EFFECTS OF SELECTIN-SIALYL LEWIS^x BLOCKADE ON MESENTERIC MICROVASCULAR PERMEABILITY ASSOCIATED WITH CARDIOPULMONARY BYPASS

Charles S. Cox, Jr, MD
Steven J. Allen, MD
Henning Sauer, MD
John Frederick, BS

Objectives: Cardiopulmonary bypass is associated with an inflammatory response that is associated with a neutrophil-mediated microvascular barrier injury. We studied the effects of blocking neutrophil-endothelial tethering on microvascular permeability and edema formation during cardiopulmonary bypass. Using a selectin antagonist that prevents interactions with their ligands, we hypothesized that there would be less neutrophil infiltration into the tissue and a reduction in microvascular permeability and edema formation.

Methods: A canine mesenteric lymphatic fistula was created to measure Starling forces and to determine microvascular permeability. Normothermic, atrial-femoral cardiopulmonary bypass was initiated (70-90 mL · kg⁻¹ · min⁻¹). Intestinal tissue water was determined with microgravimetry. Ileal tissue myeloperoxidase was measured as an index of neutrophil tissue infiltration. One experimental group received the selectin antagonist TBC 1269 before the initiation of bypass, and the control group received saline solution.

Results: There was a modest increase in microvascular permeability in both groups, as evidenced by significantly increased transvascular protein clearance and a trend toward a decrease in reflection coefficient. There were no differences in the experimental group compared with the control group. Ileal tissue myeloperoxidase levels were lower in the experimental group than in the control group.

Conclusions: The selectin antagonist TBC 1269 reduces neutrophil infiltration into the ileum without altering ileal microvascular permeability or edema associated with cardiopulmonary bypass. (*J Thorac Cardiovasc Surg* 2000;119:1255-61)

Cardiopulmonary bypass (CPB) is associated with a generalized inflammatory response with splanchnic and pulmonary edema formation that is associated with microvascular barrier injury.¹⁻⁴ Recent studies suggest that, with the initiation of CPB, interactions between polymorphonuclear neutrophils (PMNs) and the endothelium are the early events in the initiation of

PMN-mediated microvascular barrier disruption, edema formation, and organ injury.⁴⁻¹⁰ Transient reduction of the post-CPB inflammatory response is potentially beneficial, minimizing post-CPB organ dysfunction.^{10,11}

The selectin family of adhesion molecules is composed of similar calcium-dependent carbohydrate-binding proteins, E-, P-, and L-selectin.¹²⁻¹⁴ The selectins are expressed on the endothelium after activation (E- and P-selectins) and on the platelets (P-selectin); L-selectin is constitutively expressed on PMNs. The selectins mediate the initial tethering and rolling of PMNs along the vascular endothelium binding to sialyl Lewis^x ligands.¹⁵ Interruption of the mechanism of initial tethering and rolling has prevented PMN adhesion on the microvasculature in vitro, and the use of selectin antagonists has reduced tissue damage in direct lung injury and ischemia/reperfusion models.¹⁶⁻¹⁸

Smith and colleagues² established the canine mesenteric lymphatic fistula as an ideal model by which to study microvascular barrier injury associated with CPB.

From the Department of Surgery, Division of Pediatric Surgery, and the Department of Anesthesiology, and the Center for Lymphatic and Microvascular Studies at the University of Texas-Houston, Medical School, Houston, Tex.

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Address for reprints: Charles S. Cox, Jr, MD, 6431 Fannin, MSB 5.246, Houston, TX 77030 (E-mail: ccox@utsurg.med.uth.tmc.edu).

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We sought to study the effects of blocking the PMN-endothelial tethering on microvascular permeability and edema formation associated with CPB. By minimizing PMN adherence, using a selectin antagonist that binds to P-, E-, and L-selectins and preventing interactions with their ligands, we hypothesized there would be less PMN infiltration into the tissue and a reduction in microvascular permeability to protein and edema formation.^{14,15} Moreover, we have chosen to study the mesenteric microvasculature because of a growing body of evidence that shows the posts ischemic gut serves as a priming bed for circulating PMNs that provoke multiorgan failure.¹⁹ Thus, in theory, a reduction of PMN adherence in the gut could potentially alter the post-CPB inflammatory response.

Methods

Animal preparation. All procedures were approved by the University of Texas Animal Welfare Committee and were consistent with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health. Conditioned mongrel dogs of either sex (30.3 ± 0.7 kg [control animals]; 31.6 ± 0.5 kg [experimental animals]) were anesthetized with intravenous administration of thiopental sodium, 25 mg/kg (Abbott Laboratories, North Chicago, Ill), intubated, and mechanically ventilated at a tidal volume of 15 mL/kg body weight, positive end-expiratory pressure of 5 cm H₂O, respiratory rate of 10 breaths/min with 100% oxygen with the use of a volume-cycled respirator (model 900C; Siemens-Elema AB, Solna, Sweden). Anesthesia was maintained with intravenous infusion of 1% thiopental sodium in Ringer solution.

Fluid-filled catheters were placed into the left femoral artery and vein and connected to pressure transducers for mean arterial pressure monitoring, arterial blood sampling, and fluid administration, respectively. A 7F thermodilution catheter (Swan-Ganz; Baxter-Edwards Critical Care, Irvine, Calif) was inserted into the pulmonary artery through the left jugular vein for central venous pressure, pulmonary artery pressure, and cardiac output determination. The right femoral artery was exposed for subsequent CPB cannulation. The pressure monitoring catheters were connected to pressure transducers (Isotec; Healthdyne Cardiovascular Inc, Irvine, Calif), and data were recorded on an 8-channel chart recorder (Grass Instrument Co, Quincy, Mass). We determined cardiac output in duplicate with the use of a cardiac output computer connected to a Swan-Ganz thermodilution pulmonary artery catheter. A 10 mL dose of ice cold Ringer solution was used as the injectate. A urinary drainage catheter was placed in the bladder at the time of laparotomy, and urine output was measured every 30 minutes. Arterial blood gas measurements were made with the use of an automated blood gas analyzer (IL-BGE; Instrumentation Laboratories, Lexington, Mass).

The experimental preparation is a modification of the Kubes/Granger model.²⁰ To obtain the measurements of lym-

phatic flow and lymph protein concentration (C_L), a midline laparotomy was performed, and a mesenteric lymphatic was cannulated with 0.025-inch inner diameter tubing (Silastic; Dow Corning, Midland, Mich). This cannula was attached to a micropipette, which was fixed in a horizontal position, level with the lymphatic vessel, to prevent hydrostatic pressure from affecting lymphatic flow rates. Lymph flow (Q_L) was measured by timing the lymph fluid meniscus movement in the micropipette with a stopwatch. A variable pressure occluder was placed circumferentially around the superior mesenteric vein and was used to elevate mesenteric venous pressure to achieve the interstitial protein "washdown." A distal mesenteric venous tributary was then cannulated with 0.025-inch inner diameter tubing and attached to a pressure transducer that was interfaced with a Grass physiologic recorder (Grass Model 7D Polygraph; Grass Instrument Co, Quincy, Mass). This was used to estimate capillary pressure according to the method of Granger and colleagues.²¹ Capillary pressure was measured by the venous occlusion technique, which results in a rapid rise in the venous pressure to capillary pressure, after which the increase in pressure is more gradual. This was graphically measured by increasing the chart speed on the chart recorder; the inflection point represents capillary pressure. C_L and plasma protein concentration (C_P) were determined with a refractometer (American Optical, Buffalo, NY). Colloid oncotic pressure was calculated from protein concentrations with the equation of Navar and Navar.²² The reflection coefficient is a surface area independent coefficient that represents the ability of the membrane to selectively limit the passage of macromolecules. A reflection coefficient of 1 represents an impermeable membrane, and a reflection coefficient of 0 represents no barrier function. Transvascular protein clearance was calculated as $Q_L \times C_L/C_P$ and used in conjunction with reflection coefficient as a marker of microvascular permeability to protein. The description of these variables and the rationale for their use is described further in the appendix.

Intestinal water content determination. For intestinal water content determination, we modified a gravimetric technique, originally developed for cerebral tissue.²³ Intestinal water content was determined by specific density measurement of small ileal tissue samples with a linear density gradient. If the specific density of an ileal tissue sample is known, the percent gram water per gram tissue can be calculated.

Experimental drug. TBC 1269 is a selectin antagonist that acts by binding to the selectin adhesion molecules, preventing interactions with their cognate ligands.^{14,15} It is a dimeric synthetic mannosylated-biphenyl class compound. It has a half-life of approximately 2 hours in rats, and doses of 25 mg/kg have minimized hepatic ischemia/reperfusion injury by reducing PMN infiltration. The compound operates by inhibiting PMN recruitment to the site of inflammation by blocking the initial rolling phase of PMN recruitment. The drug was given 10 minutes before the initiation of CPB in a dose of 25 mg/kg, and a continuous infusion of 5 mg/kg was subsequently administered for the duration of the experimental period. The control group received the same volume of

0.9% saline solution vehicle. This dose was chosen because of its efficacy in preventing myocardial ischemia/reperfusion injury. We have performed studies that demonstrate a peak serum level of $171 \pm 10 \mu\text{g/mL}$ in 5 dogs. These studies confirmed adequate drug levels with this dosing regimen.

CPB techniques. After preparation, heparin (300 IU/kg) was given intravenously for systemic anticoagulation. Additional doses of heparin, 75 IU/kg, were administered every 60 minutes throughout the experiment. We introduced a 16F arterial perfusion cannula into the prepared right femoral artery. A 2-stage (34F/38F) venous cannula (model TAC2; DLP Inc, Grand Rapids, Mich) was inserted into the right atrium and inferior vena cava through a median sternotomy. No cardioplegia, left ventricular vent, or aortic crossclamp was used. We primed the extracorporeal circuit and the membrane oxygenator (Cobe HVRF-3700; Cobe Cardiovascular, Arvada, Colo) with 800 mL of Ringer solution and 1000 IU of heparin. A rectal temperature probe was placed, and the body temperature was maintained at 37°C during extracorporeal circulation with the use of a heat exchanger (Sarns heater-cooler; Sarns/3M, Ann Arbor, Mich). We maintained CPB flow between 70 and 90 $\text{mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ and mean systemic perfusion pressure between 60 and 80 mm Hg. Lactated Ringer solution was added to the reservoir to maintain a constant level of 100 mL.

Experimental protocol. Animals were randomly assigned to either the control or experimental groups. Experimental animals ($n = 6$ animals) received 25 mg/kg pretreatment with TBC 1269 followed by a constant infusion of 5 mg/kg thereafter. Control animals ($n = 6$ animals) received the same volume of only vehicle. After instrumentation, we recorded baseline measurements of cardiac output, mean arterial pressure, pulmonary artery pressure, and right atrial pressure (central venous pressure). Lymph flow rate, lymph and plasma protein determinations, and capillary pressure measurements were made. Once baseline measurements were completed, mesenteric venous pressure was elevated in 1 step to 30 ± 1 mm Hg in control animals and to 30 ± 1 mm Hg in experimental animals to obtain a minimum C_L/C_P . Once a steady state was achieved as evidenced by 2 similar C_L measurements 15 minutes apart, CPB was initiated. Previous experiments of 3 to 4 hours failed to demonstrate further "washdown" with reduction in C_L . All other variables were measured at 30-minute intervals for 2 hours of CPB. At the conclusion of the CPB period, the CPB flow was reduced, and the dog was weaned and separated from CPB. Ileal tissue samples were taken at baseline and steady-state every 30 minutes for gravimetric tissue water determinations.

Assay of tissue myeloperoxidase. Myeloperoxidase tissue levels were analyzed as an index of neutrophil infiltration in the tissue. The presence of myeloperoxidase, an enzyme specific for neutrophils, was determined in ileal tissue by the method described by Bradley and colleagues²⁴ and modified by Mullane and colleagues.²⁵ To determine myeloperoxidase activity, we subjected ileal tissue samples to a spectrophotometric assay. In brief, samples from the ileum were snap frozen in liquid nitrogen and stored at -70°C until processed.

Fig 1. Q_L increases with the increase in mesenteric venous pressure. There is no statistical difference in Q_L between groups after the initiation of CPB. *Statistical significance with respect to wash down in control group ($P = .02$ at 30 minutes of CPB compared with wash down).

Samples were pulverized, then homogenized (10% wt/vol) with a Polytron homogenizer (model PT2000; Brinkman Instruments, Inc, Westbury, NY) in 50 mmol phosphate buffer solution (pH = 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide (Sigma Chemical Co, St Louis, Mo) for 90 seconds. Homogenates were centrifuged at 12,500g for 45 minutes (4°C). The supernatant was added to 0.166 mg/mL *o*-dianisidine dihydrochloride (Sigma) and 0.0005% hydrogen peroxide in 50 nmol/L phosphate buffer (pH = 6.0). The change in absorbance was measured spectrophotometrically at 460 nm (Beckman DU 640; Beckman Instruments, Inc, Fullerton, Calif) every 5 seconds for 2 minutes. Results were expressed as units of myeloperoxidase per 100 mg tissue (wet weight), where 1 unit of myeloperoxidase activity was defined as the quantity of enzyme degrading 1 $\mu\text{mol/L}$ peroxide per minute at 25°C . The average for the duplicates was used for analysis.

Statistical analysis. All data presented are mean $\pm$ SE. We examined the time courses of each measured parameter using analysis of variance (ANOVA) for repeated measures and the Fisher least significant difference test. Time point comparisons were made using the unpaired Student *t* test.

Results

Lymph flow is shown in Fig 1. There is no significant difference between control and experimental groups. Although control results demonstrate a significant increase in Q_L ($P = .03$ at 30 minutes of CPB; ANOVA and the Fisher least significant difference test) after CPB was initiated, the absolute flow rates are not different between groups. As demonstrated graphically, this is due to the higher Q_L at washdown in experimental animals compared with control animals. As shown

Fig 2. Reflection coefficient (*Sigma*) decreases with the initiation of CPB, indicating a modest increase in the microvascular permeability to protein. There is no statistically significant difference between groups.

in Fig 2, there is no statistically significant difference in the reflection coefficient between control and experimental animals. Compared with washdown, reflection coefficient is lower with the initiation of CPB in both groups, but this does not reach statistical significance ($P = .07$; ANOVA and the Fisher least significant difference test). Transvascular protein clearance (Fig 3) significantly increases in both control and experimental animals after CPB is initiated, but there are no significant differences between groups. Ileal tissue water (Fig 4) increases in both control and experimental groups after the initiation of CPB. At the conclusion of CPB, ileal tissue water is almost identical in experimental and control animals. Ileal tissue myeloperoxidase is statistically lower ($P < .05$) in experimental (0.36 ± 0.04 U/100 mg tissue) compared with control (0.97 ± 0.09 U/100 mg tissue) animals 30 minutes after the conclusion of CPB.

Discussion

The purpose of this study was to determine whether the blockade of the initial steps of PMN adherence could affect the intestinal microvascular permeability and edema formation associated with the initiation of CPB. Our study shows that selectin blockade before CPB reduces PMN accumulation in the ileum but does not significantly affect microvascular permeability to protein or ileal edema formation.

Although we found that selectin blockade decreased CPB-induced ileal tissue PMN infiltration, selectin blockade did not affect protein permeability. Although we did not demonstrate a reduction in permeability to

Fig 3. Transvascular protein clearance increases with the initiation of CPB, indicating an increase in microvascular permeability to protein. Although transvascular protein clearance increases from wash down ($P = .01$, comparing wash down with 30-minute CPB), there is no statistical difference between groups. *Statistical significance within groups, comparing individual time points with wash down.

total protein (decreases in transvascular protein clearance), the possibility remains that a protein fractionation technique could detect more subtle microvascular permeability changes. The reduction in leukocyte sequestration without alterations in microvascular permeability initially seems contradictory but is quite similar to previous data reported from lung tissue in a canine model of CPB. Dreyer and colleagues⁴ demonstrated a significant reduction in pulmonary leukocyte sequestration with the use of a monoclonal antibody against CD18 (R15.7). However, similar to our ileal data, there was no significant reduction in lung tissue water. These data are consistent with clinical studies that show an increase in pulmonary leukocyte sequestration, with only modest deterioration in lung function with CPB.

An alternative explanation of our data is that the initial events of increased microvascular permeability are not PMN mediated; thus altering PMN adherence would not be expected to decrease the permeability changes. Indeed, numerous other events associated with blood-foreign surface interactions can increase microvascular permeability. These include mast cell degranulation, histamine release, and complement activation (independent of PMN-mediated injury).

If the PMN is the putative mediator of the tissue injury associated with CPB, then these data seem somewhat contradictory. Specifically, there are fewer PMNs within the tissue in the experimental group, but there is no demonstrable decrease in tissue injury. We

hypothesize that our data represent the initial up-regulation of adhesion molecules in the microcirculation and on the PMNs and that this results in the accumulation of PMNs within the tissue. Of critical importance, the only inflammatory stimulus in our model is the initiation of CPB. In that regard, this mimics the clinical scenario of routine procedures requiring CPB. In contrast, models that use successive inflammatory stimuli (eg, shock-endotoxin, burn-endotoxin) have shown exaggerated tissue injury when compared with singular inflammatory stimuli. Moreover, as described by numerous authors, these PMNs are probably “primed” by the initial inflammatory stimulus to increase oxygen-derived free radicals and proteases, which are subsequently released with further stimulation.²⁷ With complicated or prolonged CPB, gut epithelial barrier dysfunction has been demonstrated along with indirect evidence of bacterial/endotoxin translocation.²⁸ It has been hypothesized that this is responsible for PMN activation associated with CPB. However, without further stimulation, there is no substantial increase in PMN-mediated tissue injury. We have demonstrated this phenomenon previously using an ovine model of direct lung injury followed by extracorporeal membrane oxygenation as cardiopulmonary support. We showed an augmented pulmonary leukocyte sequestration and exacerbated lung injury after the initiation of extracorporeal membrane oxygenation.²⁷ These data support the hypothesis that a second inflammatory stimulus to a “primed” PMN results in an augmented inflammatory response and organ injury.

The systemic inflammatory response associated with CPB is related, in part, to PMN activation. PMN activation associated with CPB is due to several factors: blood–foreign surface interactions, global ischemia-reperfusion, and altered blood flow patterns. The recognition of the critical role of the PMN in the pathophysiologic response to CPB has stimulated efforts to minimize the PMN-mediated tissue injury associated with CPB. PMNs adhere to the activated venular endothelium in response to inflammatory stimuli through specific PMN-endothelial interactions.¹⁵ Once PMNs are recruited into tissue, the activated PMN releases oxygen-derived free radicals and enzymes, such as elastase. Specific efforts have focused on reducing PMN adherence to the activated venular endothelium to minimize the pulmonary microvascular injury and subsequent pulmonary edema formation associated with CPB. In the past, clinicians empirically used methylprednisolone to reduce the inflammatory symptoms seen after CPB. Clinical studies demonstrated that methylprednisolone decreased the systemic inflammatory

Fig 4. Tissue water (ileum) as measured by microgravimetrics. There is no statistically significant difference between groups.

response not only by reducing tumor necrosis factor α release but also by reducing CD11b up-regulation on the PMN surface.¹¹ More recently, specific CD11b/CD18 adhesion monoclonal antibodies and agents to prevent CD11b/CD18 adhesion molecule up-regulation have been used experimentally to reduce the PMN-mediated lung injury associated with CPB.⁴ Some concern has been raised about the potential use of these types of agents in clinical practice. Immunosuppression and impaired wound healing associated with corticosteroid use have prevented a widespread acceptance of this approach to reduce the systemic inflammatory response associated with CPB. Although monoclonal antibodies are more specific in their reduction of the inflammatory response, they expose patients to foreign proteins and possible serum sickness. For these reasons, there has been interest in a selective approach to reducing PMN-mediated tissue injury that does not require a global down-regulation of the inflammatory response or the exposure to foreign proteins.

The selectins mediate the initial rolling and tethering of PMNs to the activated venular endothelium before firm adhesion and transendothelial migration occur. There is some overlap in their function with ligands on both the PMN and endothelium such that the blockade of a single selectin does not totally prevent the initial PMN rolling. Likewise, most global inflammatory stimuli that result in increased circulating proinflammatory cytokines up-regulate all 3 selectins. In vivo approaches to block selectin-dependent inflammatory responses have been developed. These techniques consist of infusing selectin-reactive sulfated oligosaccharides that functionally bind to the up-regulated

selectins on the vascular endothelium and PMN.¹² These approaches have proved effective in reducing PMN-mediated lung injury because of smoke inhalation and cobra venom factor.^{12,16,18} These molecules are potentially useful because of their short half-life, focused effects, and lack of foreign protein exposure. These effects are the rationale for the use of a selectin antagonist with CPB.

In summary, we studied the effects of a selectin antagonist, TBC 1269, on microvascular permeability and leukocyte sequestration associated with CPB. CPB increased mesenteric microvascular permeability as measured by increased transvascular protein clearance, but this was not reduced by TBC 1269. Post-CPB ileal leukocyte sequestration was decreased by TBC 1269. These data must be considered when anti-inflammatory strategies for CPB are being developed.

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Appendix

Starling equation. Transcapillary fluid filtration rate (J_v) is determined by the Starling forces as calculated from the equation:

$$Q_L = J_v = K_f([P_C - P_i] - \sigma[\pi_c - \pi_i]) \quad (1)$$

where Q_L = lymph flow, K_f = capillary filtration coefficient, P_C = capillary pressure, P_i = interstitial pressure, σ = reflection coefficient, π_c = plasma oncotic pressure, and π_i = interstitial oncotic pressure.

Reflection coefficient, σ , was calculated by simultaneously measuring C_L and C_p , respectively, after interstitial protein “washdown” was induced by elevated mesenteric venous pressure. When C_L/C_p reached filtration independence:

$$\sigma = 1 - C_L/C_p \quad (2)$$

The Navar equation is used for the conversion of protein concentrations into oncotic pressures. It is shown as equation (3), where C is the protein concentration and π is oncotic pressure.

$$\pi = 1.4C + 0.22C^2 + 0.005C^3 \quad (3)$$

Intestinal tissue water measurements. For preparation of the density gradient, we used 2 mixtures of kerosene (specific gravity, 0.773) and bromobenzene (specific gravity, 1.484). The specific gravities of these mixtures were adjusted to 0.983 and 1.073, respectively, and the density column was generated with a gradient former (model GC-0971; Bethesda Research Laboratories, Bethesda, Md). We then calibrated

the gradient with various K_2SO_4 solutions with known specific gravities of 1.086, 1.079, 1.072, 1.067, 1.044, 1.035, 1.031, and 1.027. We carefully placed 10- μ L drops of the K_2SO_4 solutions in the gradient and recorded the equilibration depth after 1 minute. We then plotted equilibration depth versus specific gravity and confirmed the linearity of the gradient by linear least square regression analysis. The mean correlation coefficient was 0.987 ± 0.0003 ($n = 12$).

To determine the specific gravity of ileum, we sharply excised full-thickness ileal tissue samples (6-8 mm³). These samples were gently placed into the density gradient, and the equilibration depth was recorded after 1 minute. The gram water per gram ileum or ileal water content (IWC, %) was calculated with the following equation:

$$IWC = (1 - [(SG_{ileal} - 1)/(1 - 1/SG_{dry}) \cdot SG_{ileal}]) \cdot 100\% \quad (4)$$

where SG_{ileal} and SG_{dry} are the specific gravities of the ileal tissue sample and of dry ileum, respectively. At the end of the experiment, we killed the dog with intravenous thiopental sodium overdose and saturated potassium chloride. The bowel was then weighed, after which a sample was stored in an oven and dried to a constant weight at 60°C. We calculated SG_{dry} with the following equation:

$$SG_{dry} = 1/(1 - [SG_{ileal} - 1] \cdot W/[D \cdot SG_{ileal}]) \quad (5)$$

where W and D are wet and dry weights of ileum, respectively. We assumed that SG_{dry} did not change over the experimental period. All ileal tissue water content measurements were performed in triplicate.

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