Plasma levels of interleukin-8 and expression of interleukin-8 receptors on circulating neutrophils and monocytes after cardiopulmonary bypass in children

Peter Gessler, MD^a
 Juerg Pfenninger, MD^b
 Jean-Pierre Pfammatter, MD^b
 Thierry Carrel, MD^c
 Oskar Baenziger^a
 Clemens Dahinden, MD^d

CHD

Objective: Cardiopulmonary bypass induces a systemic inflammatory response that causes substantial clinical morbidity. This study sought to determine cellular and humoral variables of inflammation. We hypothesized that chemokines are a major source of stimulation of neutrophils and monocytes in pediatric cardiac surgery.

Methods: We performed an observational prospective clinical study of 20 pediatric patients before and after cardiopulmonary bypass. Plasma levels of interleukin-6, interleukin-8, myeloperoxidase, and nitric oxide were measured by immunoassays. Expression of interleukin-8 receptors (CXCR1, CXCR2) and CD14 of circulating neutrophils and monocytes was assessed by flow cytometry. Clinical evaluations included length of inotropic support and mechanical ventilation as well as oxygenation.

Results: Two hours after cardiopulmonary bypass, plasma levels of interleukin-6 and interleukin-8 were strongly increased ($P = .0001$ and $P = .0032$, respectively). Interleukin-6 and interleukin-8 concentrations correlated with the length of inotropic support, as well as with the length of mechanical ventilation ($r > .70$, $P \leq .0006$), and were inversely related to the ratio of arterial oxygen tension to fraction of inspired oxygen. There was a strong association between the postoperative levels of interleukin-6 and nitric oxide, as well as between interleukin-6 and CD14 expression on monocytes ($r > .62$, $P \leq .0031$). The expression of CXCR2 but not CXCR1 on neutrophils and monocytes correlated negatively with the levels of interleukin-8 and myeloperoxidase.

Conclusions: After cardiopulmonary bypass, impairment of cardiovascular and respiratory function correlated with the levels of interleukin-6 and interleukin-8 as mediators of an inflammatory response. The negative correlation of CXCR2 expression with interleukin-8 and myeloperoxidase indicates that myeloid cells were stimulated by CXC chemokines with Glu-Leu-Arg (ELR) motif and thereby contributed to tissue damage, leading to impairment of cardiovascular and respiratory function.

From the University Children's Hospital,^a Zurich, Switzerland; University Children's Hospital,^b Berne, Switzerland; Department of Cardiovascular Surgery,^c University Hospital, Berne, Switzerland; and Institute of Immunology, University of Berne, Berne, Switzerland.

The study was financially supported by a grant from Novartis Stiftung, Basel, Switzerland (No. 99B24). There is no conflict of interest.

Received for publication Oct 8, 2002; revisions requested Nov 25, 2002; revisions received Dec 3, 2002; accepted for publication Jan 10, 2003.

Address for reprints: Peter Gessler, MD, University Children's Hospital, Steinwiesstr. 75, CH 8032, Zurich, Switzerland (E-mail: peter.gessler@kispi.unizh.ch).

J Thorac Cardiovasc Surg 2003;126:718-25

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0022-5223/2003 \$30.00 + 0

doi:10.1016/S0022-5223(03)00685-8

Cardiac surgery with cardiopulmonary bypass (CPB) leads to an acute inflammatory response associated with activation of complement and release of cytokines and chemokines into the circulation, as well as activation of inflammatory cells.^{1,2} Elevated levels of proinflammatory cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-6, and IL-8 have been reported.^{3,4} This inflammatory response is counterbalanced by a complex system of inhibitors such as IL-10

TABLE 1. Patient demographic data and clinical characteristics

No.	Diagnosis	Age (months)	Weight (kg)	CPB (min)	Aortic crossclamp time (min)	Inotropic support (hours)	Inotropic drugs used ≤ 24 hours*	Mechanical ventilation (hours)	PaO ₂ /Fio ₂ (mm Hg)
1	Aortic stenosis	78	17.5	29	19	0		8	673
2	VSD	4	3.9	81	29	70	Dn	81	134
3	ASD	69	16.0	36	9	0		12	436
4	VSD	109	38.0	49	24	0		5	444
5	A-V canal	5	4.2	109	56	69	Do, Ep, No	98	70
6	Fallot tetralogy	18	10.5	62	36	26	Do, Dn	27	206
7	DORV	148	39.7	75	41	40	Dn	28	527
8	Cor triatriatum	19	9.5	105	27	96	Do, Dn, No	120	140
9	VSD	8	6.9	84	41	46	Dn, Ep	25	354
10	DORV	13	7.9	102	49	30	Dn, Ep	25	309
11	A-V canal	5	5.6	104	51	56	Dn	45	249
12	ASD	92	28.4	34	23	0		4	440
13	A-V canal	46	21.0	47	27	20	Dn	19	392
14	Fallot tetralogy	69	20.8	81	36	21	Dn	18	390
15	VSD	162	51.8	48	27	23	Dn	9	455
16	A-V canal	5	5.6	80	42	73	Dn, Ep, No	71	432
17	Fallot tetralogy	8	8.1	96	50	24	Dn	42	348
18	VSD	9	7.3	49	25	23	Dn	6	390
19	ASD	138	42.6	35	15	11	Dn	4	410
20	A-V canal	4	4.5	137	66	96	Dn, Ep	120	266

A-V canal, Complete atrioventricular canal; DORV, double-outlet right ventricle.

*Inotropic drugs used during the first 24 hours: dopamine (Do), dobutamine (Dn), epinephrine (Ep), norepinephrine (No).

and soluble cytokine receptors.⁵⁻⁷ Proinflammatory cytokines can induce the release of nitric oxide (NO) by endothelial cells, smooth muscle cells, and inflammatory cells through the inducible form of the enzyme NO synthase.⁸⁻¹⁰ NO is implicated in the pathophysiology of the inflammatory state and induces vasodilation, increased vascular permeability, and inhibition of myocardial contractility.¹¹ In addition, NO has a protective role by inhibiting neutrophil-endothelial cell adhesion or by blocking neutrophil accumulation in the inflammatory sites.^{12,13} Because inflammatory cells release a variety of proteases and oxidants as final mediators of tissue damage,^{14,15} reduced infiltration of the myocardium by inflammatory cells may reduce tissue injury.⁹ The process of attraction and activation of leukocytes to tissues during an inflammatory process is controlled by chemokines.¹⁶ IL-8 and other CXC chemokines (named according to the position of the first 2 cysteines separated by 1 amino acid) are potent neutrophil chemotactic and activating peptides, which are proposed to be major mediators of inflammation.¹⁷ IL-8 is produced by a variety of cell types including monocytes, T lymphocytes, neutrophils, fibroblasts, endothelial cells, and epithelial cells. The central role of CXC chemokines in neutrophil accumulation at sites of inflammation has been demonstrated in animal models of myocardial ischemia.^{18,19} Two IL-8 receptor types (CXCR1 and CXCR2) have been described on human neutrophils; both have high affinity for IL-8 but CXCR2 has additional affinity to other CXC chemokines containing a

common amino-terminal Glu-Leu-Arg (ELR) amino acid sequence.²⁰⁻²²

The proinflammatory events after CPB manifest clinically as capillary leak syndrome with generalized edema, low cardiac output, and multiple organ dysfunction.^{23,24} Monocytes and neutrophil granulocytes may undergo activation, releasing proteinases and oxidants as well as inflammatory cytokines, which may influence cardiovascular and respiratory function. The role of chemokines as a possible source of cellular activation was assessed.

Methods

Patients and Blood Sampling Protocol

The study was approved by the local ethics committee. Informed written consent from the parents of each child was obtained. The studied population included 20 consecutive patients. All patients were operated by the same 2 surgeons. Patient demographic data and clinical characteristics are shown in Table 1.

Patients were ventilated (Draeger Evita IV, Luebeck, Germany) using biphasic positive airway pressure mode. Fio₂ was adjusted to maintain a PaO₂ level of 10 kPa or greater. Ratio of arterial oxygen tension to fraction of inspired oxygen (PaO₂/Fio₂) was calculated 2 hours after CPB. Respiratory function was determined as the duration of mechanical ventilation and the ratio of PaO₂/Fio₂ (in all cases, repair of cardiac lesion resulted in complete separation of pulmonary and systemic circulations). Mechanical ventilation was discontinued according to a standard weaning protocol, which is followed for all patients before extubation.

Cardiovascular function was assessed by the length of inotropic support after CPB and the number of inotropic drugs used during the first 24 hours after CPB. Postoperative inotropic support was done according to age-adapted reference values for blood pressure and clinical variables (ie, skin perfusion, urine output) using dopamine, dobutamine, epinephrine, or norepinephrine, either as a single drug or in combination. Prescription of the drugs was done by a team of senior pediatric intensivists following written internal guidelines. The intensivists were unaware of the laboratory results done for research purpose.

Arterial blood samples for immunophenotyping of cells as well as for cytokine assays were collected from each patient before and 2 hours after CPB. Measurements of cell receptors were done immediately after sample collection. Blood samples for cytokine assays were immediately centrifuged and the plasma stored at -70°C .

CPB Management

The extracorporeal circuit (ECC) consisted of a roller pump, cardiectomy reservoir, tubing set, and oxygenator. The circuit was primed with a mixture of red blood cell concentrate, human albumin, sodium bicarbonate 8.4%, mannitol 20%, magnesium chloride, aprotinin (Bayer, Leverkusen, Germany), and heparin (Roche Pharma, Grenzach, Switzerland). It should be pointed out that aprotinin was used in all patients and the dosage was consistent in all patients. Cardioplegia solutions were the standard Buckberg potassium-based solutions (pharmacy of the Inselspital, Berne) mixed with blood in a ratio of 4:1 (blood:cardioplegia). The cardioplegia protocol included cold antegrade induction (30 mL per kg of body weight for 5 minutes) and maintenance. For reperfusion, a warm solution enriched with aspartate/glutamate was given (30 mL per kg of body weight over a period of 2 minutes, concentration of aspartate and glutamate 13 mmol/L, respectively). The ECC flow rates were between 2.4 L/min/m² and 2.8 L/min/m². Ultrafiltration was performed in all patients in a standardized manner during the rewarming period using a blood concentrator (20 mL per minute resulting in a volume of 600 ± 200 mL). Hypothermia (32°C) was maintained during surgery. The injected heparin was neutralized with protaminhydrochloride (ICN Pharmaceuticals, Frankfurt, Germany) after CPB. The aorta was clamped for 34.7 ± 14.7 minutes (mean $\pm$ SD); CPB lasted 72.2 ± 30.7 minutes (mean $\pm$ SD) (Table 1).

Immunophenotyping of Cells

Cell surface receptors were detected using a standard technique for immunophenotyping. Arterial blood anticoagulated with ethylenediaminetetraacetic acid was used and measurements were done immediately after sample collection. Fluorescent monoclonal antibodies (mAb): fluorescein isothiocyanate-conjugated mouse anti-human CD14 (Clone M5E2), anti-CDw128 (IL-8RA/CXCR1, Clone 5A12)-phycoerythrin (PE) mAb, and anti-IL-8RB (CXCR2, Clone 6C6)-PE mAb (Pharmingen, Heidelberg, Germany), isotype-matched controls (Becton Dickinson, Heidelberg, Germany). Fixation of leukocytes and lysis of erythrocytes were done with fluorescence-activated cell sorter (FACS)-lysing solution (Becton Dickinson). For estimation of the number of mAb-PE bound per cell, beads conjugated with known levels of PE were used (QuantibRITE PE, Becton Dickinson). A total of 10,000 cells were

acquired per measurement using a FACSCalibur flow cytometer (Becton Dickinson). A 2-parameter light scatter dot plot was created and a software gate was set around the monocytes and neutrophil granulocytes using the CellQuest software (Becton Dickinson). The gated cells were analyzed for their fluorescence properties, and results are expressed as relative fluorescence intensity (RFI). RFI values are directly proportional to the respective expression of receptors on cell surface.

Plasma Levels of IL-6, IL-8, Myeloperoxidase, and Total NO

Concentrations of IL-6 and IL-8 were measured using a particle-based immunoassay with fluorescent detection by flow cytometry (Cytometric Bead Array, human inflammation kit, Becton Dickinson) according to the manufacturer's instructions. Assay sensitivity for IL-6 was 2.5 pg/mL and for IL-8, 3.6 pg/mL. The range defining the minimum and maximum quantifiable level was 10 to 5000 pg/mL, respectively.

Plasma Nitrate and Nitrite

Myeloperoxidase (MPO) in plasma was measured with a commercial enzyme-linked immunosorbent assay kit (R&D Systems, Abingdon, UK). Sensitivity and dynamic range for MPO was 1.5 ng/mL, with a range of 1.5 to 100 ng/mL.

Plasma samples were ultrafiltered through a 10,000 molecular weight cutoff filter to eliminate proteins (Microcon7; Millipore, Bedford, Mass). As most of the NO is oxidized to nitrite (NO_2^-) and nitrate (NO_3^-), the concentrations of these anions are used as a quantitative measure of NO production. After the conversion of NO_3^- to NO_2^- , the spectrophotometric measurement is accomplished by using the Griess reaction (Total Nitric Oxide Assay, R&D systems). Sensitivity and range was $<1.35 \mu\text{mol/L}$ with a range of 3.12 to 100 $\mu\text{mol/L}$.

Statistical Analysis

Statistical analysis was performed by paired Wilcoxon test. Correlation trends were assessed by using Spearman rank correlation coefficient r . Data are presented as means $\pm$ SD unless otherwise noted.

Results

IL-6 and IL-8 Concentrations

Two hours after CPB, plasma levels of IL-6 and IL-8 were both strongly increased compared with preoperative values (IL-6 postoperative levels 384.4 ± 300.7 pg/mL versus preoperative levels 34.2 ± 37.0 pg/mL, $P = .00004$, and IL-8 levels 111.0 ± 99.2 pg/mL versus 21.3 ± 18.0 pg/mL, $P = .0003$, respectively). There was a statistically significant correlation between postoperative levels of IL-6 and IL-8 ($r = 0.5951$, $P = .0057$). Plasma concentrations of both IL-6 and IL-8 determined 2 hours after CPB were correlated to the duration of CPB and to aortic clamp time (IL-6 to CPB time $r = 0.7424$, $P = .0002$, and to aortic clamp time $r = 0.5774$, $P = .0077$; IL-8 to CPB $r = 0.6925$, $P = .0007$, and to aortic clamp time $r = 0.6269$, $P = .0031$, respectively). Increased levels of IL-6 and IL-8 determined

Figure 1. Correlation between plasma interleukin-6 concentrations (pg/mL) determined 2 hours after cardiopulmonary bypass to the length of inotropic support (hours) ($r = 0.7263$, $P = .0003$).

Figure 2. Correlation between plasma interleukin-8 concentrations (pg/mL) determined 2 hours after cardiopulmonary bypass to the length of inotropic support (hours) ($r = 0.7000$, $P = .0006$).

2 hours after CPB were associated with increased duration of inotropic support ($r = 0.7263$, $P = .0003$, and $r = 0.7001$, $P = .0006$, respectively) (Figures 1 and 2), as well as with prolonged need for mechanical ventilation ($r = 0.7456$, $P = .0002$, and $r = 0.7025$, $P = .0006$) and decreased $\text{PaO}_2/\text{FiO}_2$ after CPB ($r = -0.6989$, $P = .0005$, and $r = -0.7676$, $P = .0002$, respectively) (Figures 3 and 4).

Plasma Levels of MPO and Total NO

MPO is a specific marker of primary neutrophil granules, which are released in response to strong activating stimuli. Postoperative MPO levels were elevated in all patients compared with preoperative values (13.7 ± 6.5 ng/mL versus 2.9 ± 1.6 before CPB, $P = .0001$). There was a

correlation between postoperative levels of MPO and IL-8 ($r = 0.4467$, $P = .0481$), but no correlation was found between MPO and IL-6 ($r = 0.1661$, $P = .4843$). In contrast, postoperative levels of IL-6 and IL-8 were both strongly correlated to the levels of total NO ($r = 0.6295$, $P = .0030$ and $r = 0.6166$, $P = .0038$, respectively).

CD14 Expression on Monocytes

After CPB, expression of CD14 on the surface of monocytes correlated with increased levels of IL-6 ($r = 0.6267$, $P = .0031$) as well as with the plasma levels of total NO ($r = 0.4443$, $P = .0496$). In contrast, preoperatively neither IL-6 levels nor NO levels correlated with the expression of CD14 ($r = -0.4440$, $P = .0514$, and $r = 0.3786$, $P = .1100$, respectively).

Figure 3. Postoperative plasma levels of interleukin-6 (pg/mL) in relation to the ratio of $\text{PaO}_2/\text{FiO}_2$ at 2 hours after cardiopulmonary bypass ($r = -0.6989$, $P = .0005$).

Figure 4. Postoperative plasma levels of interleukin-8 (pg/mL) in relation to the ratio of $\text{PaO}_2/\text{FiO}_2$ at 2 hours after cardiopulmonary bypass ($r = -0.7676$, $P = .0002$).

CXCR1, CXCR2 on Neutrophils and Monocytes

Analysis of CXCR1 and CXCR2 using mAbs specific for the 2 IL-8 receptor subtypes showed that the expression of CXCR2, but not CXCR1, on neutrophils and monocytes was negatively correlated with the plasma levels of IL-8 (neutrophil CXCR2 to IL-8 correlation $r = -0.6724$, $P = .0012$, and monocyte CXCR2 correlation to IL-8 $r = -0.5454$, $P = .0125$). Furthermore, decreased expression of CXCR2 on neutrophils and monocytes was associated with increased levels of MPO ($r = -0.4609$, $P = .0408$, and $r = -0.6853$, $P = .0009$, respectively) (Figure 5). Again, expression of CXCR1, either on neutrophils or on monocytes, did not correlate to the plasma levels of MPO.

Discussion

After CPB, elevated levels of the proinflammatory cytokines IL-6 and IL-8 were associated with impaired respiratory and cardiovascular function. Increased levels of NO and MPO correlated with monocyte and neutrophil activation, possibly due to chemokine stimulation via CXCR2.

Increased levels of IL-6 determined 2 hours after CPB were associated with increased concentrations of NO and were followed by a prolonged need for inotropic medication. The suppression of the cardiovascular system can be attributed to a direct inhibitory effect of IL-6 on myocardial contractility.¹¹ Most nucleated cells have been shown to express and synthesize IL-6. The most prominent source

Figure 5. Postoperative plasma levels of myeloperoxidase (ng/mL) in relation to the expression of CXCR-2 on monocytes ($r = -0.6853$, $P = .0009$). *RFI*, Relative fluorescence intensity.

appears to be stimulated monocyte-macrophages and cytokine-stimulated stromal cells—fibroblasts and epithelial cells, as well as endothelial cells.²⁵ More specifically, increased expression of IL-6 was demonstrated in myocytes and interstitial fibroblasts in the failing myocardium.^{26,27} One possibility of stimulating monocyte-macrophages is mediation through binding of lipopolysaccharide to the leukocyte receptor CD14.^{28,29} CD14 has been shown to mediate responses to many pathogen-derived ligands and is one of many receptors involved in the recognition and clearance of apoptotic cells.³⁰⁻³² Cellular activation via CD14 results in the production of inflammatory cytokines, for example, TNF- α , IL-1, IL-6, IL-8, and NO as well as anti-inflammatory cytokines, and the level of expression of CD14 on monocytes is positively correlated with the grade of cellular activation.³³⁻³⁵

Here, we demonstrated a correlation between the postoperative monocyte CD14 expression and plasma levels of IL-6, IL-8, and NO. Production of cytokines like IL-6 and IL-8 as well as of NO is described as part of the inflammatory response to CPB.^{9,36,37} Moreover, coordinated production of IL-6 and IL-8 is often observed in various inflammatory conditions.³⁸ During host defense and immunologic reactions, induction of inducible NO synthase occurs and large quantities of NO are produced by activated macrophages.⁸ Additionally, NO is generated by an endothelial nitric-oxide synthase.^{10,39} NO regulates vascular tone through a cyclic guanosine monophosphate (cGMP)-dependent signaling pathway.⁴⁰ In addition, NO has cGMP-independent effects within the vasculature, such as inhibition of leukocyte adhesion, that relies on its ability to inactivate or antagonize superoxide.^{12,41} Our findings of increased CD14 expression in correlation with plasma concentrations of NO support a major role of monocytes during the early postop-

erative time after CPB. Clinically, vasodilation and impaired myocardial contractility may be due to the effects of NO and IL-6 and were followed by a prolonged need for inotropic medication. Increased vascular permeability may have contributed to a decrease of pulmonary oxygen exchange and the subsequent longer need for mechanical ventilation.

MPO is a specific marker of primary neutrophil granules and is only released in response to strong activating stimuli. MPO has both cytotoxic and antibacterial activities through its ability to interact with chloride and hydrogen peroxide to generate hydroxyl radical and hypochlorous acid.¹⁴ The high plasma levels of myeloperoxidase 2 hours after CPB indicate a profound activation of neutrophils in vivo during surgery. One of the most prominent proinflammatory cytokines for neutrophils is IL-8, which induces chemotaxis, granule release, and a respiratory burst.⁴² Following IL-8 stimulation, the IL-8 receptors are rapidly internalized and subsequently recycled to the plasma membrane or degraded.^{43,44} Down-regulation of IL-8 receptors therefore may be an indicator of in vivo exposure of the leukocyte to the corresponding agonist. Most interestingly, increased plasma levels of MPO were demonstrated together with increased levels of IL-8 and decreased expression of the IL-8 receptors CXCR2 on neutrophils. These findings indicate that neutrophils have been activated in vivo during CPB. The fact that the expression of CXCR2 but not CXCR1 was down-regulated on neutrophils and monocytes suggests production of 1 or more of other proinflammatory chemokines of the IL-8 family with higher affinity for CXCR2.^{45,46} Recently, a selective nonpeptide CXCR2 antagonist has been shown to prevent IL-8–induced neutrophil chemotaxis in vitro and margination in vivo.⁴⁷

Limitations of the study: Whereas the ratio of $\text{PaO}_2/\text{FiO}_2$ is well established to quantify disturbed lung function, documentation of compromised cardiovascular function is difficult. Measurement of cardiac output is, at least in theory, the gold standard but in most centers is not routine with pediatric cardiac surgery. Length of inotropic support was taken in this study as a surrogate of disturbed cardiovascular function. Concerning the method of CPB, a number of factors like the use of aprotinin or the performance of ultrafiltration may influence the inflammatory response. However, in this study, CPB was done in a standardized manner in all patients, thus not accounting for the individual differences.

In conclusion, CPB in children induced several humoral and cellular variables of an acute systemic inflammatory response. The strong correlation of NO and MPO, presumably generated by activated neutrophils and monocytes with the extent of impairment of cardiovascular and respiratory function, reveals novel targets of therapeutic interventions. Because stimulation of neutrophils and monocytes may at least in part be dependent on CXCR2, antagonists of this receptor could offer a new therapeutic option.

We thank A. Urwyler (Institute of Immunology, University of Berne) for technical assistance.

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