CARDIAC AND PULMONARY REPLACEMENT

THE RELATIONSHIP OF ISCHEMIA-REPERFUSION INJURY OF TRANSPLANTED LUNG AND THE UP-REGULATION OF MAJOR HISTOCOMPATIBILITY COMPLEX II ON HOST PERIPHERAL LYMPHOCYTES

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Objectives: This study was designed to examine the relationship between ex vivo preservation time of the transplanted lung and the extent of injury and to relate this to the severity of rejection with and without allogenicity. **Methods:** Single lung transplantation was performed on two groups of domestic swine. Group A ($n = 7$) and group B ($n = 6$) had ex vivo preservation times of 4 and 15 hours, respectively, at 4° C hypothermia. Group C ($n = 6$) underwent 2 hours of warm ischemia via dissection and isolation of the left lung with ligation of its bronchial artery and crossclamping of the left pulmonary artery, vein, and bronchus without explantation. Assessment measures included lung function, antioxidant enzyme activities in the plasma and lung tissue, levels of inflammatory mediators in the recipient plasma, and quantification of major histocompatibility complex II HLA-DR- β on host peripheral lymphocytes. **Results:** All groups demonstrated increases in interleukin-10, lung weight, and HLA-DR- β expression and decreases in lung-tissue antioxidant enzyme activities, gas exchange, and lung compliance. There was a strong positive correlation between ex vivo preservation time and the expression of HLA-DR- β and a negative correlation between ischemic time and lung-tissue superoxide dismutase. **Conclusions:** These results suggest that the intensity of the host immunogenic response is related to the severity of ischemia-reperfusion injury and is independent of tissue incompatibility and/or the type of ischemic insult. We conclude that the extension of ex vivo preservation time may predispose the transplanted lung to more severe rejection. (J Thorac Cardiovasc Surg 1998;115:978-89)

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Ischemia-reperfusion injury (IRI) and graft rejection are considered to be independent problems in the management of solid organ transplantation.^{1,2} Rejection of transplanted organs is thought to be solely related to the problem of tissue incompatibility.³ Recently, however, a link between IRI and organ rejection has been made.⁴ Other studies have documented the importance of proinflammatory cytokines, such as interleukin-2 (IL-2), tumor necrosis factor- α (TNF- α), and interferon- γ ,^{5,6} and the involvement of oxygen radicals^{7,8} during IRI. Additionally, changes in the graft recovering from ischemic injury have been well characterized. There is increased expression of major histocompatibility complex class II (MHC II) on graft tissue^{9,10}; the

Fig. 1. Lung function parameters. Percent changes of PO_2 (a), alveolar-arterial oxygen gradient (b), alveolar-arterial oxygen ratio (c), and volume/pressure relationships (d) are shown. The solid line represents group A (4 hours of cold ischemia); the dashed line represents group B (15 hours of cold ischemia); and the dotted line represents group C (2 hours of warm ischemia). P values with an asterisk indicate significant differences within groups, and those with a diamond sign indicate significant differences between groups. pre, Before transplantation; 30m post, 30 minutes after reperfusion; 72h post, 72 hours after reperfusion.

cells infiltrating the graft are primarily polymorphonuclear and lymphocytic in origin.^{11, 12} However, the relationship between the severity of IRI (manifested by alterations in inflammatory mediators, antioxidant enzyme activities, and lung function) and host response, as measured by the expression of MHC II HLA-DR- β on host lymphocytes, has not been previously evaluated. IRI induction of heightened lymphocyte activation and recruitment in the recipient may play an important role in the rejection process. To clearly define IRI as an etiologic factor in the rejection phenomenon, allogenicity must be entirely excluded.

The specific aims of this protocol included the following: (1) to evaluate the relationship between ex vivo preservation time, extent of lung damage, and the up-regulation of MHC II, (2) to determine whether IRI is capable of the up-regulating of MHC II independent of allogenicity, and (3) to examine whether the type of ischemic insult (warm versus

cold) differently affects MHC II up-regulation. We hypothesized that IRI induced by ex vivo preservation can up-regulate MHC II expression and prime host peripheral lymphocytes for more severe rejection of transplanted organs and that this host immunogenic response is independent of tissue incompatibility and/or the type of ischemic insult.

Methods

Experimental protocol. Thirty-two domestic swine (*Sus scrofa domestica*) weighing 27 to 35 kg were divided into three groups (A, B, and C). Single lung transplantation was performed on group A (donor = 7, recipient = 7) and group B (donor = 6, recipient = 6) with ex vivo preservation times of 4 hours and 15 hours, respectively, at 4°C hypothermia. Group C ($n = 6$) underwent 2 hours of warm ischemia by dissection and isolation of the left lung with ligation of the bronchial artery and crossclamping of the left pulmonary artery, vein, and bronchus. These surgical procedures are routinely performed within our laboratories and were preceded by sham operations on animals that showed no changes in assessment measures

Fig. 2. Tissue antioxidant enzyme activities. Means $\pm$ standard errors of SOD (a), catalase (b), GRed (c), and GPx (d) are shown before transplantation (plain bars) and after transplantation (hatched bars). Group A, 4 hours of cold ischemia; group B, 15 hours of cold ischemia; and group C, 2 hours of warm ischemia. P values were derived from paired *t* tests at 95% confidence level of before and after measurements.

(data not shown). Additionally, in this study each animal served as its own control, when pre-IRI measures to post-IRI measures were compared. This study was designed to investigate the immunogenic response as a result of IRI; therefore the use of immunosuppressive therapy was not considered. All animals were maintained in accordance with the guidelines of the Canadian Council of Animal Care under the supervision of the Animal Care Committee of the University of British Columbia.

Surgical technique. Anesthesia was induced with ketamine and maintained with isoflurane (0.5% to 2.0%). After tracheal intubation, animals were ventilated to normocapnia (Narkomed 2 Ventilator, N.A.D., Inc., Telford, Pa.) at a tidal volume of 500 ml and a rate of 12 breaths/min with an air/oxygen mixture ($\text{FiO}_2 = 0.5$ to 1.0) to maintain oxygen tension (PO_2) greater than 70 mm Hg throughout the experimental period for donors and recipients. Heart rate, electrocardiogram, and urine output were monitored continuously. Animals were instrumented, and simple left lung transplantations were performed as described previously.¹³ Group C animals were more sensitive to crossclamping; therefore the FiO_2 was kept at 1.0 throughout the experiment.

Group C animals were treated the same as the swine in groups A and B until the harvesting of the lung. At this point, the left lung was dissected free and isolated, followed by ligation of the bronchial artery and crossclamping of the left pulmonary artery, vein, and bronchus.

During this period, the animal received injections of pancuronium (2 mg/ml) and fentanyl (500 mg) to complement the isoflurane, which was decreased to a lower rate. Warm ischemia was maintained for 2 hours, after which the crossclamp was released and the isoflurane was returned to the previous rate. Blood sample collection and lung mechanics were performed at the same times in all groups (before crossclamping and 30 minutes and 72 hours after reperfusion).

After lung implantation or reperfusion, the chest was closed, at which time a chest tube was left for drainage; a portable catheter (Port-A-Cath implantable access system, SIMS Deltec, Inc., St. Paul, Minn.) was implanted in the jugular vein. All animals received injections of buprenorphine (300 mg/ml as analgesic) and calcicane (20,000 IU/0.8 ml as anticoagulant) and the antibiotic procaine penicillin G (300,000 IU/ml, Ethacilin, Rogar/STB, London, Ontario, Canada; and 150,000 IU/ml; Penlong XL [procaine penicillin G and benzathine penicillin], Rogar/STB). At 72 hours after reperfusion the animals were reanesthetized. A median sternotomy was performed for catheterization; the final postoperative functional assessments were made, and biologic specimens were harvested. The animals were killed at the end of the experiment by sodium pentobarbital overdose.

Lung function. Hemodynamic and lung functional parameters were examined at three time intervals: before transplantation, in the donor animal 1 hour before the

Table I. Lung functional parameters and plasma antioxidant enzyme activities

Group	Time	Lung function parameters			Plasma antioxidant enzymes			
		Po ₂ (mm Hg)	AaG (mm Hg)	AaR	Volume/ pressure (ml/mm Hg)	SOD (U/mg protein)	GRed (nmol/min/ mg protein)	GPx (nmol/ min/mg protein)
4 Hr of cold ischemia (n = 7)	Before transplantation	140 ± 4.0	178 ± 4.2	0.440 ± 0.01	31.2 ± 1.8	0.07 ± 0.01	1.61 ± 0.15	5.2 ± 0.41
	30 Min. after transplantation	112 ± 15	556 ± 15*	0.167 ± 0.02*	27.6 ± 1.2	0.09 ± 0.01	1.57 ± 0.11	5.0 ± 0.56
	72 Hrs after transplantation	138 ± 11	168 ± 8.7	0.4501 ± 0.03	29.1 ± 1.7	0.05 ± 0.01*	1.67 ± 0.06	5.1 ± 0.41
15 Hr of cold ischemia (n = 6)	Before transplantation	159 ± 10	160 ± 12	0.499 ± 0.03	31.1 ± 3.5	0.13 ± 0.01	2.0 ± 0.16	5.4 ± 0.41
	30 Min. after transplantation	116 ± 17*	550 ± 18*	0.174 ± 0.03*	24.7 ± 2.2	0.13 ± 0.01	2.0 ± 0.15	5.5 ± 0.51
	72 Hrs. after transplantation	166 ± 6.6	149 ± 5.6	0.527 ± 0.02	26.8 ± 2.9	0.11 ± 0.01	1.6 ± 0.18	5.6 ± 0.23
2 Hr of warm ischemia (n = 6)	Before transplantation	464 ± 43	189 ± 25	0.601 ± 0.08	30.6 ± 1.6	0.10 ± 0.02	1.0 ± 0.06	3.8 ± 0.26
	30 Min. after transplantation	419 ± 87	332 ± 45†	0.570 ± 0.10†	26.1 ± 1.5	0.09 ± 0.01	1.1 ± 0.06	4.2 ± 0.27
	72 Hrs. after transplantation	323 ± 74†	274 ± 60	0.499 ± 0.07	24.7 ± 1.4*	0.08 ± 0.01	0.92 ± 0.07	4.0 ± 0.15

All values are given as mean ± standard error. AaG, Alveolar-arterial oxygen gradient; AaR, alveolar-arterial oxygen ratio.

*Significant difference within groups.

†Significant difference between groups.

harvesting (used as the baseline control), 30 minutes after reperfusion, and 72 hours after reperfusion in the recipient animals. Hemodynamic parameters (systolic, diastolic, and mean values for systemic arterial, pulmonary arterial, right atrial, and pulmonary capillary wedge pressures) were monitored continuously during transplantation and 72 hours after reperfusion. Assessment of pulmonary function included serial measurements of Po₂ and percent oxygen saturation. The alveolar-arterial oxygen gradient and alveolar-arterial oxygen ratio were calculated as previously described.¹³ Dynamic pulmonary compliance was assessed with subdivisions of volume/pressure relationships. Lung hemodynamics and compliances were then assessed for both lungs; for the left lung by occlusion of the right pulmonary artery and right bronchus.

Biochemistry

Antioxidant enzymes. The activities of antioxidant enzymes in the plasma and lung tissue were measured before transplantation, 30 minutes after reperfusion, and at 72 hours after reperfusion. Before the left lung was harvested from the donor animal, arterial blood samples and right lung tissue were collected. Blood was collected in glass tubes containing sodium heparin and centrifuged for 15 minutes at 1500 rpm and 4° C; the plasma was collected, and red blood cells were washed twice with sterile cold saline solution and stored at -80° C until analysis. Tissue blocks from the upper lobe of the donor right lung (assumed equal to left lung at baseline) were flash frozen in liquid nitrogen and used to provide an estimate of baseline tissue antioxidant activities. Blood samples (red blood cells and plasma) were also collected from the recipient animal. Blocks of tissue were collected from the transplanted left lung. The activities of superoxide dismutase (SOD), glutathione reductase (GRed), glutathione peroxidase (GPx), and catalase (tissue only) were quantitated by enzyme bioassay as described previously.¹⁴⁻¹⁷

Inflammatory mediators. Blood samples were collected from the left atrium of the recipient animal before trans-

plantation, 30 minutes after reperfusion, and 72 hours after reperfusion for the quantitation of IL-2, IL-4, IL-10, TNF-α, and thromboxane (TxB₂).

Blood samples for TxB₂ assay were collected in siliconized glass tubes containing ethylenediaminetetraacetic acid and indomethacin (INN: indometacin; 10 μg/ml). Plasma was aliquoted with polypropylene pipette tips and stored at -80° C until analysis. The plasma samples were acidified to pH 3 with citric acid and applied to C2 minicolumns (Amprep, Amersham Life Science, Cleveland, Ohio) prewashed with methanol and water. Impurities were washed away with diethyl ether, and samples were eluted with methyl formate and dried under a stream of nitrogen. The purified TxB₂ fraction was reconstituted in assay buffer and quantitated by enzyme immunoassay (Amersham Life Science) against a known standard concentration.

The IL and TNF-α blood samples were collected in siliconized glass tubes containing sodium heparin. The blood was centrifuged, and plasma aliquots were stored at -80° C until quantitation. The levels of IL-2, IL-4, IL-10, and TNF-α were determined by a competitive enzyme-linked immunosorbent assay with labeled cytokine (Cyt Immune Sciences Inc.). In brief, recipient plasma was directly added to 96-well plates precoated with secondary antibody and the anti-cytokine antibody, cytokine conjugate (labeled), streptavidin complex, substrate, and amplifier were applied in successive steps. Color development was monitored by optical density at 490 nm.

MHC expression

Host peripheral lymphocytes

CELL PREPARATION. Venous blood samples (21 ml) were collected before transplantation and 24, 48, and 72 hours after reperfusion and fractionated with Hypaque-Ficoll (Pharmacia) at 1500 rpm for 30 minutes. One sample was kept separate and uncollected for complete blood cell count. The buffy coat was removed and washed once with phosphate-buffered saline solution and twice with 2% culture media (TC199, Fisher Scientific Interna-

Fig. 3. Plasma cytokine levels. Percent changes of IL-2 (a), IL-4 (b), IL-10 (c), and TNF- α (d) are shown. The solid line represents group A; the dashed line represents group B; and the dotted line represents group C. *P* values with an asterisk depict significant differences within groups, and those with a diamond sign depict significant differences between groups. *pre*, Before transplantation; *30m post*, 30 minutes after reperfusion; *72h post*, 72 hours after reperfusion.

tional, Pittsburgh, Pa.) at 1500 rpm for 15 minutes. Isolated cells were then counted and frozen in TC199 at 10% dimethylsulphoxide and 20% fetal calf serum and stored at -70°C until flow cytometry analysis (Epic Profile, Coulter Corporation, Hialeah, Fla.).

FLOW CYTOMETRY. Two-color flow cytometry with swine anti-HLA-DR- β (MSA3, 1:20 dilution) and anti-CD3 (8E6, 1:20 dilution) primary antibodies (VMRD Inc., Pullman, Wash.) was performed on isolated lymphocytes. Bitmapping of the lymphocyte population was performed with swine anti-CD45 (Pan-Leuc, 1:40 dilution) and anti-monocytic antibodies (1:20 dilution). Two secondary goat anti-mouse antibodies labeled with phycoerythrin (1:100 dilution) and fluorescein isothiocyanate (1:40 dilution) were used for staining the lymphocytes bound to primary antibodies. Cells were incubated with primary antibodies at 4°C for 30 minutes and then stained with secondary antibodies at 4°C for 30 minutes. After each incubation, cells were washed with 2% TC199 for 15 minutes and centrifuged at 1500 rpm. Stained cells were fixed with 1% formaldehyde and kept at 4°C before flow cytometry analysis (Epic Profile).

Graft tissue. At the same sampling times as pathology specimens, sections of the upper lobes of the donor right lung before transplantation and the left transplanted lung after harvesting from the recipient were taken, immersed in optimal cutting temperature compound (Tissue-Tek, Miles Inc., Division of Bayer Corp., Tarrytown, N.Y.), and frozen in blocks by immersion in 2-methylbutane cooled

by liquid nitrogen and stored at -70°C . Immunohistochemistry for MHC II expression was performed on 5 mm thick cryostat sections fixed for 10 minutes in 4°C acetone. The sections were rehydrated in TRIS buffer (0.05 mol/L, pH 7.6) for 4 minutes, and excess fluid was removed by blotting. Non-immune serum (normal goat serum 1:20 dilution, Cedar Lane Laboratories Limited, Hornby, Ontario, Canada) was added for 10 minutes at room temperature; when removed, the primary antibody (mouse anti-swine MHC II HLA-DR, 1:1000 dilution; VMRD Inc.) was applied for 60 minutes at room temperature. The sections were then washed twice with TRIS buffer for 5 minutes, and endogenous peroxidase activity was blocked by the application of TRIS- H_2O_2 solution 1:1 for 25 minutes at room temperature. After a 5-minute wash with TRIS buffer, the secondary peroxidase conjugated antibody (goat anti-mouse IgG hematoxylin and eosin, 1:200 dilution; Jackson Immune Research Laboratories Inc.) was applied for 80 minutes at room temperature. Washes with TRIS buffer for 5 minutes and acetate buffer (0.1 mol/L, pH 5.2) for 3 minutes were performed, and freshly prepared substrate (3-amino-9-ethyl carbazole; Sigma Chemical Co., St. Louis, Mo.) was added for 7 minutes. Finally, the sections were washed 1 minute under tap water, counterstained with Carazzi's hematoxylin, and crystal mounted. The MHC II staining was graded on a semiquantitative scale of 1 to 4 $^{+}$.

Pathologic analysis. The weight of the left lung was measured after 4 or 15 hours of ex vivo preservation

Table II. Plasma cytokine levels

Group	Time	Plasma cytokine levels				
		IL-2 (ng/ml)	IL-4 (ng/ml)	IL-10 (ng/ml)	TNF α (ng/ml)	TxB ₂ (pg/ml)
4 Hr of cold ischemia (n = 7)	Before transplantation	2.3 $\pm$ 0.66	3.5 $\pm$ 0.94	0.54 $\pm$ 0.10	0.41 $\pm$ 0.07	8.7 $\pm$ 1.3
	30 Min. after transplantation	1.7 $\pm$ 0.26	2.5 $\pm$ 0.72	0.63 $\pm$ 0.20	0.85 $\pm$ 0.22	13.7 $\pm$ 3.6
	72 Hrs. after transplantation	1.3 $\pm$ 0.17	3.6 $\pm$ 0.75	2.1 $\pm$ 0.61*†	0.66 $\pm$ 0.08	6.9 $\pm$ 1.1
15 Hr of cold ischemia (n = 6)	Before transplantation	0.77 $\pm$ 0.14	1.4 $\pm$ 0.27	0.35 $\pm$ 0.05	0.48 $\pm$ 0.22	17.9 $\pm$ 5.5
	30 Min. after transplantation	0.6 $\pm$ 0.14	1.3 $\pm$ 0.19	0.28 $\pm$ 0.09	0.19 $\pm$ 0.06	22.1 $\pm$ 6.5
	72 Hrs. after transplantation	1.1 $\pm$ 0.16	2.1 $\pm$ 0.44	0.54 $\pm$ 0.09	0.38 $\pm$ 0.06	21.3 $\pm$ 4.7
2 Hr of warm ischemia (n = 6)	Before transplantation	0.35 $\pm$ 0.06	0.10 $\pm$ 0.03	0.38 $\pm$ 0.04	0.64 $\pm$ 0.16	27.9 $\pm$ 2.1
	30 Min. after transplantation	0.43 $\pm$ 0.13	0.25 $\pm$ 0.18	0.47 $\pm$ 0.12	0.80 $\pm$ 0.37	24.8 $\pm$ 5.0
	72 Hrs. after transplantation	0.77 $\pm$ 0.19	0.14 $\pm$ 0.09	0.79 $\pm$ 0.10*	0.62 $\pm$ 0.10	26.8 $\pm$ 4.0

All values are given as mean $\pm$ standard error.

*Significant difference within groups.

†Significant difference between groups.

Table III. MHC II HLA-DR- β expression on CD3⁺ host peripheral lymphocytes

Group	Time	Host peripheral lymphocytes	
		MHC II intensity	MHC ⁺ CD3 ⁺ cells (no.)
4 Hr of cold ischemia (n = 7)	Before transplantation	5.694 $\pm$ 0.674	2797 $\pm$ 120
	24 Hrs. after transplantation	4.985 $\pm$ 0.164	2367 $\pm$ 90†
	48 Hrs. after transplantation	8.209 $\pm$ 0.690*	2461 $\pm$ 117*
	72 Hrs. after transplantation	6.845 $\pm$ 0.479	2366 $\pm$ 44*
15 Hr of cold ischemia (n = 6)	Before transplantation	5.337 $\pm$ 0.369	706 $\pm$ 19
	24 Hrs. after transplantation	7.582 $\pm$ 0.504†	611 $\pm$ 8*
	48 Hrs. after transplantation	13.702 $\pm$ 1.514†	575 $\pm$ 29*
	72 Hrs. after transplantation	8.205 $\pm$ 0.930*	649 $\pm$ 9
2 Hr of warm ischemia (n = 6)	Before transplantation	5.428 $\pm$ 0.787	1698 $\pm$ 197
	24 Hrs. after transplantation	7.450 $\pm$ 0.589	1305 $\pm$ 41†
	48 Hrs. after transplantation	19.163 $\pm$ 0.955†	493 $\pm$ 96†
	72 Hrs. after transplantation	10.106 $\pm$ 1.754†	895 $\pm$ 55

All values are given as mean $\pm$ standard error.

*Significant difference within groups ($p = 0.03$).

†Significant difference within groups ($p = 0.05$).

before implantation and at 72 hours after reperfusion, immediately after the transplanted lung was harvested from the recipient. In group C, the weight of the left lung could only be measured at 72 hours after reperfusion.

From groups A and B, lung tissue was collected from the discarded donor right lung at organ harvest and the transplanted left lung for pathologic analysis. In group C, lung tissue was collected both before and after cross-clamping. The lung tissue was perfused with 10% neutral buffered formalin and stored for 24 hours. Tissue blocks were collected in a systematic fashion from the upper, middle, and lower lobes and processed for light microscopy with hematoxylin and eosin staining.

Statistics. Analysis of variance with repeated measures and standard errors were performed on the raw data of lung functional parameters. For presentation purposes only, the results are shown as percent change from

baseline with significance levels indicated by denoted symbols. Similarly, the raw data of the plasma antioxidant enzymes and plasma cytokines were analyzed by analysis of variance with repeated measures and standard errors. Tissue antioxidant enzymes and lung weights were analyzed by paired t tests (donor before transplantation versus after transplantation). MHC II HLA-DR- β expression on host peripheral lymphocytes was analyzed by nonparametric Mann-Whitney tests.

Results

Lung function. Lung functional parameters (PO₂ and alveolar-arterial oxygen gradient and ratio) depicted in Fig. 1, *a*, *b*, and *c* demonstrated a reduction in oxygen exchange for all three

Fig. 4. Expression of MHC II HLA-DR- β on CD3⁺ host peripheral lymphocytes. **a**, MHC II HLA-DR- β intensity per MHC⁺CD3⁺ cell. **b**, The number of host peripheral lymphocytes (B and T cells). The solid line represents group A; the dashed line represents group B, and the dotted line represents group C. *P* values were derived by nonparametric Mann/Whitney tests. **c**, Regression analysis of MHC II intensity per cell versus ischemic time. **d**, An example of a bitmap generated by flow cytometry analysis – quadrant 2 denotes MHC⁺CD3⁺ cells. *pre*, Before transplantation; *24h post*, 24 hours after reperfusion; *48h post*, 48 hours after reperfusion; *72h post*, 72 hours after reperfusion; *PE*, phycoerythrin; *FITC*, fluorescein isothiocyanate; *2h post*, 2 hours after reperfusion.

groups after 30 minutes of reperfusion, but this was significant only in group B ($p = 0.02$) for PO_2 . At 72 hours after reperfusion, however, these gas exchange parameters returned to their preischemic levels for animals in groups A and B after 4 and 15 hours of hypothermic storage and allotransplantation, respectively. In contrast, for group C, in which the animals were subjected to 2 hours of warm ischemia by simple crossclamping, gas exchange deteriorated and at 72 hours after reperfusion PO_2 and alveolar-arterial oxygen gradients and ratios were significantly decreased. Lung compliance measured by the volume/pressure relationship indicated similar changes at 30 minutes after reperfusion for all groups (Fig. 1, *d*). The recovery of lung compliance, however, was not complete at 72 hours for all groups and was significantly ($p = 0.03$) less for group C. Results of the functional parameters are presented as percent change in Fig. 1, and their real values $\pm$ standard errors are presented in Table I.

Biochemistry. Fig. 2 shows the postischemic antioxidant enzyme status of the lung tissue after 72 hours after reperfusion compared with the pretransplantation donor right lung (control) of the same animal. A significant ($p = 0.02$) decrease in SOD in groups A and B was observed, and this was positively correlated with increased ex vivo preservation time ($r^2 = 0.76$). In group C, however, SOD did not show a significant reduction. Tissue catalase (groups A and B) and GRed (groups A and C) were also significantly depleted. The activity of GPx was unchanged in all groups.

The activities of plasma antioxidant enzymes (SOD, GRed, and GPx) were measured in the recipient before transplantation and after 30 minutes after reperfusion and 72 hours after reperfusion (Table I). Within group A, which underwent the shortest storage time, SOD was the only plasma antioxidant enzyme that was significantly decreased ($p = 0.01$) 72 hours after reperfusion.

The levels of cytokines IL-2, IL-4, IL-10, TNF- α , and TxB₂ were assayed in the plasma of the recipi-

Fig. 5. Histologic comparison of lung tissue from allografts undergoing 4 hours (a) and 15 hours (b) cold ischemic preservation time, and 2 hours of warm, in situ ischemia (c). a, Expansion of the interlobular septal tissue by lymphatic dilation (L) and tissue edema (*). There is a sparse alveolar infiltrate (arrow). b, Alveolar infiltrates are more dense (*) and are composed of mononuclear and polymorphonuclear inflammatory cells. c, Foci of acute alveolar hemorrhage are seen (arrow), in addition to the inflammatory infiltrate. (Hematoxylin and eosin; original magnification $\times 125$, all panels.)

ent before transplantation and after 30 minutes after reperfusion and 72 hours after reperfusion (Fig. 3, a through d). IL-2, IL-4, and TNF- α levels did not change significantly with respect to the duration and/or type of ischemic damage. The level of IL-10, however, was increased significantly in all groups at 72 hours after reperfusion. TxB₂ levels were also increased in group A but had a tendency to decrease in groups B and C. The levels of plasma cytokines are presented as percent change in Fig. 3; the absolute values $\pm$ standard errors of both plasma cytokines and TxB₂ are given in Table II.

MHC II expression

Host peripheral lymphocytes. Table III presents the fluorescence-activated cell sorter analysis as total MHC II intensity and the number of MHC II⁺ CD3⁺ counted in the periphery. These results showed that MHC II HLA-DR- β expression on host peripheral CD3⁺ T lymphocytes had a tendency to increase in the first 24 hours after reperfusion (Fig. 4, a). At 48 hours, the MHC II intensity per cell reached its peak for all groups. At 72 hours after reperfusion, the values had generally begun to decline toward baseline levels. The peak levels of MHC II expression on individual CD3⁺ T lymphocytes were significantly ($p = 0.01$) higher after 15 hours than after 4 hours of storage. Fig. 4, c, also

shows that if the relationship of cold ischemic time to MHC II intensity per cell were linear, then the rate of MHC II expression per hour would have been 0.499 channel intensity per hour of ex vivo preservation based on the illustrated correlation (coefficient = 0.88). The data from group C also indicate that an ischemic insult itself without the complication of tissue incompatibility is capable of intensifying the expression of MHC II on peripheral T lymphocytes. Two hours of warm ischemia affected the expression of MHC II more severely than allotransplantation after 4 or 15 hours of ex vivo preservation. In addition, the number of lymphocytes (T and B) in the periphery shows a significant ($p = 0.05$) decrease for groups B and C in the first 48 hours after reperfusion (from complete blood cell count, Fig. 4, b). Results were expressed as mean fluorescence intensity of MHC⁺ CD3⁺ cells and the number of MHC⁺ CD3⁺ cells (Table III).

Pathologic evidence. Lung weights increased significantly ($p < 0.0001$) after transplantation in groups A and B compared with pretransplantation values; this was reflected histologically by expansion of pleural and interlobular septal lymphatics with edema that often contained significant quantities of inflammatory cells. There was no gross evidence of intimal thrombosis at either the site of

Fig. 6. Immunohistochemistry of lungs undergoing 2 hours of warm in situ ischemia: **a**, preischemic biopsy; **b**, lung at time of death. In **a**, basal, or constitutive expression of MHC II is identified on vascular endothelium (*arrow*); there is no alveolar inflammatory infiltrate (*). In **b**, there is a large increase in MHC II tissue expression contributed to by an influx of MHC II⁺ inflammatory cells and increased expression of MHC II on capillary and larger vessel endothelium (*arrow*). (Hematoxylin and eosin; original magnification $\times 125$, both panels.)

arterial crossclamping or at the anastomotic suture line. Intraalveolar infiltrates were identified in all groups at the time of death and consisted of a polymorphous mixture of neutrophils, alveolar macrophages, lymphocytes, and plasma cells (Fig. 5). Qualitative analysis identified a greater inflammatory component in group B, which underwent the longest ischemic time. The infiltrates in groups A and C tended to be more patchy and less dense. In all groups, there were focal areas of diffuse alveolar damage that were accompanied by microvascular thrombosis, which was presumed to be the result of direct microvascular injury from IRI. Contralateral (native) lungs also demonstrated edema and alveolar infiltrates, although they were obviously reduced relative to the operated lungs.

Immunohistochemical analysis confirmed a post-operative up-regulation in the expression of MHC II antigen in all groups (Figs. 6 and 7). Positive staining was observed on endothelium (large vessels and alveolar capillaries) and on inflammatory cells. Increased expression with the tissue sections was seen in concert with the increase in inflammatory infiltrates in each group and thus paralleled the findings of the routine light microscopic sections. Increased density of antigen, as measured semiquantitatively on endothelial surfaces, was not believed to be differ-

ent among groups A, B, and C; however, this observation may reflect the small size of the tissue sample and the patchy nature of the inflammatory process.

Discussion

For many years IRI has been suggested to be one of the etiologic factors in the rejection of transplanted organs.^{1, 2, 4} Initial clinical observations reported more persistent and more severe rejection of organs that had extended ex vivo preservation times in comparison to organs transplanted within expected preservation times.³ These observations triggered the interest of investigators to determine whether factors in addition to tissue incompatibility are involved in organ rejection. It was hypothesized that IRI itself might play a role in the cause of organ rejection after transplantation. Reperfusion of the transplanted organ triggers a cascade of events, including the generation of oxygen radicals, the inactivation of antioxidant enzymes, and the release of inflammatory mediators, ultimately leading to organ damage and subsequent rejection.¹⁸ Most experimental work in this area has focused on the expression of MHC class II on transplanted donor organs.^{9, 10} The present experimental protocol was designed to investigate the up-regulation of MHC not only

Fig. 7. Immunohistochemistry of lungs undergoing 4 hours of cold, ex vivo preservation before implantation. **a**, Tissue before transplantation shows low basal expression of MHC II antigen on vascular endothelium (*arrow*). **b**, In tissue after transplantation, endothelial MHC II expression is increased (*arrow*). (Hematoxylin and eosin; original magnification $\times 125$, both panels.)

in the transplanted tissue but also in circulating host T lymphocytes and to correlate this MHC II up-regulation with functional, biochemical, and morphologic impairment of ischemic-reperfused organs with and without allotransplantation.

Comparison of groups A and B, which underwent 4 or 15 hours of cold ischemic storage, respectively, demonstrated a clear relationship between ex vivo preservation time, degree of tissue damage, and MHC II up-regulation. Reduction of lung functional capacity, as reflected in decreased oxygen exchange and compliance used as a measure of IRI, has been observed in our previous experiments^{19,20} and in those of others.^{21,22} However, the present report further suggests that with increased ex vivo preservation time the loss of functional capacity becomes more severe. This relationship between ex vivo preservation time and functional impairment is confirmed by our histologic analyses to be associated with intraalveolar and interstitial edema formation, tissue hemorrhage, and leukocyte infiltration. This loss of function and of morphologic integrity of lung has been postulated to be due to the damaging effect of oxygen-derived free radicals produced during postischemic reperfusion.^{23,24} Because direct measurement of oxygen-derived free radicals is extremely difficult in an in vivo model such as ours, we measured the plasma and tissue antioxidant enzymes that serve as the first lines

of defense against the oxygen-derived free radical-induced damage.⁷ Significant inhibition of antioxidant enzymes such as SOD ($p < 0.05$), catalase ($p < 0.05$), and GRed ($p = 0.001$) within transplanted lung tissue suggested that the functional and morphologic impairment observed might be due to the effect of oxygen-derived free radicals and lipid peroxidation during ischemia and reperfusion.^{18,25} Alternatively, it could be suggested that the observed antioxidant alterations might be a consequence of IRI.

Results of the flow cytometry analyses of host peripheral CD3⁺ T lymphocytes in the allotransplantation study demonstrated the up-regulation of MHC II HLA-DR- β to correlate positively (coefficient = 0.88) with the time of ex vivo preservation and with functional, biochemical, and morphologic changes. The MHC II up-regulation on peripheral T lymphocytes also correlates with the transplanted lung tissue MHC up-regulation as detected by immunohistochemistry. Although it has been shown by other investigators that IRI increases MHC expression within the graft tissue,^{9,10} to our knowledge, this is the first documentation of a relationship of ex vivo preservation time with MHC II up-regulation on host peripheral CD3⁺ lymphocytes and correlation of this up-regulation to functional, pathologic, and biochemical manifestations of IRI in transplanted lung.

To explain the effect of IRI alone without allogeneity, we also created a model of ischemic injury by dissection of the left lung, simple occlusion of the left pulmonary artery, vein, and bronchus, and ligation of the bronchial artery. It is well accepted that cold storage is a preservation technique for reducing the degree of ischemic damage; therefore a pilot study with different ischemic time periods was performed (data not shown) to allow the identification of a warm ischemic time that closely produced injury comparable to that induced by 4 to 15 hours of cold storage. On the basis of the pilot study, 2 hours of warm ischemia was chosen to examine the relationship of IRI and host response in a system that eliminated tissue incompatibility. In group C, the biochemical, functional, and morphologic results were very similar to the cold preservation measurements; in some cases these were even enhanced. In particular, the MHC up-regulation was greater than 100% from baseline, demonstrating that the IRI may directly contribute to host immunogenic response and subsequent organ rejection. It is also interesting to note that the chosen durations of warm and cold ischemic conditions produced very similar functional and biochemical results. Moreover, the specific effect of IRI on the MHC up-regulation with and without cold storage has not to our knowledge been reported elsewhere.

Knowing that IRI can produce functional and morphologic deterioration and that MHC II up-regulation can lead to subsequent rejection, we tried to identify the factors that may be responsible for MHC II up-regulation in both ischemic models. Other studies have demonstrated the link between the production of IL-2, TNF- α , and interferon- γ and allogeneic rejection caused by tissue incompatibility.^{6,28} However, it is not known to what extent these same cytokines are involved in IRI induction of MHC II up-regulation as in antigenic up-regulation. In our experimental protocol, the levels of IL-2 and TNF- α in the peripheral blood were examined as the primary cytokines implicated in MHC II up-regulation associated with tissue incompatibility.^{5,26} In addition, the proliferation-associated cytokine IL-4 and the immunoregulatory cytokine IL-10 were investigated to identify the role of other molecules in the process of MHC up-regulation, possible inhibitory feedback mechanisms, and rejection caused by IRI. The results of this study demonstrated that IL-2 and IL-10 were the two cytokines that may have a direct effect on MHC II up-regulation triggered by IRI. Although levels of IL-2

did not increase significantly, they did show a tendency to increase as the severity of damage increased. IL-10, on the other hand, demonstrated a significant rise; but the degree was inversely proportional to the extent of lung damage. TNF- α and IL-4 showed no significant changes, which would appear to detract from their possible involvement in this mechanism of MHC II up-regulation.

In summary, results of this study suggest that IRI can up-regulate the MHC II HLA-DR- β in host peripheral lymphocytes and in transplanted lung tissue and that this is increased by lengthening the ex vivo preservation time. Our findings also suggest that this MHC II up-regulation is independent of tissue incompatibility and is related to the degree of damage caused by IRI. The mechanism by which this phenomenon occurs has yet to be investigated; however, one or more distinct regulatory cytokines may be involved.

This study is relevant to the realm of clinical transplantation. Although IRI cannot be considered the most important factor in rejection of transplanted organs, it clearly can be a pivotal factor in priming the recipient response and enhancing the rejection of transplanted organs. Thus renewed effort has to be directed to improving preservation of all donor organs.

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REFERENCES

1. Toronto Lung Transplant Group. Experience with single lung transplantation for pulmonary fibrosis. *JAMA* 1988;259:2258-62.
2. Kawahara K, Ikari H, Hisano H, et al. Twenty-four-hour canine lung preservation using UW solution. *Transplantation* 1991;51:584-7.
3. Lung transplantation: report of the ATS Workshop on Lung Transplantation. *Am Rev Respir Dis* 1993;147:772-6.
4. Howard TK, Klintmalm GBG, Cofer JB, Husberg BS, Goldstein RM, Gonwa TA. The influence of preservation injury on rejection in the hepatic transplantation recipient. *Transplantation* 1990;49:103-7.
5. Serrick C, Adoumie R, Giaid A, Shennib H. The early release of interleukin-2, tumor necrosis factor- α and interferon- γ after ischemia reperfusion injury in the lung allograft. *Transplantation* 1994;58:1158-62.
6. Serrick C, La Franchesca S, Giaid A, Shennib H. Cytokine interleukin-2, tumor necrosis factor- α and interferon- γ release after ischemia/reperfusion injury in a novel lung autograft model. *Am J Respir Crit Care Med* 1995;152:277-82.

7. Nezu K, Kushibe K, Tojo T, et al. Protection against lipid peroxidation induced during preservation of lungs for transplantation. *J Heart Lung Transplant* 1994;13:998-1002.
8. McCord JM. Oxygen-derived free radicals in post-ischemic tissue injury. *N Engl J Med* 1985;312:159-63.
9. Chang SC, Hsu HK, Perng RP, Shiao GM, Lin CY. Increased expression of MHC class II antigens in rejecting canine lung allografts. *Transplantation* 1990;49:1158-63.
10. Shackleton CR, Ettinger SL, McLoughlin MG, Scudamore CH, Miller RR, Keown PA. Effect of recovery from ischemic injury on class I and class II MHC antigen expression. *Transplantation* 1990;49:641-4.
11. Adoumie R, Serrick C, Gaiad A, Shennib H. Early cellular events in the lung allograft. *Ann Thorac Surg* 1992;54:1071-7.
12. Crim C, Keller CA, Dunphy CH, Maluf HM, Ohar JA. Flow cytometric analysis of lung lymphocytes in lung transplant recipients. *Am J Respir Crit Care Med* 1996;153:1041-6.
13. Qayumi AK, English JE, Duncan S, et al. Extended lung preservation using PAF-antagonist TCV-309 in combination with prostaglandin PGE₁. *J Heart Lung Transplant* 1997;16:946-55.
14. Winterbourne CC, Hawkins RE, Brian M, Carriel RW. The estimation of red cell superoxide dismutase activity. *J Lab Clin Med* 1975;85:337-41.
15. Long WK, Carson PE. *Biochem Biophys Res Commun* 1961;5:394-9.
16. Paglia DE, Valentine NW. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med* 1967;70:158-69.
17. Aebi H. Catalase. In: Bergmeyer HV, editor. *Methods of enzymatic analysis*. Weinheim, Germany: Chemie; 1974. p. 673.
18. Qayumi AK, Jamieson WRE. Pathophysiology of reperfusion injury in organ transplantation. In: Das DK, editor. *Pathophysiology of reperfusion injury*. Boca Raton [FL]: CRC Press; 1993. p. 325-62.
19. Qayumi AK, Jamieson WRE, Poostizadeh A. Effects of platelet-activating factor antagonist CV-3988 in preservation of heart and lung for transplantation. *Ann Thorac Surg* 1991;52:1026-32.
20. Qayumi AK, Jamieson WRE, Godin DV, et al. Response to allopurinol pretreatment in a swine model of heart-lung transplantation. *J Invest Surg* 1990;3:331-40.
21. Allison RC, Kyle WK, Adkins VR, Prasad JM, McCord JM, Taylor AE. Effect of ischemia reperfusion or hypoxia-reoxygenation on lung vascular permeability and resistance. *J Appl Physiol* 1990;69:597-603.
22. Yamazaki F, Yokomise H, Keshavjee SH, et al. The superiority of an extracellular fluid solution over Euro-Collins solution for pulmonary preservation. *Transplantation* 1990;49:690-4.
23. Connor HD, Gao W, Nukina S, Lemasters JJ, Mason RP, Thurman RG. Evidence that free radicals are involved in graft failure following orthotopic liver transplantation in the rat: an electron paramagnetic resonance spin trapping study. *Transplantation* 1992;54:199-204.
24. Kennedy T, Rao N, Hopkins C, Tolley E, Hoidal J. Reperfusion injury occurs in the lung by free radical mechanisms. *Chest* 1988;93(3 suppl):1495.
25. Roza AM, Pieper G, Moore-Hilton G, Johnson CP, Adams MB. Free radicals in pancreatic and cardiac allograft rejection. *Transplant Proc* 1994;26:544-5.
26. Sundaresan S, Alevy YG, Steward N, et al. Cytokine gene transcripts for tumor necrosis factor- α , interleukin-2, and

interferon- γ in human pulmonary allografts. *J Heart Lung Transplant* 1995;14:512-8.

Discussion

Dr. Nasser K. Altorki (New York, N.Y.). Have you measured the MHC expression before reperfusion?

Dr. Qayumi. Yes. We measured the MHC II expression before reperfusion and then at 1 day, 2 days, and 3 days after the reperfusion; the most expression was seen at 48 hours after the reperfusion.

Dr. Chi-Ming Wei (Baltimore, Md.). Do you have data to show immunohistochemical staining for TNF- α and IL-10?

Dr. Qayumi. We did not look for the protein expression in the lung tissue, but we looked at the level of cytokines in plasma that we thought would be involved in the mechanism of this expression. And, as you know, in allogenicity, IL-2, TNF- α , and interferon- γ are involved. We thought we should look at some other regulatory cytokines, such as IL-4 and IL-10 in particular; we found that the involvement of cytokines like IL-2 or TNF- α were not significant. But we found that IL-10 was significantly different, and that difference is, as I said, inverse because it is a regulatory cytokine.

Dr. William A. Cook (North Andover, Mass.). Could you show the slide that had the microscopic sections on it [Figs. 5 to 7]? What I was going to try to point out to you is that in the left-hand side of the slide what you have is a congested-looking interalveolar septum. What we were not seeing, looking at this on microscopic flow, was these clumps sticking to the endothelial cells. At that time, the whole concept was that these changes happened because the endothelium was somehow abnormal. What we actually did see was these increasingly large clumps of cells, and we literally saw them bounce off from the endothelium many times in many fields so that it did not appear to be a stickiness in the endothelial cells. Instead, these clumps would build up and keep packing into the smaller vessels until enough of the flow through the lungs was eradicated and just shut down completely. Then, of course, you would get injury because the tissue oxygenation was poor.

Your last slide was very provocative. We took some of these clumps out and dissected them, and there appeared to be some sort of a lymphocyte or plasmocyte or something in the middle with sticky red cells all clumped around it.

Your last slides suggested to me that perhaps the effect you are describing is, in fact, an intravascular cellular response that leads to the sort of phenomenon we observed looking at it microscopically.

Dr. Qayumi. I agree with you, it could be that intravascular phenomenon as well. But I would believe that it is the mechanism of IRI with respect to the ischemic damage to the endothelial cells and also to the adhesion of white blood cells and lymphocytes and so on. So I believe it could be.

Dr. Cook. Is that the secondary effect? In other words, if the vessel gets plugged up, then of course you are going to have ischemic injury.

Dr. Qayumi. I would personally believe that first the ischemic injury occurs, then as a result of that, the blood vessel would be plugged as you described. But that is my personal opinion.