al.* 1999). The induction of systemic inflammation is caused by several factors including exposure of blood to the extracorporeal circuit, post-ischemic reperfusion of the heart and lungs as well as surgical trauma (Dernek *et al.* 1999). Mediators released during an inflammatory response, such as pro-inflammatory cytokines and complement activation products, promote polymorphonuclear cell adhesion to the endothelial surface, evoke their infiltration in tissues and cause the release of reactive oxygen species (ROS) and enzymes (Lojek *et al.* 1992, Starkopf *et al.* 1997, Boyle *et al.* 1997, Defraigne *et al.* 2000).

The recruitment of white blood cells into tissues

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requires a stepwise interaction between adhesion molecules on the surface of leukocytes and their corresponding receptors on the luminal surface of inflamed endothelium (Vinten-Johansen 2004). Various adhesion molecules are involved in this process: (1) selectins, which mediate the initial attachment and the rolling of leukocytes along the vessel wall under shear stress changes, (2) β2 integrins, which mediate firm adhesion of leukocytes to endothelium, and (3) adhesion molecules of immunoglobulin superfamily expressed on the endothelial side which, in conjunction with the β2 integrins, regulate a firm adhesion and transendothelial migration of activated phagocytes (Asimakopoulos *et al.* 2000). This study investigated the expression of CD62L (L-selectin), CD11b/CD18 (Mac-1) and CD31 (PECAM-1) on polymorphonuclear leukocytes (PMNL) and monocytes during open heart surgery. These surface antigens together with the production of ROS were selected as markers of phagocyte activation.

The activation of complement pathways significantly promotes phagocyte adhesion and migration while contributing to the systemic inflammation connected to open heart surgery (Gu *et al.* 1998, Struber *et al.* 1999). Therefore the complement activity in the plasma was also determined. To further describe the process of blood leukocyte activation during open heart surgery with CPB, the alterations in gene expression associated with a systemic inflammatory response were examined using cDNA microarray analysis.

The aim of this study was to clarify the mechanisms of activation of blood phagocytes during open heart surgery and their contribution to the induction of systemic inflammatory response induced by CPB.

Methods

Patients

A group of 30 patients who had had open heart surgery was examined in the study. Heart surgery was performed for ischemic heart disease (n=19), aortic valve replacement (n=5) and mitral valve replacement (n=6). Cold crystalloid cardioplegic solution (St. Thomas Hospital solution) was used in a total volume of 1000-1500 ml depending on the crossclamping time. Three patients (males: 56, 69 and 69 years) with ischemic heart disease (IHD) without peri- and postoperative complications were chosen for gene expression analysis using the cDNA microarray technique. The study was approved by the local ethics committee.

Sampling

Heparinized peripheral blood samples were taken from the patients before surgery, at the beginning of ischemia, at the start and after 30 min, 4 h and 24 h of reperfusion. Cell counts and the oxidative burst of phagocytes were determined immediately. Total number of leukocytes in the blood and their relative differentiation counts were determined using a Coulter counter STKS (Coulter, England) and in stained blood smears, respectively. Whole blood (12.5 ml) was collected according to the manufacturer's protocol (PAXgene^{TM} Blood RNA tubes, PreAnalytiX, Germany), before and 4 h after the operation for cDNA microarray analysis. Plasma samples were frozen and stored at –20 ^{o}C.

Oxidative burst of phagocytes

Luminol-enhanced chemiluminescence (CL) of whole blood phagocytes was measured using Luminometer LMT-01 (Immunotech, Czech Republic) as described previously (Pečivová *et al.* 2004). The principle of the method is based on luminol interaction with the phagocyte-derived ROS which results in large light emission. Opsonized zymosan particle (OZP)-activated CL was measured for 60 min at 37 ^{o}C. The assays were run in duplicates. Spontaneous CL measurements in samples containing all substances except the activator were included in each assay. The integral value of the CL reaction, which represents total ROS production by blood phagocytes, was corrected for 10^3 neutrophil granulocytes.

Determination of the expression of adhesion molecules

The measurements were performed according to the manufacturer's protocol (Caltag Laboratories, USA) using unfixed whole blood with minor modifications as described previously (Gallová *et al.* 2004). Briefly, blood samples were incubated with anti-CD11b, anti-CD62L, and anti-CD31 monoclonal antibodies. Phycoerythrin- or fluorescein isothiocyanate-conjugated murine immunoglobulins of the same isotype were used as the negative controls. Ten thousand PMNL selected on the basis of their typical scattering characteristics were analyzed by a flow cytometer FACSCalibur (Becton Dickinson, USA) and the median of relative fluorescence was determined.

Determination of complement activity

Complement activity was determined by a bioassay using an *E. coli* JM109 strain carrying luciferase

plasmid pCSS962 (a gift of Prof. E.M. Lilius, University of Turku, Finland). Cultivation of bacteria and the determination of complement activity was described previously (Virta *et al.* 1998, Nikoskelainen *et al.* 2002). Briefly, the bacteria cultivated overnight to the log phase in L broth (1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl; pH 7.0) and containing appropriate antibiotics, were collected by centrifugation (1500 x *g*, 10 min), washed with Hanks' balanced salt solution (HBSS), resuspended in HBSS, and diluted to a concentration of 10^7 cells/ml with HBSS. Complement reactions were carried out by mixing the plasma with bacteria suspension at a ratio of 1:5. Reaction mixtures were incubated for 90 min at 37 °C without shaking.

The reactions were stopped by placing the samples on ice for 10 min prior to luminometric analysis. The reaction mixture was then mixed with 50 µl of luciferin solution (1 mmol/l D-luciferin in 100 mmol/l sodium citrate, pH 5.0), and luminescence was monitored with a luminometer LMT-01 for 10 min at 37 °C. The assays were run in duplicates. The luminescence data were converted to a percentage of the reference sample containing human serum without any detectable complement activity.

cDNA microarrays

Pure total RNA was isolated from whole blood by PAXgene^{TM} Blood RNA Kit (PreAnalytiX, Germany). The quality of total RNA was examined by agarose gel electrophoresis. The quantity of total RNA was determined spectrophotometrically at 260 nm using NanoDrop spectrophotometer (NanoDrop Technologies, USA). The labeling of cDNA of the samples and subsequent hybridization to the arrays were done according to the manufacturer's manual (SIRS-Lab, Germany). Briefly, 20 µg of total RNA of leukocytes was used for analysis. mRNA was annealed to an anchored Oligo-dT18V primer and used to generate cDNA utilizing the Superscript II reverse transcription**.** For labeling, the reaction was spiked with Cy5-dUTP (4 h after operation) or Cy3-dUTP (before operation) fluorescence-tagged nucleotides and then analyzed using a Lab-arraytor Human-500 (SIRS-Lab, Germany). Fluorescence detection of Cy-3 and Cy-5 fluorophores was performed using a glass slide scanner GenePix4000B (Axon Instruments, USA). Relative rates of gene expression were determined with "AIDA Array Evaluation" software.

Statistical analysis

A standard two-sample t-test was applied for inspection of differences among variants using Statistica for Windows 5.0 (StatSoft. Inc., USA). Data are expressed as means and standard errors of the mean.

Results

Total leukocyte number was significantly increased from the start of reperfusion till 24 h after operation. The sharp increase in total number of leukocytes was associated with a relative increase in neutrophils (both bars and segments) (Table 1). The changes in leukocyte numbers correspond with the activation of blood phagocytes. A significant increase in both spontaneous and OZP-activated production of ROS from the start of reperfusion to 24 h after operation was also observed (Table 2).

Table 1. Changes in the numbers of blood leukocyte

	Before operation	Initiation of CPB	End of CPB	30 min after CPB	4 hours after CPB	24 hours after CPB
Total leukocyte [x10^3]	4.9±0.3	5.1±0.4	7.8±0.6 *	10.3±0.9 *	11.3±0.7 *	11.3±0.7 *
Neutrophil granulocytes - segmented [%]	59.0±2.2	58.5±1.6	62.7±1.2 *	64.9±1.4 *	69.6±1.2 *	70.9±1.6 *
Neutrophil granulocytes - bars [%]	5.9 ±0.7	6.3±0.9	13.6±1.4 *	14.8±1.0 *	14.1±1.5 *	15.0±1.1 *

Total leukocyte counts and percentage of segments and bars (not fully maturated neutrophils) at different sampling times ([before operation, before the initiation of CPB, at the time of reperfusion (the end of CPB), 30 min, 4 h and 24 h after reperfusion (after the end of CPB)]. Asterisks mark the sampling times when the values differed significantly from the pre-operational values ($p<0.05$).

Table 2. Changes in the ROS production by blood phagocytes

	Before operation	Initiation of CPB	End of CPB	30 min after CPB	4 hours after CPB	24 hours after CPB
Spontaneous CL	18±3	26±5	60±11 *	54±9 *	43±6 *	112±17 *
OZP-activated CL	647±70	641±60	1622±201 *	2120±239 *	2010±182 *	2162±174 *

Spontaneous and OZP activated chemiluminescence of whole blood at different sampling times [before operation, before the initiation of CPB, at the time of reperfusion (the end of CPB), 30 min, 4 h and 24 h after reperfusion (after the end of CPB)], expressed as RLU*s*10^3 cells. Asterisks mark the sampling times when the values differed significantly from the pre-operational values ($p<0.05$).

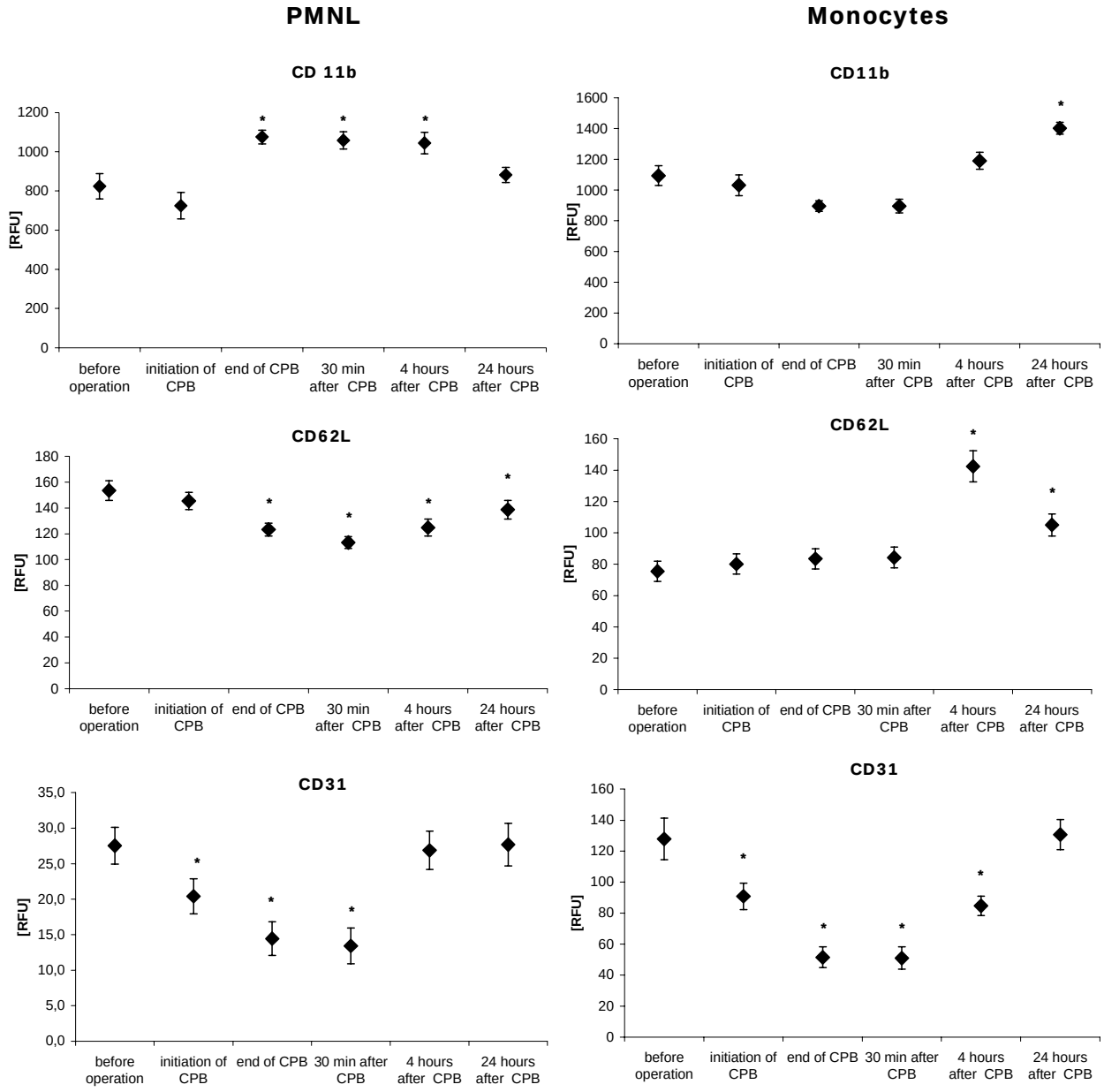

Fig. 1. Changes in the expression of leukocyte adhesion receptors. The expression of CD11b, CD62L and CD31 on blood phagocytes – PMNL and monocytes at different sampling times [before operation, before the initiation of CPB, at the time of reperfusion (the end of CPB), 30 min, 4 h and 24 h after reperfusion (after the end of CPB)]. Asterisks mark the sampling times when the values of a specified parameter differed significantly from the pre-operational values ($p<0.05$).

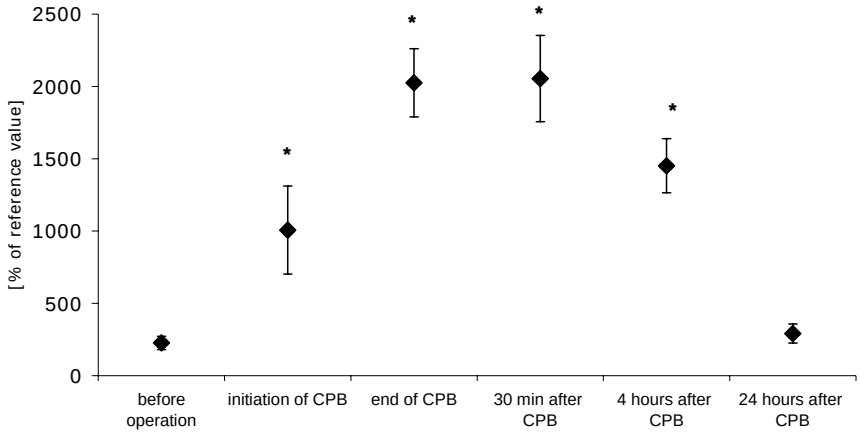

Fig. 2. Changes in the plasma complement activity. The activity of complement in plasma at different sampling times [before operation, before the initiation of CPB, at the time of reperfusion (the end of CPB), 30 min, 4 h and 24 h after reperfusion (after the end of CPB)]. Asterisks mark the sampling times when the values differed significantly from the pre-operational values ($p<0.05$).

Table 3. Changes in the blood leukocyte gene expressions - list of genes up-regulated 4 h after reperfusion.

	HUGO	NameMean	fold change
Cytokine receptors	IFNAR1	interferon (alpha, beta and omega) receptor 1	2.8
	IL1R2	interleukin 1 receptor, type II	3.8
	IL4R	interleukin 4 receptor	3.7
Cytokine-induced molecules	TNFAIP6	tumor necrosis factor, alpha-induced protein 6	5
Chemokines	CX3CL1	chemokine (C-X3-C motif) ligand 1	4.9
	S100A12	S100 calcium-binding protein A8 (calgranulin A)	3.7
	S100A8	S100 calcium-binding protein A12 (calgranulin C)	13.3
CD and adhesion molecules	CD14	CD14 antigen	3
	DPP4	dipeptidylpeptidase IV (CD26, adenosine deaminase complexing protein 2)	5
	ITGA5	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	3.3
Cell cycle and apoptosis	BCL2A1	BCL2-related protein A1	7.5
	IER3	immediate early response	3.5
Signal transduction	HPRT1	hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome)	4.6
	IL6ST	interleukin 6 signal transducer (gp130, oncostatin M receptor)	2.9
	MAPK14	mitogen-activated protein kinase 14	4.2
	MKNK1	MAP kinase-interacting serine/threonine kinase 1	3
Eicosanoid signaling pathway	ALOX5AP	arachidonate 5-lipoxygenase-activating protein	3.9

HUGO`s (The Human Genome Organization) gene abbreviations were used. The table summarizes data from three cDNA microarray experiments.

The activation of PMNL from the end of CPB was confirmed by significant changes in the expression of selected surface receptors. The upregulation of CD11b on PMNL surface from the end of CPB to 4 h after reperfusion was accompanied by a downregulation of the CD62L antigen. The downregulation of the CD31 antigen, a process that is also connected to phagocyte activation, had already started at the start of reperfusion and lasted up to 3 h after reperfusion (Fig. 1). In contrast, monocytes, the second major group of blood phagocytes, have been activated to a lesser extent compared to PMNL during open heart surgery. A significant increase in the CD11b antigen expression on monocytes was not observed until 24 h after the surgery (Fig. 1). The expression of CD62L on monocytes was increased 4 h and 24 h after reperfusion. On the other hand, a significant decrease in the CD31 expression (from the start reperfusion up to 4 h after reperfusion) was found on monocytes.

The increased number of blood phagocytes, along with their activation, was preceded by a significant increase in complement activity. The complement activity in the plasma significantly increased at the beginning of CPB and reached its maximum at the time of reperfusion and 30 min after reperfusion (Fig. 2).

Gene array analysis allowed us to investigate mRNA expression of 340 genes, among them membrane ligands and their receptors, kinases, phosphatases, molecules induced by inflammatory stress, transcription factors and transcription modulators. It was found that the expressions of 17 genes (ALOX5AP, BCL2A1, CD14, CX3CL1, DPP4, HPRT1, IER3, IFNAR1, IL1R2, IL4R, IL6ST, ITGA5, MAPK14, MKNK1, S100A12, S100A8, TNFAIP6) were upregulated significantly (2.5-fold or more) in all three patients (Table 3). No decrease was found in the expression of any gene.

Discussion

Several inflammatory pathways become activated during open heart surgery with cardio-pulmonary bypass and subsequent generalization of inflammatory response occurs. The systemic inflammatory response is assumed to be one of the major factors responsible for the diffuse tissue damage in the lung, myocardium, kidney and brain as described in patients undergoing open heart surgery (Baue *et al.* 1998, Wan and Yim 2001). Blood phagocyte activation is one of the causative mechanisms of this processes and leads to the increased production of ROS by phagocytes and the consequent oxidative stress, further adhesion and migration of phagocytes to tissues after ischemia/reperfusion.(Baue *et al.* 1998, Kotani *et al.* 2000, Čížová *et al.* 2004)

In our study, the significant increase in leukocyte numbers was observed from the start of reperfusion up to 24 h after surgery. The sharp increase in the total number of leukocytes was associated with a relative increase in both forms of neutrophils – bars and segments. The increase in circulating leukocyte count during and after operation in patients undergoing CPB has also been observed by other investigators (Ashraf *et al.* 1998, Gu *et al.* 1998, Kotani *et al.* 2000, Rothenburger *et al.* 2002). This systemic leukocytosis is thought to be caused by a combined effect of complement activation and the release of proinflammatory cytokines resulting in the mobilization of leukocytes from the marginating pools and bone marrow.

The changes in leukocyte numbers corresponded with the activation of blood phagocytes as was determined by various methods. One of them was the determination of spontaneous as well as OZP-activated blood phagocyte ROS production which increased remarkably. The increased ROS production induces lipid peroxidation which was described in patients undergoing open heart surgery with CPB (Lojek *et al.* 1992, Davies *et al.* 1993, Partrick *et al.* 1999, Kubala *et al.* 2002). The activation of blood phagocytes during open heart surgery was also supported by the determination of selected surface receptors involved in the adhesion and migration of leukocytes. CD62L antigen, which is constitutively expressed on non-activated leukocytes, is responsible for leukocyte rolling and margination and is rapidly downregulated by chemotactic stimulation (Venturi *et al.* 2003). The subsequent firm adhesion of leukocytes to activated endothelium is mediated by the upregulation of the β2-integrin complex, particularly CD11b/CD18 (Alonso *et al.* 1999, Asimakopoulos *et al.* 2000, Rinder *et al.* 2003). Downregulation of CD62L and upregulation of CD11b are indicators of neutrophil activation (Ilton *et al.* 1999, Gallova *et al.* 2004). Similarly, CD31 is implicated in the transendothelial migration of leukocytes, angiogenesis and integrin activation (Buckley *et al.* 1996). We observed a significant upregulation of CD11b expression on the surface of PMNL in early post-operative intervals. The downregulation of CD62L and CD31 expressions on PMNL was observed during the same time period. Upregulation of CD11b/CD18 and

downregulation of CD62L expressions on PMNL during CPB were also observed by other authors, but there are also contrary reports which did not observe any changes in CD62L expression (Galinanes *et al.* 1996, Le Deist *et al.* 1995, 1996). A significant decrease in CD31 expression, which also confirms the complex activation of PMNL during open heart surgery, was found on monocytes similar to that on PMNL. The expression of CD11b on monocytes also increased, but only after 24 h of reperfusion. Similarly to our findings, no significant increase in the expression of CD11b on monocytes during open heart surgery was reported by Rinder *et al.* (2003). While neutrophils demonstrated a downregulation of CD62L expression, its expression on monocytes was increased. These findings suggest that when compared to PMNL, monocytes are activated to a lesser extent during and immediately after the operation. We can consequently speculate that the immune response to surgical trauma is mediated particularly by PMNL.

Complement activation is one of the most important factors for leukocyte activation and leukocytosis during open heart surgery and it is considered to be a "trigger" of CPB-induced inflammatory response (Struber *et al.* 1999). A significant increase in plasma complement activity was determined early after the beginning of the operation (at the time of the start of CPB). In contrast, significant changes in the number of circulating leukocytes and activation of blood phagocytes were observed later during surgery. This supports the idea that the complement is the key initiator of the immune response during open heart surgery. The main suggested pathway of complement activation is the exposure of blood to the artificial surface of the CPB circuit (Gu *et al.* 1998, Cartier 2003). Nevertheless, in our study, a significant increase in complement activity was determined prior to the contact of blood with the artificial surface in the CPB circuit. From these results it could be concluded that the extensive surgical procedure itself activates the complement even before CPB begins. This is an important finding especially in view of the current trend in heart surgery where CPB is not used to avoid the induction of inflammatory responses (Cartier 2003). Our data suggest that significant complement activation could be expected even during heart surgery without the use of CPB.

Leukocyte activation is a complex process that is accompanied by changes in the expression of a wide range of genes participating in activation and regulation of cells. Studying the variation in leukocyte gene expression can therefore provide essential information about this sophisticated process. We have used gene expression profiling with cDNA microarrays to investigate the spectrum of genes related to the systemic inflammatory response. Of the 340 genes evaluated, the expression levels of 17 genes were increased while no genes were repressed 4 h after surgery. The over-expressed genes belong to the groups of cytokine receptors, cytokine-induced molecules, chemokines, adhesion molecules, cell cycle and apoptosis related molecules, signal transductors, and eicosanoid signaling pathway related molecules.

The mRNA expression of some proinflammatory cytokines appears to be of particular interest. In a previous study we reported a significant increase in plasma levels of the proinflammatory cytokine IL-6 during heart transplantation and open heart surgery with CPB, where the maximal level was observed 4 h after CPB (Kubala *et al.* 2002). Interestingly, a cDNA micro-array assay showed that mRNA of this cytokine was not upregulated at this time. Therefore, we would suggest that IL-6 present in the blood 4 h and later is not of leukocyte origin, but could be produced by cells from organs such as the liver, lung and heart which undergo ischemia or partial ischemia with consequent reperfusion. This conclusion concurs with Wan *et al.* (1997) who suggested that the myocardium is an important source of IL-6. The absence of any overexpressed cytokines in leukocytes 4 h after CPB was in accordance with the good clinical outcome in all three patients.

In conclusion, open heart surgery with cardiopulmonary bypass was associated with a rapid and pronounced activation of blood phagocytes. The findings suggest that the complement is one of the key "triggers" of the inflammatory response in patients undergoing open heart surgery and its activation begins before the onset of cardiopulmonary bypass. Consequently, phagocytes produce high amounts of reactive oxygen species and express the surface molecules required for adhesion and the recruitment of leukocytes in the vascular endothelium. On the basis of our results, we can speculate that the immune response to surgical trauma is mediated in particular by activated polymorphonuclear leukocytes.

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