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Endotoxin Desensitization of Human Mononuclear Cells after Cardiopulmonary Bypass

Role of Humoral Factors

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Background: **The ability of leukocytes to release proinflammatory cytokines on lipopolysaccharide stimulation in vitro is impaired after cardiopulmonary bypass (CPB). This study tested contribution and interaction of humoral factors in altered leukocyte responsiveness to lipopolysaccharide.**

Methods: **Whole blood and isolated peripheral-blood mononuclear cells (PBMCs) from 10 patients obtained after induction of anesthesia (T_1) and 20 min (T_2) and 24 h (T_3) after CPB were cultured in the absence or presence of lipopolysaccharide and assessed for release of tumor necrosis factor α (TNF-α) and interleukin (IL)-1β and their functional antagonists, IL-1 receptor antagonist (IL-1ra) and IL-10. In addition, dose–response characteristics and interaction of IL-10 and norepinephrine as modulators of TNF-α release were studied.**

Results: **Cardiopulmonary bypass induced release of antiinflammatory (T_2: IL-10: median 25 pg/ml, 25th–75th percentile 9–42; IL-1ra: median 1,528 pg/ml, 25th–75th percentile 1,075–17,047; $P < 0.05$ compared with T_1) but failed to induce proinflammatory cytokines (T_2: TNF-α: median 0 pg/ml, 25th–75th percentile 0–6; IL-1β: median 1 pg/ml, 25th–75th percentile 0–81; nonsignificant). Removal of plasma at T_2 increased TNF-α response to lipopolysaccharide (+83.8%; $P < 0.05$), whereas it suppressed IL-10 (−36.8%; $P < 0.05$). Similarly, incubation of PBMCs (T_1) with plasma obtained after CPB (T_2) as well as addition of IL-10 or norepinephrine in concentrations present in plasma after CPB led to a reduced lipopolysaccharide-stimulated TNF-α and an increased IL-10 response. Coadministration of norepinephrine and IL-10 had synergistic effects. Although pretreatment with an anti–IL-10 antibody and labetalol before addition of plasma obtained at T_2 largely restored the TNF-α response *in vitro*, their addition post-treatment failed to restore the monocytic TNF-α response.**

Conclusions: **Plasma contains interacting factors that inhibit the release of TNF-α and increase the release of IL-10, presumably attenuating the inflammatory response to CPB. Although norepinephrine fails to induce a cytokine response in the absence of other stimuli, its administration seems to augment the antiinflammatory IL-10 response while attenuating the TNF-α response. (Key words: Cardiac surgery; catecholamines; cytokines; inflammation; stress response.)**

CORONARY artery bypass grafting (CABG) with cardiopulmonary bypass (CPB) is associated with an ischemia–reperfusion injury to the heart and lungs and induces a complex inflammatory response not only affecting lungs and heart but also remote organs such as kidney, gut, and brain.[1,2] This response may contribute to organ dysfunction and morbidity. Among the different mediators involved in the regulation of the inflammatory response, proinflammatory and antiinflammatory cytokines seem to be of particular importance.[3]

Although excessive release of proinflammatory cytokines may contribute to acute and chronic cardiovascu-

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lar dysfunction as well as to organ injury,[4] their production is necessary for the host defense response and wound healing in surgical patients.[5] Previous work from our laboratory as well as others has indicated a reduced capacity of cultured whole blood obtained after trauma and major surgery to synthesize proinflammatory cytokines, most notably tumor necrosis factor α (TNF-α) on lipopolysaccharide stimulation.[6–9] Consistent with a requirement of TNF-α for the host defense response under stress conditions, impaired TNF-α responsiveness correlated with an unfavorable postoperative course in these patients.[7] The molecular mechanisms responsible for the impaired lipopolysaccharide responsiveness after major surgery may involve cellular and humoral factors. Regarding the latter, release of interleukin (IL)-10 in response to surgical stress has been proposed to reflect a key mechanism for immunosuppression in these patients.[9,10]

Interleukin 10 can be released by monocytes on various stimuli, such as bacterial toxins, but also catecholamines, and seems to play a significant role in the monocytic feedback regulation terminating the cytokine response after contact with infectious stimuli.[9–11] However, its role in endotoxin tolerance is controversial.[12] This term describes the diminished release of proinflammatory cytokines by leukocytes on repeated exposure to lipopolysaccharide, which resembles the observed refractory state of leukocytes after major surgery.[13] In the present study, we investigated contribution of humoral factors to the observed hyporesponsiveness of leukocytes in their intact environment to lipopolysaccharide stimulation after CPB. In particular, the role of catecholamines and IL-10 in mediating this response was addressed with special emphasis on their interaction.

Materials and Methods

All chemicals, including lipopolysaccharide, were obtained from Sigma Chemicals (St. Louis, MO) if not otherwise specified.

Patients and Study Design

After obtaining approval from the local ethics committee and written informed consent, 10 male patients scheduled for elective CABG were enrolled in the study. Patients with previous CABG surgery, coexisting congestive or valvular heart disease, myocardial infarction during the last 3 months, or evidence of concomitant malignant or immunologic diseases were excluded. Additional exclusion criteria were antecedent medication with corticosteroids, methylxanthines, or intraoperative use of aprotinine or tranexamic acid. Oral premedication consisted of 2 mg flunitrazepam 1.5 h before anesthesia. Anesthesia was induced and maintained with midazolam and fentanyl, because previous results showed that both anesthetics did not interfere with the cytokine response of cultured whole blood *in vitro*.[14] Pancuronium was used to provide neuromuscular blockade. Ventilation with oxygen-enriched air (fraction of inspired oxygen, 0.5) was adjusted to maintain normocapnia. Nitroglycerin was titrated for blood pressure control if necessary. The extracorporal circuit consisted of roller pumps, a right atrial two-stage cannula for venous drainage, a cardiotomy reservoir, a membrane oxygenator, and a 40-mm arterial line filter with arterial return to the ascending aorta. The circuit was primed with 1 l Ringer's lactate and 0.5 l of a standard gelatin preparation (Gelafundin, Braun, Melsungen, Germany). CPB was performed with moderate hypothermia (31–32°C rectal temperature) and acid base balance correction. The perfusion volume was maintained at $2.4\ l \cdot min^{-1} \cdot m^{-2}$. Myocardial preservation was achieved by antegrade application of cooled St. Thomas' solution into the aortic route after aortic cross-clamping. After discontinuation of CPB, inotropic support consisted of dopamine ($2–3\ mg \cdot kg^{-1} \cdot min^{-1}$) in all patients and, if necessary, norepinephrine ($0.05–0.15\ mg \cdot kg^{-1} \cdot min^{-1}$) for treatment of inadequate low systemic vascular resistance. One patient required epinephrine for apparent low output syndrome during weaning from CPB.

Processing of Blood Samples and Experimental Protocol

Whole Blood Culture Experiments. Blood samples were taken after induction of anesthesia but before surgery (T_1) and 20 min (T_2) and 24 h (T_3) after the discontinuation of CPB. Samples were collected aseptically into a pyrogen-free citrate containing system for culture (Sarstedt Monovette, Muembrecht, Germany) and processed immediately as described by Wilson *et al.*[15] with minor modifications as described in detail previously.[7,8,14] Briefly, blood samples from each patient were diluted 1:5 with cell culture medium Roswell Park Memorial Institute (RPMI) 1640 and recalcified by addition of 1 ml of a 250-mM calcium chloride ($CaCL_2$) stock solution per 100 ml diluted blood after addition of heparin (2 units/ml; tested for endotoxin < 5 pg/ml). Aliqouts of diluted blood were placed in sterile polypro-

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pylene tubes (Falcon; Becton Dickinson, Lincoln Park, NY) and incubated in a humidified atmosphere with 5% CO_2 at 37°C for 24 h in the absence or presence of 1 μg/ml lipopolysaccharide (*Escherichia coli* O111:B4), a dose that induces a maximum release of all cytokines studied. Parallel cultures containing a neutralizing monoclonal anti–IL-10 antibody (rat antihuman–IL-10 antibody, Biosource International, Camarillo, CA) or serotype-matched control immunoglobulin were performed for each time point and each lipopolysaccharide-stimulated aliquot. Based on our previous results on IL-10 plasma levels during CABG,[8] a concentration of 25 ng anti–IL-10 monoclonal antibody per milliliter of culture medium (reflecting approximately a 500–1,000-fold molar excess of the antibody over immunoreactive IL-10 plasma levels) was added to the cultures. This concentration of the neutralizing antibody did prevent the inhibitory effect of exogenous IL-10 up to 5,000 pg/ml on lipopolysaccharide-stimulated TNF-α response (data not shown). At 24 h after onset of culture, the samples were removed and centrifuged at 1,900 *g* for 15 min. The plasma was then removed and stored immediately at −80°C until it was assayed for cytokines. In addition, blood obtained from healthy volunteers was used to study dose–response characteristics of the modulation of the cytokine response by IL-10 and norepinephrine. These cultures were treated identically as previously described for the patient samples obtained at the various time points during the surgical procedure.

Determination of Epinephrine, Norepinephrine, Antidiuretic Hormone, Adrenocorticotropic Hormone, and Cortisol. Samples were taken into pyrogen-free EDTA- (for assessment of epinephrine and norepinephrine) or heparin- (for assessment of antidiuretic hormone, adrenocorticotropic hormone [ACTH], and cortisol) containing systems (Sarstedt Monovette, Muembrecht, Germany). The samples were kept on ice and centrifuged within 10 min after collection. Supernatants were subsequently frozen in liquid nitrogen and stored at −80°C until analysis. Epinephrine and norepinephrine were measured in supernatants from EDTA plasma using a commercially available kit (Clin Rep; Recipe, Munich, Germany) by means of high-performance liquid chromatography. Antidiuretic hormone, ACTH, and cortisol were determined in supernatants of blood samples anticoagulated with heparin using commercially available kits and standards according to the manufacturers instructions (Vasopressin Direct, Buehlmann Laboratories, Basel, Switzerland; ACTH Double Antibody and Coat-A-Count, Diagnostic Products Corp., Los Angeles, CA). Detection limits, recovery rates, and coefficients of variance for the assays for our laboratory have been reported in detail previously.[16]

Culture Assays Using Isolated Peripheral-blood Mononuclear Cells. Additionally, peripheral-blood mononuclear cells (PBMCs) were isolated from aliquots of blood obtained at each time point by density gradient centrifugation over Ficoll-Hypaque (d = 1.077) at 680*g* for 20 min, as described previously.[14] Briefly, the PBMC pellet was rinsed repeatedly with phosphate-buffered saline (Life Technologies, Grand Island, NY) to remove humoral factors. The cell count was adjusted to 1.5×10^6 cells/ml, and cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (both obtained from Life Technologies). Isolated PBMCs were cultured in the absence or presence of lipopolysaccharide (1 μg/ml) for 24 h as previously described for the whole blood culture system. Because removal of plasma increased TNF-α and decreased IL-10 response (see below), in additional experiments the impact of humoral factors in plasma obtained after CPB on lipopolysaccharide-stimulated cytokine response by unstimulated PBMCs was studied. Patients for these experiments (n = 10) met the same inclusion criteria and were comparable regarding biometric data and patient characteristics (data not shown). In these control experiments, PBMCs were obtained from patients at T_1 and were cultured in RPMI medium until 20 min after the end of CPB. Additionally, PBMCs from healthy donors were used. The inhibitory effect of plasma obtained at T_2 was observed irrespective of whether autogenic PBMCs or allogenic PBMCs from volunteers were subjected to culture. Plasma obtained at T_1 or T_2 was added to aliquots of PBMCs obtained at T_1 or to PBMCs from volunteers. The use of PBMCs from volunteers allowed a large number of parallel incubations while limiting the amount of blood withdrawn in patients at T_1 and T_2. These cultures were stimulated with lipopolysaccharide after 1 h of preincubation in plasma–RPMI.

Experiments Addressing the Role of Interleukin 10 and Norepinephrine as Humoral Mediators of Endotoxin Desensitization. To further clarify which humoral factors might contribute to the observed inhibitory effect of plasma obtained at T_2, two series of experiments were conducted. First, the effects of exogenous IL-10 and norepinephrine as well as their interaction on lipopolysaccharide-stimulated TNF-α and IL-10 response were studied, because both factors had independently been shown previously to downregulate TNF-α *in vitro*.[17,18] After an incubation period of 1 h in

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the presence of norepinephrine 0, 10, 100, and 1,000 ng/ml and in the absence or presence of the α_1- and β-adrenergic receptor blocking agent labetalol (10^{-5} M), whole blood cultures from healthy volunteers were stimulated with 1 μg/ml lipopolysaccharide. The effect of exogenous IL-10 was similarly studied by adding IL-10 0, 5, 50, 5,000, or 50,000 pg/ml. In addition, the effect of coadministration of IL-10 and norepinephrine was exemplary studied, because both factors are present after termination of CPB and might act synergistically. In these experiments, norepinephrine 0, 10, 100, and 1,000 ng/ml was added in the presence of IL-10 0 or 50 pg/ml or 50 ng/ml to the whole blood culture system. After preincubation for 1 h, 1 μg/ml lipopolysaccharide was added.

In a second series of experiments, PBMCs (1.5×10^6 cells/ml) from volunteers were preincubated with neutralizing anti–IL-10 antibodies, labetalol, or a combination thereof. After preincubation with the antagonists, plasma obtained at T_2 was added. PBMCs cultured identically after addition of allogenic plasma obtained at T_1 served as controls. One hour after addition of the plasma, 1 μg/ml lipopolysaccharide was added. Supernatants were obtained after a culture period of 24 h in a humidified atmosphere at 37°C and 5% CO_2.

Supernatants of all experiments were stored for less than 2 months at −80°C until assessment of cytokine concentrations by means of enzyme-linked immunosorbent assay (Medgenix/Biosource, Ratingen, Germany). A detailed protocol including interassay and intraassay coefficients of variance for our laboratory has been published previously.[14]

Statistical Analysis

Data are reported as median and 25th–75th percentile or mean ± SD and range. Statistical differences from baseline were determined by one-way repeated-measures analysis of variance followed if significant by *post hoc* Student-Newman-Keuls test using raw data. To allow comparison between whole blood assays and cultures of isolated PBMCs at the time points T_2 and T_3, data were normalized to their respective baseline values (T_1) and compared by Student *t* test. When criteria for parametric testing (equivalency of variances or normal distribution as assessed by Levene mediane test and Kolmogorov-Smirnov test, respectively) were violated, the appropriate nonparametric tests (*i.e.*, Friedman repeated-measures analysis of variance on ranks or Mann–Whitney U test) were used. For correlation analysis, the Pearson product moment correlation was used. Statistical tests were performed using the SigmaStat software package (Jandel GmbH, Erkrath, Germany). All *P* values reported are two-sided and are considered significant if $P \leq 0.05$.

Results

Biometric Data, Blood Cell Counts, and Circulating Stress Hormones

Biometric data and patient characteristics are summarized in table 1. Although erythrocyte and platelet counts reflect a hemodilution as a result of CPB, a substantial concomitant increase of circulating leukocytes was observed (table 2).

Discontinuation of CPB was paralleled by increased serum catecholamine, antidiuretic hormone, ACTH, and cortisol concentrations (table 3). The high catecholamine concentrations early after CPB primarily reflect the inotropic support with norepinephrine in all patients studied and with epinephrine in one patient presenting with low output syndrome. The stress hormones norepinephrine, antidiuretic hormone, and ACTH decreased on the first postoperative day, whereas cortisol and epinephrine exhibited persistent high serum concentrations (table 3).

Unstimulated and Lipopolysaccharide-stimulated Release of Interleukin 1β, Interleukin 1ra, Tumor Necrosis Factor α, and Interleukin 10 into Whole Blood Culture

The unstimulated and lipopolysaccharide-stimulated cytokine response of cultured whole blood is summarized in table 4. TNF-α and IL-1β were produced in trace

Table 1. Demographic Data, Left Ventricular Function, Aortic Cross-clamp, Bypass Times, and Medication of Patients Enrolled

No. of patients	10
Age (yr)	60.8 ± 11.3 (37–76)
Weight (kg)	81.8 ± 7.3 (68–94)
Height (cm)	171.5 ± 4.6 (162–180)
Cardiopulmonary bypass time (min)	91.9 ± 38.0 (42–181)
Aortic cross-clamp time (min)	54.3 ± 19.1 (25–90)
Ejection fraction (%)	57.1 ± 11.9 (38–81)
No. of patients receiving	
Angiotensin-converting enzyme inhibitors	4
Nitrates	10
β-Blockers	5

Where appropriate, values are mean ± SD (range).

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Table 2. Cell Counts during the Course of Coronary Artery Bypass Grafting Surgery and on the First Postoperative Day

	After Induction of Anesthesia (T_1)	20 min after End of CPB (T_2)	24 h after CPB (T_3)
Leukocyte count ($\times 10^3/\mu l$)	4.6 ± 1.42 (2.6–7.0)	12.7 ± 4.88* (5.7–19.1)	10.8 ± 2.36* (5.0–14.2)
Erythrocyte count ($\times 10^6/\mu l$)	4.3 ± 0.69 (3.72–6.15)	3.1 ± 0.31* (2.54–3.61)	3.7 ± 0.48*† (2.76–4.44)
Hemoglobin (g/dl)	12.8 ± 1.48 (11.2–16.6)	9.3 ± 0.98* (7.6–10.7)	11.1 ± 1.49† (9.0–14.0)
Hematocrit (%)	36.9 ± 4.23 (33.0–48.2)	27.0 ± 2.66* (22.8–31.1)	32.1 ± 3.85† (25.5–37.9)
Platelets ($\times 10^3/\mu l$)	177.2 ± 39.36 (125–235)	135.5 ± 29.56* (93–197)	145.6 ± 46.11* (88–223)

Data are mean ± SD (range).

* $P < 0.05$ *versus* after induction of anesthesia (T_1).

† $P < 0.05$ *versus* 20 min after end of cardiopulmonary bypass (T_2).

CPB = cardiopulmonary bypass.

amounts during the culture period even in the absence of lipopolysaccharide. This basal release was not consistently affected by CPB. Although IL-10 was barely detectable in cultured blood obtained at T_1 or T_3, a significant production of IL-10 was observed in samples obtained after CPB (T_2) and subsequently cultured in the absence of lipopolysaccharide. Similarly, unstimulated production of IL-1ra significantly increased in the culture system when blood was collected after CPB. Lipopolysaccharide-stimulated TNF-α, IL-1β, and IL-10 response of cultured whole blood at T_2 was reduced by 89% (TNF-α), 84% (IL-1β), and 68% (IL-10) as compared with values observed in cultures of blood drawn at T_1. Release of TNF-α and IL-1β by leukocytes to lipopolysaccharide stimulation at T_3 returned to values not significantly different from those obtained in cultures of blood drawn at T_1, whereas IL-10 responsiveness was even increased at T_3. In contrast, the IL-1ra response was significantly augmented at T_2 and further increased in lipopolysaccharide-stimulated cultures of blood drawn on the first postoperative day (T_3).

Contribution of Humoral Factors to Lipopolysaccharide Desensitization of Cultured Leukocytes

The contribution of humoral factors to the observed hyporesponsiveness of PBMCs to mount a cytokine response on lipopolysaccharide stimulation was further studied for TNF-α and IL-10. Comparison of the lipopolysaccharide-stimulated TNF-α response of cultured whole blood with the response observed in washed, isolated PBMCs revealed a significant increase of TNF-α release in the absence of plasma at T_2 and T_3 (fig. 1A). In contrast, lipopolysaccharide-stimulated IL-10 response was further decreased in the absence of plasma in the early (T_2) and late phase (T_3) after CPB (fig. 1B). Similarly, addition of plasma obtained at T_2 to PBMCs isolated at T_1 significantly decreased lipopolysaccharide-stimu-

Table 3. Endocrine Stress Response*

	After Induction of Anesthesia (T_1)	20 min after End of CPB (T_2)	24 h after CPB (T_3)
Epinephrine (pg/ml)	8 (4–13)	72 (33–242)†	114 (50–203)†
Norepinephrine (pg/ml)	105 (60–158)	5,987 (2,180–23,665)†	649 (504–1270)‡
ADH (pg/ml)	8 (4–11)	46 (40–66)†	12 (9–35)‡
ACTH (pg/ml)	11 (9–13)	74 (36–167)†	19 (6–40)‡
Cortisol (ng/ml)	96 (72–113)	221 (156–259)†	247 (196–378)†

Data are median (25th–75th percentile).

* Concentrations of epinephrine, norepinephrine, antidiuretic hormone, adrenocorticotropic hormone, and cortisol during and after coronary artery bypass grafting.

† $P < 0.05$ *versus* after induction of anesthesia (T_1).

‡ $P < 0.05$ *versus* end of cardiopulmonary bypass (T_2).

ACTH = adrenocorticotropic hormone; ADH = antidiuretic hormone; CPB = cardiopulmonary bypass.

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Table 4. Concentrations of Interleukin 1β, Interleukin 1ra, Tumor Necrosis Factor α, and Interleukin 10 in Whole Blood Obtained before Surgery (T_1), after Discontinuation of Cardiopulmonary Bypass (T_2), and 24 h after Cardiopulmonary Bypass (T_3)

	After Induction of Anesthesia (T_1)	20 min after End of CPB (T_2)	24 h after CPB (T_3)
Unstimulated concentration of IL-1β (pg/ml)	0 (0–63)	1 (0–81)	0 (0–64)
Lipopolysaccharide-stimulated concentration of IL-1β (pg/ml)	7,794 (5,911–11,249)	1,241 (819–1,886)*	6,552 (1,360–11,947)
Unstimulated concentration of IL-1ra (pg/ml)	676 (476–1,048)	1,528 (1,075–1,747)*	1,846 (1,138–2,687)†
Lipopolysaccharide-stimulated concentration of IL-1ra (pg/ml)	20,123 (15,806–25,572)	37,584 (16,479–55,652)*	71,926 (51,639–111,944)*†
Unstimulated concentration of TNF-α (pg/ml)	0 (0–16)	0 (0–6)	8 (0–25)
Lipopolysaccharide-stimulated concentration of TNF-α (pg/ml)	12,723 (10,288–14,998)	1,306 (797–1,709)*	8,882 (5,886–13,610)†
Unstimulated concentration of IL-10 (pg/ml)	0 (0–1)	25 (10–42)*	0 (0–0)†
Lipopolysaccharide-stimulated concentration of IL-10 (pg/ml)	907 (406–1,216)	215 (102–374)*	1,154 (757–1,488)*†

Supernatants were assayed for cytokines after a 24-h culture period in the absence or presence of lipopolysaccharide (1 μg/ml). Data are corrected for 1:5 dilution with RPMI 1640. Data are median (25th–75th percentile).

* $P < 0.05$ *versus* after induction of anesthesia (T_1).

† $P < 0.05$ *versus* end of cardiopulmonary bypass (T_2).

CPB = cardiopulmonary bypass; IL = interleukin; ra = receptor antagonist; TNF-α = tumor necrosis factor α.

lated TNF-α response (−58%), whereas the IL-10 response was maintained (+13%). Thus, plasma from patients undergoing CABG contains factor(s) that inhibit the production of the proinflammatory TNF-α and increase the release of the antiinflammatory IL-10 on stimulation. A similar inhibitory effect of plasma obtained at T_2 as compared with plasma T_1 was observed when PBMCs from healthy volunteers were cultured in the presence of plasma from patients undergoing CABG. In these experiments, pretreatment with anti–IL-10 monoclonal antibody and, in particular, with a combination of anti–IL-10 monoclonal antibody and labetalol significantly increased the lipopolysaccharide-stimulated TNF-α response in the presence of plasma T_2, almost to levels observed in the presence of plasma T_1 (fig. 2). Although higher plasma concentrations of cortisol tended to correlate with lower lipopolysaccharide-stimulated TNF-α concentrations (r^2 for cortisol and lipopolysaccharide-stimulated TNF-α response: 0.21), none of the other stress hormones measured correlated with the cytokine response.

Comparative and Interactive Effects of Norepinephrine and Interleukin 10 on Lipopolysaccharide-stimulated Cytokine Response

The modulatory role of norepinephrine and IL-10 was further studied by addition of either exogenous norepinephrine or IL-10 to whole blood cultures of healthy volunteers. To address potential interactive or synergistic effects of humoral factors, the impact of the combined addition of norepinephrine and IL-10 was exemplarily studied. Although norepinephrine failed to induce either TNF-α or IL-10 in the absence of lipopolysaccharide (data not shown), its presence significantly increased TNF-α while attenuating IL-10 on lipopolysaccharide stimulation. This effect was saturated at doses of norepinephrine exceeding 100 ng/ml, where no further decrease of TNF-α or increase of IL-10 on lipopolysaccharide stimulation was observed (figs. 3A and 3B). Both effects were receptor mediated because pretreatment with the α_1- and β-adrenergic receptor blocking agent labetalol prevented the increase in IL-10 (fig. 3B) and substantially attenuated the decrease of the stimulated TNF-α response (fig. 3A). It is noteworthy that plasma norepinephrine concentrations as observed in the present study at T_2 (*i.e.*, approximately 10 ng/ml) were paralleled by an approximately 30–35% decrease of the stimulated TNF-α response and a similar increase in IL-10 (figs. 3A and 3B). In contrast to the saturable effect observed for norepinephrine, addition of high amounts of IL-10 (approximately 1,000-fold, exceeding the measured concentration in patient plasma) completely suppressed the lipopolysaccharide-stimulated TNF-α release (fig. 4). Norepinephrine and IL-10 synergistically inhibited the TNF-α response, and the combined addition of these factors at concentrations observed in the patient plasma at T_2 led to an approximately 60% inhibition of lipopolysaccharide-stimulated TNF-α response (fig. 4).

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Fig. 1. Effect of removal of plasma on lipopolysaccharide-stimulated tumor necrosis factor α (TNF-α) and interleukin (IL)-10 response. A significant increase of the prototypical proinflammatory cytokine TNF-α and concomitant further decrease of IL-10 production was observed in the absence of plasma factors. Blood was obtained from patients undergoing bypass surgery after induction of anesthesia but before surgery (T_1) and at 20 min (T_2) or 24 h after discontinuation of CPB (T_3). The cytokine response to lipopolysaccharide stimulation of diluted whole blood (whole blood culture) was compared with the cytokine release by isolated mononuclear cells in the absence of plasma factors (isolated peripheral-blood mononuclear cells [PBMC]). PBMCs were isolated and washed before culture in fetal calf serum–supplemented culture medium; the cell count of these cultures was adjusted to 1.5×10^6 cells/ml to correct for changes in cell count during surgery. Lipopolysaccharide (1 μg/ml) served as stimulus to elicit a TNF-α (*A*) and IL-10 (*B*) response in both assays. Baseline values of TNF-α at T_1 [median (range)]: whole blood culture 2,544.5 (1,336.8–5,083.4) pg/ml, isolated PBMC: 2,711.0 (1,483.1–4,641.4) pg/ml; baseline values of IL-10 at T_1: whole blood culture 181.3 (72.7–276.6) pg/ml, isolated PBMC: 380.4 (308.6–1,660.3) pg/ml. Box plot represents median and 25th–75th percentile; whiskers reflect 10th and 90th percentiles. *P < 0.05 for isolated PBMCs compared with whole blood culture. #P < 0.05 as compared with respective baseline (T_1); §P < 0.05 as compared with end of cardiopulmonary bypass (T_2).

Figure 2: Effect of plasma obtained before cardiopulmonary bypass (CPB; T_1) or plasma after CPB (T_2) on lipopolysaccharide-stimulated tumor necrosis factor α (TNF-α) response of peripheral-blood mononuclear cells (PBMCs) obtained from healthy donors. Addition of plasma obtained after CPB (T_2) to PBMCs from healthy donors significantly attenuated the lipopolysaccharide-stimulated TNF-α response to lipopolysaccharide. Parallel time-matched incubations of PBMCs with plasma obtained at T_1 served as controls. For details, see Materials and Methods. Pretreatment with anti–interleukin (IL)-10 monoclonal anitibody and, in particular, a combination of anti–IL-10 monoclonal antibody and labetalol attenuated the inhibitory effect of plasma obtained at T_2 on TNF-α response. Box plot represents median, 25th–75th percentile; whiskers reflect 10th and 90th percentiles. *P < 0.05 as compared with addition of plasma T_1. #P < 0.05 as compared with addition of plasma T_2 (control).

Modulation of Lipopolysaccharide Desensitization of Cultured Human Whole Blood by the α_1- and β-adrenergic Receptor Blocking Agent Labetalol or a Neutralizing Monoclonal Anti–interleukin 10 Antibody

To assess further the role of catecholamines and IL-10 as potential humoral factors responsible for the observed lipopolysaccharide desensitization, naive PBMCs were cultured after addition of a neutralizing monoclonal anti–IL-10 antibody, labetalol, or a combination thereof. Pretreatment with these antagonists synergistically prevented the decrease in TNF-α response when plasma T_2 was added (fig. 2). However, when applied to whole blood cultures from samples obtained at T_2 as a posttreatment, both antagonists, anti–IL-10 monoclonal antibody and labetalol, failed to restore the TNF-α response (*e.g.*, at T_2: 14.5% of baseline as compared with 13.5% in samples cultured in the absence of anti–IL-10 monoclonal antibody). Similarly, chronic administration of β blockers (5 of 10 patients) did not substantially affect the stimulated TNF-α response *ex vivo* as compared with cultures of blood obtained from patients not receiving β blockers (at T_2: lipopolysaccharide-stimulated TNF-α re-

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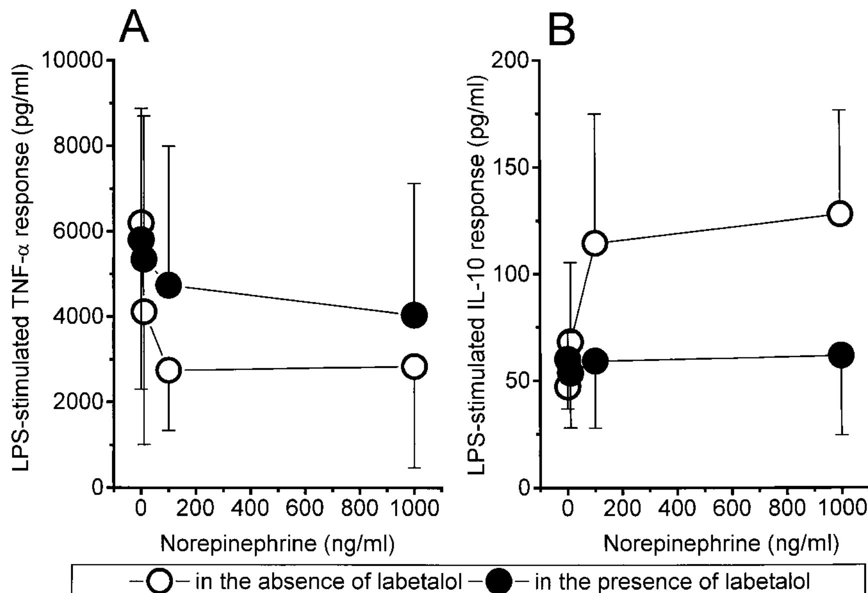

Fig. 3. Modulation of lipopolysaccharide-stimulated cytokine response in cultured whole blood assays by norepinephrine. Dose–response characteristics of norepinephrine and contribution of adrenergic receptors were studied by adding increasing concentrations of norepinephrine (0, 10, 100, 1,000 ng/ml) in the absence or presence of the α_1- and β-adrenergic receptor blocking agent labetalol (10^{-5} M) to diluted whole blood from healthy volunteers. For details see Materials and Methods. Norepinephrine decreased tumor necrosis factor α (TNF-α; *A*) while simultaneously increasing interleukin (IL)-10 response (*B*) to lipopolysaccharide stimulation. Both effects were receptor-mediated as reflected by the attenuation in the presence of labetalol. Data are mean ± SD for five individual experiments for each condition.

sponse of patients receiving β blockers: 9.7% of respective baseline value; lipopolysaccharide-stimulated TNF-α response of patients not receiving β blockers: 12.1% of respective baseline value).

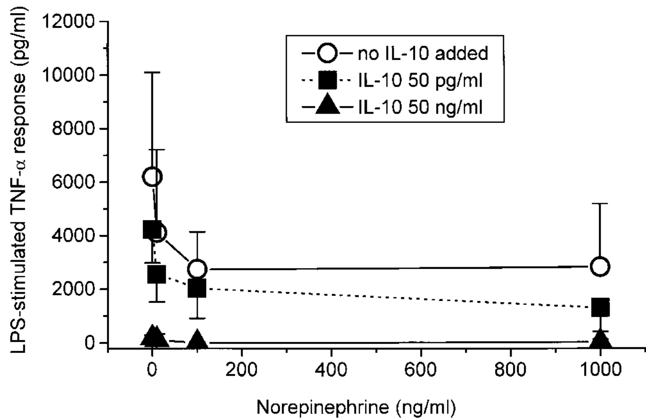

Fig. 4. Comparative and interactive effects of norepinephrine and interleukin (IL)-10 on lipopolysaccharide-stimulated tumor necrosis factor α (TNF-α) response of cultured whole blood. Norepinephrine was added in the absence or presence of IL-10 (50 pg/ml or 50 ng/ml) to diluted whole blood from healthy volunteers. Although inhibition of lipopolysaccharide-stimulated TNF-α response by norepinephrine is saturable, a complete blockade of lipopolysaccharide-stimulated TNF-α response could be evoked by addition of high amounts of exogenous IL-10. Combined addition of IL-10 and norepinephrine in concentrations measured after cardiopulmonary bypass (*i.e.*, IL-10 50 pg/ml and norepinephrine 10 ng/ml, respectively) produced an approximately 60% inhibition of lipopolysaccharide-stimulated TNF-α response. Data are mean ± SD for five individual experiments for each condition.

Discussion

In the present study, we investigated the role of humoral factors for attenuation of the proinflammatory cytokine response of leukocytes obtained from patients undergoing CABG, including CPB to lipopolysaccharide stimulation. Our data indicate that plasma from these patients obtained after separation from CPB contains sufficient amounts of humoral factors to downregulate the proinflammatory cytokine response of PBMCs to lipopolysaccharide stimulation. Although pretreatment with anti–IL-10 monoclonal antibody, either alone or in concert with the α_1- and β-blocking agent labetalol, substantially attenuated the downregulating effect of plasma obtained after CPB (T_2) on naive PBMCs, posttreatment with these antagonists failed to restore the lipopolysaccharide-stimulated cytokine response in whole blood cultures from samples obtained after CPB (T_2). Thus, although these patients clinically show signs of the systemic inflammatory response syndrome,[19] the ability of whole blood or of leukocytes obtained after separation from CPB to mount a proinflammatory cytokine response to lipopolysaccharide stimulation is remarkably diminished,[8] whereas antiinflammatory cytokines, such as IL-10 and IL-1ra, are less affected or even increased.

Although systemic inflammatory response syndrome and uncontrolled hyperinflammation have attracted considerable interest as a pathway mediating multiple organ dysfunction syndrome,[1,2,19] recent evidence suggests

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that activation of a compensatory antiinflammatory response syndrome occurs simultaneously.[20] Although circulating cytokines such as IL-6, *e.g.*, induced by gut-derived endotoxemia, may initiate an inflammatory response characterized by increased leukocyte counts and a hepatic acute phase response,[21] our data suggest that after the end of CPB, PBMCs are likely to release primarily antiinflammatory cytokines on contact with (gut-derived) lipopolysaccharide or presumably other stimuli, such as the extracorporal circuit. Thus, the unstimulated release of IL-1ra and IL-10 observed after culture in the absence of lipopolysaccharide in the present study, as well as our previous results indicating primarily circulating IL-6 and IL-10 in a similar group of patients after CPB while neither TNF-α nor IL-1β were detectable,[8] are likely to reflect this change in pattern of cytokines produced to (endogenous) endotoxin stimulation. As a result, a critical balance of upregulated proinflammatory and antiinflammatory cytokines may develop,[20] because inadequate responsiveness of effector cells of the immune system to bacterial stimuli may contribute to either systemic inflammation and remote tissue injury or to increased susceptibility to infectious complications, reflecting the leading cause of morbidity and mortality of patients recovering from major surgery. Consistent with this concept, recent work from our laboratory provided correlational evidence that the perioperative suppression of lipopolysaccharide-stimulated TNF-α response after abdominal aortic aneurysm repair is associated with an unfavorable postoperative course.[7]

Generally, the observed altered pattern of the cytokine response of cultured whole blood may involve cellular and/or humoral mechanisms. Regarding the latter, various soluble factors such as cortisol, epinephrine, norepinephrine, dopamine, or IL-10, which had been previously shown *in vitro* to interfere with the proinflammatory cytokine response of monocytes,[17,18,22–25] were documented in the current study to be present in concentrations after CPB that could attenuate the proinflammatory TNF-α and increase the antiinflammatory IL-10 response.

To assess the net contribution of soluble factors present in plasma of patients undergoing CABG, PBMCs were isolated by density gradient centrifugation, and their cytokine response was compared with parallel whole blood cultures, *i.e.*, a system in which soluble–humoral factors are present. In addition, plasma obtained at T_2 was added to PBMCs isolated before CPB or PBMCs obtained from healthy volunteers. These experiments revealed the presence of factor(s) inhibiting the release of the prototypical proinflammatory cytokine TNF-α while simultaneously stimulating the production of the prototypical antiinflammatory cytokine IL-10. This plasma activity was present early, *i.e.*, at 20 min, as well as late, *i.e.*, 24 h, after discontinuation of CPB.

Interleukin 10 has been shown to substantially attenuate organ dysfunction and lethality in various models of systemic inflammation.[12,26] Such a systemic inflammatory response characterized by circulating endotoxin, increased inflammatory mediators, and leukocytes has long been recognized to occur with cardiac surgery involving CPB. Because elective CABG overall has a low postoperative mortality and morbidity despite the consistent development of a systemic inflammatory response,[1,2,8] it is tempting to speculate that this plasma factor(s)–activity attenuates the inflammatory response initiated by CPB. However, whether this activity can be attributed to a single factor–protein remains open. As previously discussed, a variety of factors, including cytokines such as IL-10 or stress hormones that are upregulated during major surgery and specifically on initiation of CPB, are known to inhibit the monocytic TNF-α response *in vitro*. Because many of these factors can induce IL-10,[27] we hypothesized that IL-10 release may be the common final pathway mediating the suppression of the TNF-α response. Consistent with this notion, norepinephrine in concentrations measured in plasma of our patients augmented the IL-10 release and attenuated the TNF-α response. Furthermore, pretreatment of PBMCs with anti–IL-10 antibody diminished the inhibitory effect of plasma obtained after CPB on stimulated TNF-α response significantly. However, addition of a molar excess of a neutralizing antibody against IL-10 failed to restore the impaired proinflammatory cytokine response after CPB as a post-treatment. Thus, although IL-10 has been suggested as a key mediator of monocytic desensitization in surgical patients,[9] neutralizing antibodies to IL-10 seem not be sufficient to restore the monocytic cytokine response as a therapeutic strategy.

Similarly, addition of the α_1- and β-adrenergic blocking agent labetalol, which was shown in the present study to prevent upregulation of IL-10 or downregulation of TNF-α when administered before norepinephrine, failed to prevent the altered cytokine pattern as a post-treatment in whole blood cultures obtained at T_2. Both observations would be consistent with the notion that signal transduction through these mediators had already taken place, that altered cellular responsiveness contributes to lipopolysaccharide desensitization, or that these factors (and presumably others) synergize with each other, and blockade of a single mediator is not sufficient

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to restore responsiveness of leukocytes to lipopolysaccharide. Thus, to further characterize synergistic effects, whole blood assays were performed in which IL-10 and norepinephrine were coadministered. The results of these experiments demonstrated that IL-10 and norepinephrine can synergize and, in concentrations measured in the plasma of patients after CPB, can confer approximately a 60% inhibition of the TNF-α response. Furthermore, pretreatment with labetalol and anti–IL-10 antibody attenuated the decreased TNF-α response mediated by addition of plasma obtained at T_2 to naive PBMCs. Interestingly, although the dose–response characteristics of norepinephrine *in vitro* suggest that the effect of adrenergic receptor agonists is saturable, this was not the case for IL-10. The concentrations of IL-10 to produce an approximately 90% inhibition of the TNF-α response as observed in our patients are, however, substantially higher than concentrations detected in patient plasma. Thus, although IL-10 is released spontaneously and the increased IL-10 levels after major surgery have been proposed to mediate the suppression of the proinflammatory cytokine response observed in these patients,[9] its functional significance in mediating the refractory state of leukocytes cultured in their intact environment after CPB seems to be limited.

The results of the present study would suggest that no single humoral factor or cytokine, but rather a complex activity resulting from various mediators as well as cell-associated factors, contribute to the refractory state of leukocytes to mount a proinflammatory cytokine response. From a clinical point of view, the modulatory role of adrenergic agonists is of particular significance. Although norepinephrine did not evoke a TNF-α or IL-10 response in the absence of secondary stimuli, its presence affected the cytokine response to bacterial lipopolysaccharide. Although pretreatment with β blockers did prevent the downregulatory effect of norepinephrine on the lipopolysaccharide-stimulated TNF-α response in mice,[28] preoperative chronic administration of β blockers in 5 of 10 of our patients did not affect the cytokine response to lipopolysaccharide *ex vivo*. Furthermore, the *ex vivo* response in whole blood obtained from our patients at T_2 might have been affected by coadministration of dopamine along with norepinephrine. Evidence suggests that D_2 receptors present on macrophages may interfere with lipopolysaccharide-stimulated TNF-α response. However, the effect of D_2 receptors remains controversial because both agonists as well as antagonists were shown to inhibit lipopolysaccharide-stimulated TNF-α response.[24] Although the present results as well as others confirm direct effects of catecholamines on immunocompetent cells, additional systemic effects, such as the direct action of dopamine on the anterior pituitary gland, are likely to contribute to the alterations in cytokine response *in vivo*.[29,30] These effects restricted to the intact organism may also explain the sometimes divergent results observed for plasma cytokines and lipopolysaccharide-stimulated cytokine response *ex vivo*.

In summary, CABG and initiation of CPB induces a spontaneous production of antiinflammatory cytokines such as IL-10 and IL-1ra, whereas only trace amounts of the prototypical proinflammatory cytokines TNF-α and IL-1β were measured. Although a desensitization of leukocytes cultured in their intact environment to release TNF-α, IL-1β, and IL-10 to *ex vivo* stimulation with Gram-negative lipopolysaccharide was observed, release of the antiinflammatory IL-1ra was substantially increased, reflecting a maintained ability to synthesize cytokines. Plasma of these patients contained factors suppressing the release of the prototypical proinflammatory cytokine TNF-α, while simultaneously increasing the antiinflammatory cytokine IL-10. This antiinflammatory plasma activity seems, in part, to result from high circulating cathecholamines and could contribute to the attenuation of the systemic inflammatory response to CPB. Although this plasma activity is significant, even complete removal of humoral factors failed to restore the cytokine response early after CPB. This would suggest additionally a contribution of cellular factors to the observed diminished cytokine response of leukocytes obtained after CPB and cultured in their intact environment.

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