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Interleukin 6 G–174C Polymorphism Influences Outcome Following Coronary Revascularization Surgery

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ABSTRACT

Background: Levels of the proinflammatory cytokine interleukin 6 (IL-6) increase after surgery. The functional polymorphism in the IL-6 promoter region, G–174C, is associated with an increased risk of coronary heart disease. We investigated the genetic predisposition in IL-6 response to coronary revascularization and studied the association between the G–174C polymorphism, IL-6 levels, and clinical outcomes of surgery.

Methods: DNA was obtained from 96 consecutive patients who underwent elective coronary revascularization. Patients were genotyped for the IL-6 G–174C polymorphism by means of sequence-specific primer–polymerase chain reaction analysis. IL-6 levels were measured with an enzyme-linked immunosorbent assay on serum samples taken 3 hours postoperatively. IL-6 levels and genotypes (CC, CG, and GG) were correlated with perioperative clinical data.

Results: The prevalences of the CC, CG, and GG IL-6 –174 genotypes were 8%, 54%, and 38%, respectively. Patients homozygous for the C allele had higher circulating levels of IL-6 postoperatively than the patients with the CG and GG genotypes (P = .09). Patients homozygous for the G allele had a significantly lower incidence of postoperative atrial fibrillation (P = .032) and a shorter hospital stay (P = .005). This result remained statistically significant following risk stratification. The severity of coronary artery disease and a higher number of bypass grafts were associated with a significant increase in IL-6 level postoperatively (P = .028, and P = .005, respectively). Higher levels of IL-6 were associated with increased blood loss postoperatively (P = .016)

Conclusions: The C allele is associated with higher postoperative IL-6 levels and a less favorable clinical outcome. The G–174C polymorphism is related to the outcome after coronary revascularization.

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INTRODUCTION

Cardiac surgery is accompanied by a systemic inflammatory reaction. The magnitude of the inflammatory response is an important outcome determinant. In its mildest form, this response has no clinical impact. However, at the severe end of the spectrum, the inflammatory response is characterized by increased capillary permeability, increased interstitial fluid, peripheral vasodilatation, pyrexia, myocardial edema, diffuse cerebral edema, and a bleeding diathesis [Rothenburger 2001].

The inflammatory cascade may be stimulated preoperatively by unstable cardiac syndromes (ischemia or infarction) and intraoperatively by general anesthesia, heparinization, surgical trauma, cardiopulmonary bypass (CPB), protamine sulfate administration, and reperfusion [Butler 1993, Misoph 1997, Wei 2001].

Inflammatory cells produce cytokines that mediate various aspects of inflammation and are capable of stimulating many cells, including smooth muscle cells, fibroblasts, and endothelial cells [Pannen 1995]. Many researchers have focused on the role of cytokines in the systemic inflammation caused by coronary revascularization with or without the use of CPB. One such cytokine is interleukin 6 (IL-6), which plays an important role in attracting polymorphonuclear cells and T-lymphocytes in the reperfused tissues and organs [Liebold 1999].

IL-6 is a pleiotropic cytokine involved in the regulation of the acute-phase response [Papanicolaou 1998]. It is synthesized by macrophages, T-lymphocytes, fibroblasts, and endothelial cells that have been stimulated by tumor necrosis factor α and IL-1β. Higher IL-6 levels may cause cellular damage, such as oxidative stress [Baeuerle 1996]. Coronary revascularization is a well-characterized inflammatory stimulus that causes a substantial rise in circulating IL-6 levels that peak 3 to 6 hours after surgery [Brull 2001].

A polymorphism within the IL-6 gene at position –174 is associated with increased IL-6 levels. We have investigated whether the concentration of IL-6 in the serum in response to coronary artery surgery depends on the presence of a certain allele in the functional polymorphism at position –174. We also studied the role of the G–174C polymorphism and postoperative IL-6 levels in determining the outcomes of patients who undergo coronary revascularization.

Table 1. Primer Sequences Used for the IL-6 Sequence-Specific Primer Genotyping Method*

Primer Position	Primer Sequence	Tm
IL-6 –174C (sense)	5′-CCCCTAGTTGTGTCTTGCC-3′	60°C
IL-6 –174G (sense)	5′-CCCCTAGTTGTGTCTTGCG-3′	
Generic (antisense)	5′-GCCTCAGAGACATCTCCAGTCC-3′	
Human growth hormone (sense)	5′-GCCTTCCCAACCATTCCCTTA-3′	60°C
Human growth hormone (antisense)	5′-TCAGGATTTCTGTTGTGTTTC-3′	

*Tm indicates melting temperature.

METHODS

Study Patients

A prospective study of 96 patients who underwent first-time elective coronary revascularization was conducted between March 2001 and March 2002 at Wythenshawe Hospital (Manchester, UK). Patients with unstable angina, recent myocardial infarction (<30 days), preexisting autoimmune diseases, or renal failure were excluded from the study. Patients on immunosuppressive therapy or anti-inflammatory agents were also excluded from the study. Antiplatelet therapy was routinely stopped 7 days prior to surgery. Patients gave informed consent for the collection and storage of blood, isolation of DNA, and evaluation of the cytokine gene polymorphism. Ethical approval was obtained from the South Manchester Medical Research Ethics Committee.

Surgical Procedure

Surgery in all cases was performed through a midline sternotomy by consultant-grade surgeons. CPB was instituted in 82 patients via cannulation of the right atrium and ascending aorta. Myocardial protection was provided by intermittent antegrade blood cardioplegia with or without retrograde cardioplegia. Normothermia or mild systemic hypothermia was used. In 14 patients, the coronary revascularization was performed without the use of CPB. Perioperative anticoagulation treatment with heparin was reversed after CPB with the use of protamine sulfate.

Sample Collection

Blood samples were collected at 2 stages, preoperatively in the outpatient clinic and 3 hours after surgery in the intensive care unit. Blood samples were mixed with EDTA for anticoagulation and stored at –80°C prior to further processing.

Sequence-Specific Primer–Polymerase Chain Reaction

DNA was obtained from EDTA-anticoagulated blood with the double-lysis method. The specific oligonucleotide primers used (Sigma-Genosys, Haverhill, UK) were based on the published IL-6 sequence (GenBank accession no. AF 005485). The primer sequences used for IL-6 genotyping are shown in Table 1.

DNA was amplified in a 10-μL polymerase chain reaction as previously described [Perrey 1999]. In summary, 25 to 100 ng DNA was mixed with a cytokine-specific (A or G) primer mix (final concentration, 5 μM each primer), 0.25 μM human growth hormone control primer mix, 200 μM of each deoxynucleoside triphosphate, and 1.25 U ThermoprimePlus DNA Polymerase, in 1× AS reaction buffer with 2.0 mM $MgCl_2$ (all ABgene, Epsom, UK). Cycling conditions included an initial denaturation step of 95°C for 1 minute followed by 10 cycles of 95°C for 15 seconds, 65°C for 50 seconds, and 72°C for 40 seconds and then 20 cycles of 95°C for 50 seconds, 59°C for 50 seconds, and 72°C for 50 seconds. Amplified products were identified under UV light following electrophoresis on a 2% agarose gel (Life Technologies, Rockville, MD, USA) containing 0.5 μg/mL ethidium bromide (Life Technologies).

Enzyme-Linked Immunosorbent Assay

Serum levels of IL-6 were measured by means of a solid-phase sandwich enzyme-linked immunosorbent assay. A monoclonal IL-6 antibody was coated onto the wells of a 96-well plate, and standards of known IL-6 concentration were then added along with control samples and patient serum. After washing, a biotinylated polyclonal IL-6 antibody was added, and the plate was incubated. After washing, streptavidin-coupled peroxidase was added. Following incubation, a wash step was performed to remove a chromogenic peroxidase. Finally, tetramethylbenzidine substrate was added; this substrate changed color in direct proportion to the amount of IL-6 present. The color (absorbance) was then measured with a Dynex Technologies MRX plate reader (Dynex Technologies, Chantilly, VA, USA) at a primary wavelength of 450 nm and a reference wavelength of 620 nm.

Postoperative Arrhythmia Evaluation

Routine continuous monitoring of heart rhythm for the first 48 to 72 hours postoperatively was performed in all patients. A 12-lead electrocardiographic evaluation was performed routinely on admission to the intensive care unit, day 1 and 4 postoperatively.

Statistical Analysis

The χ^2 statistic was used to analyze categorical data. The nonparametric Mann-Whitney *U* and Kruskal-Wallis tests were used to compare continuous data. Nonparametric testing was chosen because the data did not follow a normal distribution. Statistical significance was established at a *P* value <.05. IL-6 levels are given as median and range values. A χ^2 test was used to compare the observed numbers of each genotype with those expected for a population to establish if the genotypes were in Hardy-Weinberg equilibrium. A *P* value <.05 was considered statistically significant.

RESULTS

We recruited 96 patients who underwent elective coronary revascularization. The patients' baseline characteristics are shown in Table 2. All procedures were completed, and the patients left the operating theater in sinus rhythm without signs of ischemia. The median number of grafts per patient was 3 (range, 1-5), the mean (±SD) aortic cross-clamp time was 52 ± 31 minutes, and the mean CPB time was 80 ± 50 minutes. The postoperative data demonstrated no significant differ-

Table 2. Patients' Preoperative Characteristics*

	With CPB	Without CPB	*P*
Age, y	62 ± 9.5	62 ± 12.5	.8
Male sex, n	67 (82%)	10 (72%)	.4
Previous MI, n	38 (46%)	6 (43%)	1.0
Hypertension, n	40 (49%)	6 (43%)	.7
Diabetes mellitus, n	18 (22%)	5 (35%)	.3
COPD, n	10 (12%)	2 (14%)	.6
Stroke, n	4 (5%)	1 (7%)	.5
PTCA, n	12 (15%)	5 (35%)	.1
Smoking, n			.3
Ex-smoker	60 (73%)	12 (86%)	
Current	11 (13.5%)	1 (7 %)	
Never	11 (13.5%)	1 (7 %)	

*Age data are presented as the mean ± SD. CPB indicates cardiopulmonary bypass; MI, myocardial infarction; COPD, chronic obstructive pulmonary disease; PTCA, percutaneous transluminal coronary angioplasty.

ences between the patients who underwent operation with CPB and those who underwent operation without CPB, in terms of ventilation time, blood loss, intensive care unit stay, hospital stay, and the rate of complications. The 2 in-hospital deaths were attributed to myocardial infarction and to adult respiratory distress syndrome.

IL-6 levels were not detected preoperatively in a random sample of 20 patients. All patients had detectable levels 3 hours postoperatively. IL-6 levels were comparable between the patients who underwent operation with the use of CPB (617 pg/mL; range, 54-2143 pg/mL) and those who underwent operation without the use of CPB (405 pg/mL; range, 40-1000 pg/mL) (*P* = .162).

The prevalences of the CC, CG, and GG IL-6 –174 genotypes in the study were 8%, 54%, and 38%, respectively. Patients with the IL-6 CC or CG genotype had, as predicted, higher postoperative circulating IL-6 levels than the patients with the GG genotype (CC + CG, 632 pg/mL [range, 40-1364 pg/mL]; GG, 406 pg/mL [range, 50-2134 pg/mL]) (*P* = .09; Figure 1). Patients with the GG genotype experienced a lower incidence of atrial fibrillation (AF) (*P* = .032; odds ratio, 3.79; 95% confidence interval, 1.019-14.109) and a shorter hospital stay (*P* = .005). These results remained statistically significant following risk adjustment for age, sex, blood loss, ventilation time, duration of CPB, and aortic cross-clamp time (Figures 2 and 3, Tables 3 and 4).

The severity of coronary artery disease and a higher number of bypass grafts were associated with a significant increase in IL-6 level postoperatively (*P* = .028, and *P* = .005, respectively; Figure 4). Higher levels of IL-6 were found in patients who had increased blood loss postoperatively (*P* = .016), and this result remained statistically significant after adjusting for possible confounding factors, including age, sex, duration of CPB, and aortic cross-clamp time (Figure 5).

DISCUSSION

IL-6 is a proinflammatory and regulatory cytokine of the acute-phase response. It has been the focus of many studies

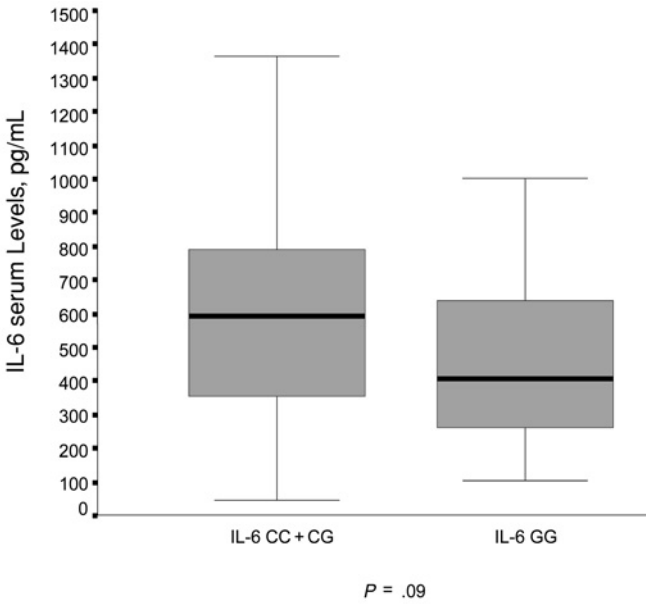

Figure 1. Postoperative interleukin 6 (IL-6) levels in the different genotype groups.

over the last few years. Increased IL-6 serum levels have been observed in various pathologic conditions, such as rheumatoid arthritis, multiple myeloma, cardiac myxoma, and liver cirrhosis. Blockade of the IL-6 receptor or inhibition of IL-6 by antibodies may become the future therapy for a certain disease or syndrome.

Systemic inflammation is a cause and a consequence of ischemic heart disease [Ridker 2000a, 2000b, Jenny 2002]. IL-6 plasma levels are a marker of the magnitude of this inflammatory response and of the severity of cardiovascular disease [Basso 2002]. Different stimuli, such as general anesthesia and surgical wounds, are thought to play a major role in the genesis of this response in addition to the role of CPB.

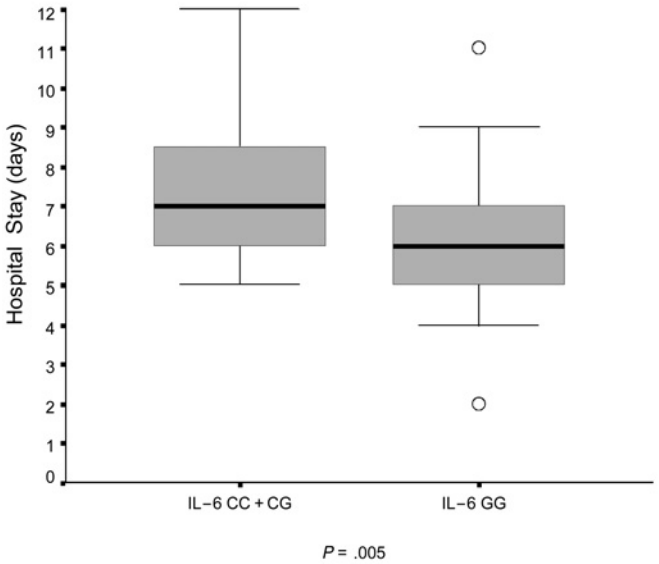

Figure 2. Hospital stay in relation to interleukin 6 (IL-6) genotype.

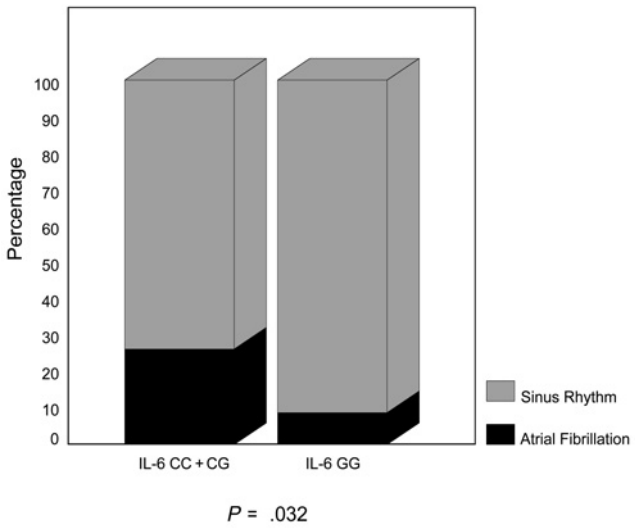

Figure 3. The incidence of postoperative atrial fibrillation in correlation to interleukin 6 (IL-6) genotype.

Higher levels of IL-6 play an important role in attracting polymorphonuclear cells and T-lymphocytes in the reperfused tissues and organs [Liebold 1999] and lead to cellular damage [Finkel 1993, Abe 1994, Baeuerle 1996]. Coronary revascularization is a well-characterized inflammatory stimulus that causes a substantial rise in circulating IL-6 levels, which peak 3 to 6 hours after surgery [Brull 2001].

This study has addressed aspects of the relationship between IL-6 genotype, IL-6 levels, and surgical outcome following coronary revascularization. We have determined that the IL-6 genotype distribution for the ischemic heart disease patients who underwent revascularization surgery is comparable with the distribution predicted for Hardy-Weinberg proportions.

In this cohort, the relationship between the different –174 IL-6 genotypes and the IL-6 response was examined. The –174 C allele was associated with increased postoperative IL-6 levels. Previous studies of patients with atherosclerotic disease who had undergone surgery found a significant association between the –174 C allele and increased levels of IL-6 [Brull 2001, Jones 2001]. Our results add weight to the existing evidence that the C rather than the G allele is associated with a greater IL-6 response.

Table 3. Preoperative Characteristics: Demographic Data by the Genotype Groups*

	CC + CG (n = 61)	GG (n = 35)	*P*
Age, y	62 ± 9.8	62 ± 10.1	.6
Male sex, n	47 (77%)	30 (85%)	.2
Previous MI, n	29 (47%)	15 (42%)	.4
Hypertension, n	29 (47%)	17 (48%)	.5
Diabetes mellitus, n	18 (29%)	5 (14%)	.07
COPD, n	7 (11%)	5 (14%)	.45
Stroke, n	4 (6%)	1 (3%)	.3

*Age data are presented as the mean ± SD. MI indicates myocardial infarction; COPD, chronic obstructive pulmonary disease.

Table 4. Postoperative Outcome: Demographic Data by the Genotype Groups*

	CC + CG (n = 61)	GG (n = 35)	*P*
Blood loss, mL	635 (120-3500)	620 (200-2160)	.87
Ventilation time, h	11 (2-86)	11 (0-23)	.32
Intensive care unit stay, h	24 (24-408)	24 (24-96)	.84
Hospital stay, d	7 (4-17)	6 (2-12)	.005
Atrial fibrillation, n	16 (26%)	3 (8%)	.03

*Data are presented as the median (range) where appropriate.

A previous investigation of the role of the IL-6 G–174C promoter polymorphism in healthy males found the –174 C allele to be associated with a higher systolic blood pressure and a generally increased cardiovascular risk [Humphries 2001]. In the WOSCOPS trial [Basso 2002], the –174 CC genotype was identified as a risk factor for cardiovascular disease. In our study, the C allele was predominant in patients with a history of hypertension and previous myocardial infarction. The C allele was also associated with a longer ventilation time, more blood loss, and a longer stay in the intensive care unit.

Hospital stay is influenced by many factors. In this study, patients homozygous for the G allele experienced a shorter hospital stay than patients with the CC or CG genotype. Using multivariate analysis to adjust for age, sex, left ventricular dysfunction, and postoperative blood loss, we identified the GG genotype as the only independent predictor of postoperative length of stay in the hospital. Gaudino et al [2003b] found in a similar study that the IL-6 G–174C polymorphism was a predictor of hospital length of stay after coronary artery bypass grafting. These workers reported that possessing the GG genotype predisposed a patient toward a higher production of IL-6 and found an association between the GG genotype and a prolonged hospital stay. Our findings agree with

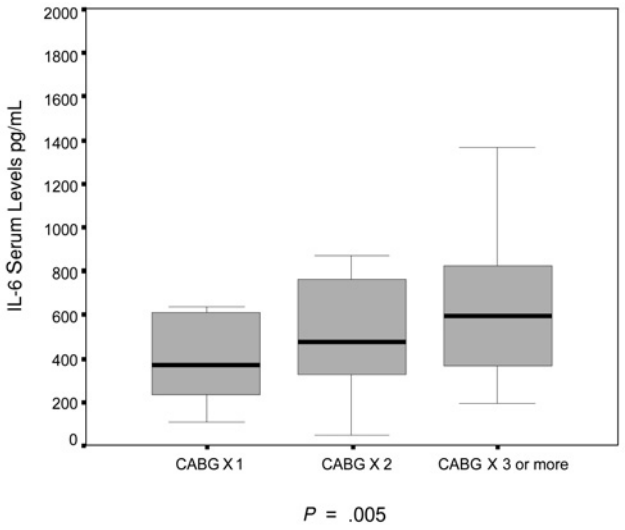

Figure 4. The association between the number of coronary artery bypass grafts (CABG) and serum levels of interleukin 6 (IL-6).

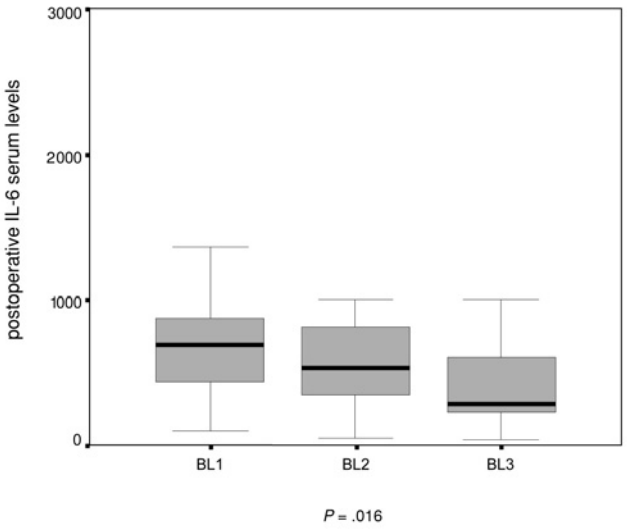

Figure 5. Postoperative blood loss and its relation to serum levels of interleukin 6 (IL-6). Blood loss (BL) groups are indicated as follows: BL1 >1000 mL; BL2 >500 mL and <1000 mL; and BL3 <500 mL.

the Gaudino et al findings that high levels of IL-6 are associated with a prolonged hospital stay. However, the uncertainty about the high-producer genotype at the –174 locus is still the subject of ongoing debate. A number of researchers have demonstrated that the CC genotype is the high producer of IL-6 [Brull 2001, Jones 2001, Basso 2002].

All patients were in sinus rhythm prior to surgery. However, 20% of the patients developed AF during the postoperative course. The median duration of AF was 24 hours (range, 4-192 hours). All patients were treated medically with amiodarone, digoxin, or sotalol. A multivariate analysis identified the G–174C polymorphism as an independent predictor of postoperative AF, with a significantly lower incidence of AF found in the patients with the GG genotype. These data support the theory of an inflammatory cause for postoperative AF and suggest that the incidence of AF is genetically influenced. Gaudino et al also found that the G–174C variation in the IL-6 gene promoter influenced the development of postoperative AF [Gaudino 2003a].

The degree of reperfusion will vary with the characteristics of the myocardial substrate (size of the vascular bed, state of the myocardium) and the number of target vessels; thus, it should not be surprising that the IL-6 response is determined by the extent of coronary artery disease and the number of bypass grafts. This point has not been raised in previous studies. In our study, patients who had triple-vessel disease and received 3 or more grafts had higher postoperative levels of IL-6. This finding remained statistically significant following risk stratification.

The severity of the inflammatory response is associated with bleeding diathesis, and one of the important findings of this study is a significant positive correlation between postoperative IL-6 level and the amount of blood loss postoperatively.

Is the reduction of postoperative IL-6 levels important? This question has frequently been asked [Morrow 2000, Basso 2002, Giomarelli 2003]. Previous reports showed that significantly increased levels of IL-6 in patients during and after coronary revascularization are associated with morbidity after cardiac surgery [Speziale 2000, Burzotta 2001]. We agree with previous studies that suggest that these patients could benefit from appropriately timed IL-6 inhibition.

CONCLUSIONS

There is a close association between the G–174C polymorphism and IL-6 serum levels. The postoperative course of recovery and the outcome of coronary revascularization are influenced by the G–174C polymorphism and postoperative IL-6 levels.

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REVIEW AND COMMENTARY

Editorial Board Member IE521 writes:

a) Off-pump and on-pump patients are mixed. As the inflammatory reaction is different if you operate with or without the heart-lung machine, it is not ideal to mix the 2 techniques. Even though there is no statistically significant difference, there is a trend that IL-6 levels were higher if the pump was used.

b) There is a contradiction that "all procedures were completed . . . without signs of ischemia." Later on, 2 patients who died of myocardial infarction are mentioned. This needs clarification.

c) Also, the claim is made that the genotypes are different with a P level of .09, which is not different by the criteria stated in the methods. This needs to be explained, because the other findings satisfy the $P < .05$ threshold.

Authors' Response by Dr. Mohamad N. Bittar:

a) Strong advocates of off-pump coronary artery bypass surgery frequently try to promote this alternative technique on the basis of reduced inflammatory response, but the evidence to date shows that both on- and off-pump techniques generate clinically significant inflammatory responses. The profile of the inflammatory cascade and the timing and magnitude of response are the focus of many ongoing studies.

b) This perceived anomaly was unintentional. The causes of death were myocardial infarction and adult respiratory distress syndrome.

On return from the theater, all patients had a normal 12-lead electrocardiographic evaluation. However, the 2 mortalities occurred in the postoperative period. The first patient developed postoperative bleeding, profound hypotension, and left ventricular dysfunction. Despite reexploration, increased pharmacologic support, and insertion of an intra-aortic balloon pump, the patient died on day 2. A recent myocardial infarction was demonstrated at post mortem. The second patient died following development of adult respiratory distress syndrome.

c) Many studies have shown that IL-6 CC is the high-producer genotype [Brull 2001, Jones 2001, Basso 2002]. However, in our study the number of patients carrying the CC IL-6 genotype was small—only 8 patients—and this fact of course influenced the statistical difference between the 3 genotype groups.