ENHANCED ISOLATED LUNG FUNCTION AFTER ISCHEMIA WITH ANTI-INTERCELLULAR ADHESION MOLECULE ANTIBODY

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The binding of leukocytes to intercellular adhesion molecules expressed on endothelial surfaces during ischemia and subsequent reperfusion initiates leukocyte-mediated reperfusion injury. Interruption of this leukocyte-endothelium interaction may therefore prevent reperfusion injury. In an isolated, ventilated, blood-perfused rabbit lung preparation, we studied the effect of a monoclonal anti-intercellular adhesion molecule antibody on lung function during reperfusion. Lungs were harvested with 50 ml/kg cold Euro-Collins flush and 30 μ g prostaglandin E₁ before storage for 18 hours at 4° C. Experimental groups received low-dose (100 μ g) or high-dose (200 μ g) anti-intercellular adhesion molecule antibody added to the pulmonary flush at harvest and to the initial perfusate. Eighteen-hour control preparations were preserved for 18 hours and received saline solution vehicle. Immediate control preparations were harvested and immediately reperfused. The oxygen tension in the recirculated pulmonary venous effluent was measured after 30 minutes of reperfusion. Histologic specimens were graded by blinded observers for degree of leukocyte infiltration (0, normal, to 4, severe infiltration). The mean oxygen tensions ($\pm$ standard error of the mean) were 138.29 ± 6.23 , 58.86 ± 9.14 , 86.87 ± 11.32 , and 139.33 ± 16.15 mm Hg in immediate control preparations, 18-hour control preparations, low-dose antibody group, and high-dose antibody group, respectively ($p = 0.0001$). The leukocyte grades (mean $\pm$ standard error of the mean) were 1.5 ± 0.723 , 3.0 ± 0.955 , 1.9 ± 0.899 , and 1.2 ± 0.834 , respectively ($p = 0.0002$). We conclude that anti-intercellular adhesion molecule antibody added to the pulmonary flush and initial perfusate results in a dose-dependent enhancement of the reperfused lung's ability to oxygenate blood, possibly as a result of decreased leukocyte sequestration. (*J Thorac Cardiovasc Surg* 1996;111:941-7)

The sequestration of activated leukocytes within pulmonary allografts has been established as a significant cause of pulmonary reperfusion injury

after lung transplantation.¹ Correspondingly, leukocyte depletion of the recipient by means of filtration methods has been shown to attenuate reperfusion injury in experimental models.^{2,3} Practical difficulties with effective leukocyte filtration and concerns about leukopenia in already immunocompromised patients, however, have prevented the routine clinical application of leukocyte depletion in lung transplant centers.^{1,4} Newer methods of limiting leukocyte-mediated reperfusion injury that exploit our growing understanding of the molecular mechanisms of the leukocyte-endothelium interaction during reperfusion have been proposed. For example, the leukocyte membrane glycoprotein CD18 binds to the endothelium-based intercellular adhesion molecule-1 (ICAM-1), initiating leukocyte demargination early in reperfusion.^{5,6} Monoclonal antibodies have been developed that bind to the ICAM-1 receptor and prevent this interaction.⁷⁻⁹

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We hypothesized that the administration of an anti-ICAM-1 monoclonal antibody to pulmonary allografts before reperfusion would decrease leukocyte sequestration and thus attenuate reperfusion injury.

Materials and methods

We developed a model of isolated, ventilated, and blood-perfused rabbit lung function, which was employed in this protocol. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institute of Health (NIH Publication No. 86-23, revised 1985).

Harvest procedure. Forty New Zealand White rabbits weighing 3.0 to 3.5 kg were used as lung donors. Each animal was anesthetized with intramuscular ketamine (50 mg/kg) and xylazine (5 mg/kg). A tracheostomy was performed and mechanical ventilation was instituted with a constant-pressure ventilator (RSP1002; Kent Scientific Corporation, Litchfield, Conn.) on room air and a rate of 20 breaths/min. A median sternotomy was then performed and thymectomy was carried out. The two superior venae cavae and one inferior vena cava were loosely encircled with ligatures, and the pericardium was opened. Both the pulmonary artery (PA) and the aorta were dissected free and similarly encircled. A purse-string suture was then placed in the free wall of the right ventricle, and the rabbit was heparinized (500 U/kg). After injection of 30 μ g prostaglandin E_1 into the PA, the venae cavae were interrupted and the onset of ischemia was noted. The PA was then cannulated through the right ventricular purse-string, and both the right ventricular and PA ligatures were tied around the cannula. After venting of the left ventricle and ligation of the aorta, 50 mg/kg of saline solution at 4° C was infused into the PA from a height of 30 cm. Topical cooling was achieved with cold saline solution slush. During PA flush, the left atrium was cannulated through a left ventricular purse-string. After completion of the PA flush, the inflow and outflow cannulas were clamped. Care was taken to leave the pleurae intact until the completion of the flush to avoid parenchymal injury. The tracheostomy tube was then clamped at end-inspiration and the heart-lung block was excised. The lungs were stored at 4° C in saline solution.

Reperfusion procedure. Forty-five heparinized and anesthetized New Zealand White rabbits served as venous blood donors. Saline solution was added to each aliquot of blood to achieve a hematocrit of 28% to 30%. During reperfusion, the lungs were suspended by a force transducer in a warmed, humidified chamber. Room air ventilation at 20 breaths/min was reestablished with a constant-pressure ventilator. The initial maximum inspiratory pressure was set to achieve a tidal volume of 10 ml/kg of donor rabbit weight and was subsequently held constant. Any lung with evidence of air leakage was excluded. The inflow and outflow cannulas were then connected to a venous blood-filled perfusion circuit, with care taken to

avoid the introduction of air bubbles. The circuit (Kent Scientific Corporation) was designed to recirculate 200 ml warmed blood with a roller pump (7521-40; Cole-Parmer Instrument Company, Niles, Ill.) and a blood filter (2C7600; Baxter Healthcare Corp., Deerfield, Ill.) at a rate of 40 ml/min. Continuous recording of PA pressure, pulmonary venous pressure, lung weight, airway flow, and airway pressure was carried out with a dynamic data-acquisition program (Workbench PC; Strawberry Tree, Inc., Sunnydale, Calif.) run on a personal computer (470A; Compaq Prolinea, Houston, Texas). This program allowed immediate calculation of tidal volume, pulmonary vascular resistance (Pulmonary vascular resistance = $80 \times [\text{PA pressure} - \text{Pulmonary venous pressure}] / \text{Flow}$), and dynamic airway compliance (Dynamic airway compliance = $\text{Tidal volume} / \text{Airway pressure}$). The pulmonary venous pressure was maintained between 5 and 8 mm Hg by changing the height of an outflow reservoir in the circuit. Pulmonary venous blood samples were collected for blood gas analysis (Corning 178 pH/Blood Gas Analyzer; Corning Inc., Corning, N.Y.) at 1, 10, 20, and 30 minutes after the start of reperfusion. Oxygen contact with exposed blood surfaces inside reservoir containers was minimized by the continuous passive infusion of 100% nitrogen.

Experimental protocol. Ten double-lung blocks were perfused immediately after harvest, serving as immediate control (IC) preparations. For our ischemic groups, we chose a storage interval of 18 hours, after a series of preliminary experiments in which 24 hours of cold storage was associated with a rapid hemorrhagic demise of the lung before completion of 30 minutes of reperfusion. By contrast, 6- and 12-hour ischemic intervals produced little measurable lung injury. Ten lungs were stored for 18 hours at 4° C and then reperfused, serving as 18-hour control preparations. Two treatment groups were similarly harvested and stored at 4° C for 18 hours but received low- or high-dose antibody therapy, respectively. Ten lungs in the low-dose group received 100 μ g purified anti-ICAM-1 monoclonal antibody (RR1; Boehringer Ingelheim Corp., Ridgefield, Conn.) administered intravascularly with the last 20 ml of Euro-Collins flush during harvest and a second 100 μ g dose intravascularly in the initial reperfusion. Ten lungs in the high-dose group received 200 μ g anti-ICAM-1 antibody administered in a similar manner, both during harvest and again before reperfusion. All lungs were reperfused for 30 minutes.

At the completion of the study, samples of the left lower lobe were weighed and dried for calculation of wet-to-dry weight ratios. Histologic specimens were taken from the right lower lobe and placed in formalin. The histologic specimens were graded for the degree of leukocyte sequestration by three blinded observers according to a 5-point scale modified from the method of Yousem and coworkers.¹⁰ The grading classification is outlined in Table I.

Statistical analysis. Statistical analysis was performed for the four groups by means of analysis of variance on the software STATISTICA (StatSoft, Inc., Tulsa, Okla.). Significant differences were determined by means of Tukey's Honestly Significant Difference test. The data are reported as mean $\pm$ standard error of the mean.

Table I. Grading of leukocyte sequestration

Grade	Description
0	No evidence of leukocyte sequestration; normal lung
1	Less than 25% of interstitium infiltrated by leukocytes
2	50% of interstitium infiltrated by leukocytes
3	75% of interstitium infiltrated by leukocytes
4	100% of interstitium infiltrated by leukocytes

Results

The capacity of the room air-ventilated lungs to oxygenate blood at the completion of the 30-minute period of reperfusion revealed an expected impairment in 18-hour control lungs compared with the IC group. Administration of anti-ICAM-1 monoclonal antibody markedly enhanced the oxygenation capacity in a dose-dependent manner (Fig. 1). By contrast to the IC and 18-hour control groups, the lungs receiving low- and high-dose anti-ICAM-1 antibody were capable of generating oxygen tensions of 86.87 ± 11.32 and 139.33 ± 16.15 , respectively ($p = 0.0001$). The degree of leukocyte sequestration was also increased in the 18-hour control group compared with the IC group. Administration of anti-ICAM-1 antibody was associated with decreased histologic evidence of leukocyte sequestration, again in a dose-dependent manner (Fig. 2). The leukocyte grade (mean $\pm$ standard error of the mean) was 1.5 ± 0.723 in the IC group versus 3.0 ± 0.955 in the 18-hour control group. In lungs receiving antibody, the mean leukocyte grade was improved at 1.9 ± 0.899 in the low-dose group and 1.2 ± 0.834 in the high-dose group ($p = 0.0002$). Representative histologic specimens taken from each cohort of reperfused lungs revealed a marked reduction in leukocyte sequestration associated with the administration of blocking antibody (Fig. 3). Measures of PA pressure, pulmonary vascular resistance, airway compliance, lung weight, and wet-to-dry weight ratios were not improved by the administration of anti-ICAM-1 antibody. These data are presented in Table II.

Discussion

The complex molecular events that orchestrate adhesion between leukocytes and vascular endothelium during inflammatory responses have been well characterized.^{5,6} Leukocyte adhesion to endothelial cells is mediated by a group of glycoproteins expressed on the surface of leukocytes known as β_2 integrins. Each integrin is comprised of a common β -subunit (CD18) bound to one of 3 α -subunits (CD11a, CD11b, and CD11c) forming three integrin

Fig. 1. Oxygenation capacity (partial pressure of oxygen in millimeters of mercury) after 30 minutes of reperfusion. $p = 0.0001$ for 18-hour control group versus low-dose group, 18-hour control group versus high-dose group, low-dose versus high-dose group, and IC group versus 18-hour control group.

heterodimers: CD11a/CD18, CD11b/CD18, and CD11c/CD18.⁷ The CD11a/CD18 and CD11b/CD18 molecules bind to an endothelium-based receptor called ICAM-1, initiating the process of leukocyte demargination. Various proinflammatory mediators released in response to ischemia both increase the expression of these adhesion molecules and enhance their binding effectiveness.^{8,11} Reperfusion of the ischemic pulmonary vasculature with whole blood is therefore associated with a profound sequestration of leukocytes within the allograft. Subsequent allograft damage caused by leukocyte-generated reactive oxygen species and proteolytic enzymes¹¹⁻¹⁴ can result in acute lung dysfunction after transplantation.

Because the adhesion of activated leukocytes to damaged endothelium is thought to be an early and pivotal event in the pathophysiology of reperfusion injury after ischemia, a number of investigators have tried to reduce solid organ reperfusion injury by blocking this initial leukocyte-endothelium interaction. Antibodies directed against the leukocyte CD18 complex (anti-CD18)¹⁵ and against the endothelial ICAM-1 receptor (anti-ICAM-1)¹⁶ have been shown to minimize neurologic deficits during reperfusion after ischemia of the rabbit central nervous system. Anti-ICAM-1 therapy has been shown to be protective in a model of kidney ischemia-reperfusion.¹⁷ Leukocyte antiadhesion therapy also appears to limit the size of myocardial infarction,¹⁸ minimize the severity of systemic inflammation after cardiopulmonary bypass,^{19,20} and prevent

Fig. 2. Degree of leukocyte sequestration after 30 minutes of reperfusion. $p = 0.0002$ for 18-hour control group versus low-dose group, 18-hour control group versus high-dose group, and IC group versus 18-hour control group.

myocardial stunning, low reflow, and edema formation after experimental heart transplantation.²¹

Evidence that preventing leukocyte adhesion within the pulmonary capillary bed protects the lung from the effects of ischemia and reperfusion has also been reported. Schueler and coworkers³ demonstrated that leukocyte depletion in a porcine model of double-lung transplantation improves the ischemic tolerance of the lung, with excellent lung function noted after 24 hours of preservation. Kapelanski and colleagues⁷ reported that an anti-CD18 monoclonal antibody was capable of improving gas exchange and decreasing lung water accumulation after 4 hours of preservation and 6 hours of reperfusion in a model of canine single-lung transplantation. Interestingly, other features of lung reperfusion injury, including pulmonary hypertension, increased pulmonary vascular resistance, and reduced airway compliance, were not improved with the antibody. Horgan and colleagues^{8,9} carried out experiments in which 24 hours of *in situ* rabbit lung ischemia was followed by 2 hours of reperfusion. They showed, with antibodies directed against both CD18 and ICAM-1, that neutrophil sequestration within the pulmonary vascular microcirculation is enhanced by upregulation of the ICAM-1 receptor during ischemia and is a major determinant of lung reperfusion injury. Measures of oxygenation capacity, pulmonary vascular hemodynamics, and airway compliance during reperfusion in these experiments were not recorded.

To further evaluate the role of leukocytes in mediating lung reperfusion injury and to determine whether antagonism of the ICAM-1 receptor

could ameliorate the effects of this injury, we employed an isolated, ventilated, blood-perfused model of rabbit lung function with which we have experience. With RR1, a monoclonal anti-ICAM-1 antibody, we were able to demonstrate a dose-dependent enhancement in rabbit lung oxygenation capacity after 18 hours of cold preservation and 30 minutes of reperfusion. This improved oxygenation correlated with decreased leukocyte sequestration. Similar to the results of Kapelanski and associates,⁷ we were unable to achieve improvements in reperfusion-associated pulmonary hypertension or reduced airway compliance. These features of reperfusion injury in the lung may be mediated either wholly or in part by leukocyte-independent or ICAM-1-independent mechanisms.

All of the lungs subjected to 18 hours of ischemia, regardless of anti-ICAM-1 therapy, accumulated lung water, as measured by increased lung weight during reperfusion and by increased wet-to-dry weight ratios at the completion of reperfusion. These findings suggest that the frequently observed capillary leakage phenomenon can occur without significant infiltration of leukocytes in this model and that measures designed to prevent leakage may need to be more broadly focused than any specific antileukocyte therapy. Because the administration of an isotype-matched control monoclonal antibody directed against an adhesion-independent leukocyte antigenic determinant was not used, we cannot exclude the remote possibility that nonspecific effects of monoclonal antibody administration contributed to our results.

Although the safety and efficacy of anti-ICAM-1 monoclonal antibody in human beings has yet to be firmly established, a phase I trial of its use as an immunosuppressant in renal allograft recipients has found no significant adverse side effects.²² A second published trial of anti-ICAM-1 therapy as an anti-inflammatory agent in patients with rheumatoid arthritis has further substantiated the drug's safety, with reported clinical improvement lasting as long as 55 days after completion of a 5-day course of anti-ICAM-1 therapy.²³

In conclusion, the administration of a monoclonal anti-ICAM-1 antibody prevented the impairment of oxygenation capacity and was capable of reducing leukocyte infiltration in this model of isolated lung function. These data provide additional evidence of the detrimental effect of leukocytes during pulmonary reperfusion after ischemia. Further, these data

Fig. 3. Histologic specimens (Hematoxylin-eosin stain; original magnification $\times 125$). **A**, Reperfused immediately after harvest (IC group). Minimal interstitial infiltration with inflammatory cells. Normal architecture preserved. **B**, Reperfused after 18 hours of cold ischemia (18-hour control group). Severe infiltration with polymorphonuclear cells and erythrocytes with distortion of the microscopic architecture. **C**, Low-dose antibody and 18 hours of cold ischemia (low-dose group). Moderate infiltrate with polymorphonuclear cells and well-preserved architecture. **D**, High-dose antibody and 18 hours of cold ischemia (high-dose group). No interstitial infiltrate with inflammatory cells. Architecture well preserved.

Table II. Parameters unaffected by administration of anti-ICAM-1 antibody

	PA pressure (mm Hg)	PVR (dynes $\cdot$ sec/cm $^{-4}$)	Airway compliance (% change)	Lung weight (% change)	Wet-to-dry weight ratio
IC	21.37 $\pm$ 1.91	55.18 $\pm$ 5.45	3.92 $\pm$ 8.26	15.93 $\pm$ 8.27	4.65 $\pm$ 0.72
18-hour control	46.69 $\pm$ 9.20	87.90 $\pm$ 18.37	-26.73 $\pm$ 7.28	103.13 $\pm$ 29.92	7.30 $\pm$ 0.69
18-hour low-dose group	43.34 $\pm$ 7.38	82.75 $\pm$ 14.47	-39.04 $\pm$ 5.89	136.67 $\pm$ 26.95	6.74 $\pm$ 0.95
18-hour high-dose group	36.26 $\pm$ 4.63	69.66 $\pm$ 9.54	-23.74 $\pm$ 8.41	93.18 $\pm$ 17.90	7.79 $\pm$ 1.21
<i>p</i>	0.0396; IC vs 18-hour control	NS	0.0024; IC vs 18-hour control and IC vs 18-hour low-dose group	0.0044; IC vs 18-hour control and IC vs 18-hour low-dose group	NS

Data listed represent mean values $\pm$ standard error of the mean recorded at the end of a 30-minute period of reperfusion. PVR, Pulmonary vascular resistance; NS, not significant.

support the importance of the endothelial ICAM-1 receptor in mediating leukocyte sequestration in reperfusion-injured lungs.

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Discussion

Dr. Margaret D. Allen (*Seattle, Wash.*). This is certainly an elegant study in an ex vivo model of clinical reperfusion injury after lung transplantation, which certainly is a major clinical problem. I appreciate that this is a very difficult and sophisticated model. I do have a few questions.

First, as you know, many lung operations do not require bypass, and bypass circuitry in your experiment itself could potentially result in upregulation of cytokines, adhesion molecules, and other factors. To what extent could this have influenced your results, either pro or con?

Dr. Buchanan. There is plastic tubing to which the blood is exposed in our circuit. This blood is passed through a filter. Our leukocytes are undoubtedly activated, and our adhesion receptors are potentially upregulated. Our system—like any isolated experimental preparation—may introduce some other variables.

Dr. Allen. Second, the monoclonal antibody RR1 that you used binds only to the first and second domains of ICAM-1 and thus blocks the LFA-1 binding site but not the Mac-1 binding site, which is the most important for neutrophils. There are other anti-ICAM-1 antibodies that do block both. Could this possibly explain why you saw improvement in some parameters but not in others? Did you look at what leukocyte subpopulations were present? For instance, were macrophages present, or were you selecting out certain populations?

Dr. Buchanan. The RR1 antibody we used is known to be extremely specific, and in fact I believe this explains our lack of improvement in hemodynamics and airway function. I think you are right. If we looked at subpopulations of cells involved or the particular type of ICAM receptor involved, we would see that only a subset was blocked by our experiment.

Dr. Allen. Finally, I noticed that you did not use an isotype-matched antibody in your control preparations, and I was just wondering about your thoughts on this.

Dr. Buchanan. I am not convinced of the utility of isotype-matched antibody in this preparation.

Dr. Allen. It would be a nonfunctional antibody to control for binding to FC receptors.

Dr. Buchanan. We did not use such an antibody in this experiment, but perhaps it would be interesting to do so in the future.

Dr. Allen. I think that the approach you describe would certainly have potential for clinical application.

Dr. Alden H. Harken (Denver, Colo.). I compliment you for initiating a discussion that I think is going to be a theme of today's program. We have several papers on the program that allude to the fact that all cardiac surgeons place patients on cardiopulmonary bypass, and if we leave them there for an hour we can culture bacteria from our cardiopulmonary bypass solution. Whether or not we like to acknowledge that, we are then elaborating or provoking endotoxemia, which then may elaborate tumor necrosis factor, with or without complement, and then express the CD-11 and CD-18 neutrophil receptors and upregulate the ICAM on our endothelial cells, blowing holes in endothelial cells and transudating fluid and albumin into the lungs. Whether we acknowledge that or not, it happens in all of our patients, sometimes subclinically and sometimes not. I have two interrelated questions. One is related to your model. As I look at your data, and I agree with your interpretation completely, in your high- and low-dose antibody systems I wanted to see a relationship between the adherence of neutrophils to those endothelial cells and oxygenation. I saw no difference with the high- and low-dose antibody relative to neutrophils but I did see a significant improvement in oxygenation, and that relates to my second question. If you can break this sequence, which you and I both want to do, why have all the clinical studies looking at multiple organ failure or whatever parameter you want against antiendotoxin, anti-tumor necrosis factor, or anti-ICAM-1 been failures? I would like your interpretation of these observations.

Dr. Buchanan. I will approach the second observation first. I suspect the problems that we are seeing after bypass in endotoxic shock are multifactorial, and each of the interventions—the use of an antibody to ICAM, the use of an antibody to endotoxin—by contrast is extremely specific. Perhaps we need to combine these modalities to see some more clinically relevant response.

Regarding your first point, I think that one of the

important techniques we need to develop in the laboratory, and we are in the process of doing just that, is figuring out a way to actually look at the leukocyte binding to the endothelium, for example by means of an immunohistochemical technique. It would be interesting to see the exact numbers of leukocytes that are binding, where they are binding, and if in fact they are binding to the ICAM-1 receptor. We really do not know that.

Dr. David A. Fullerton (Denver, Colo.). I too congratulate you on a very pretty study, and I also think it is clinically relevant, which adds to its value. I have a couple of questions.

First, our interest has been in the vascular component of reperfusion injury, and I was curious as to whether you had the opportunity to discern whether your antibody might preserve vascular function after reperfusion.

Dr. Buchanan. We did not look at that in this particular experiment. It would certainly be interesting, for example, to take rings from our lungs and put them on a constriction apparatus to see whether the ability for endothelium-dependent relaxation was preserved. We are in the process of carrying out some such experiments now.

Dr. Fullerton. In addition, 18 hours of ischemia is of course a fairly large insult, and I was curious as to whether you have tried shorter periods of ischemia. If so, are those results substantially different from these, or did you choose 18 hours for some methodologic reason?

Dr. Buchanan. Preliminary data suggested that these rabbit lungs are quite sensitive to ischemia, with a 24-hour period of ischemia resulting in many of the lungs simply crashing and not surviving even the 30-minute period of reperfusion. By contrast, after just 2 or 4 hours of ischemia, lungs studied reveal little evidence of reperfusion injury. In an effort to unmask a significant but treatable injury, we settled on 18 hours.

Dr. Fullerton. Finally, is it necessary to provide the antibody both at the end of your flush and on reperfusion, or is it sufficient to provide it simply on reperfusion?

Dr. Buchanan. I cannot answer that from our data, but I would guess that if I had to administer antibody at one point or the other I would choose to do so just before reperfusion. Theoretically, these ICAM receptors are not significantly upregulated immediately after harvest or during the flush. Time is required for messenger RNA to be produced and translated to actually result in upregulation of the receptor.