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Membrane Attack Complex Contributes to Destruction of Vascular Integrity in Acute Lung Allograft Rejection[1]

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The lung is known to be particularly susceptible to complement-mediated injury. Both C5a and the membrane attack complex (MAC), which is formed by the terminal components of complement (C5b-C9), can cause acute pulmonary distress in nontransplanted lungs. We used C6-deficient rats to investigate whether MAC causes injury to lung allografts. PVG.R8 lungs were transplanted orthotopically to MHC class I-incompatible PVG.1U recipients. Allografts from C6-sufficient ($C6^+$) donors to $C6^+$ recipients were rejected with an intense vascular infiltration and diffuse alveolar hemorrhage 7 days after transplantation ($n = 5$). Ab and complement (C3d) deposition was accompanied by extensive vascular endothelial injury and intravascular release of von Willebrand factor. In contrast, lung allografts from C6-deficient ($C6^-$) donors to $C6^-$ recipients survived 13–17 days ($n = 5$). In the absence of C6, perivascular mononuclear infiltrates of $ED1^+$ macrophages and $CD8^+$ T lymphocytes were present 7 days after transplantation, but vascular endothelial cells were quiescent, with minimal von Willebrand factor release and no evidence of alveolar hemorrhage or edema. Lung allografts were performed from $C6^-$ donors to $C6^+$ recipients ($n = 5$) and from $C6^+$ donors to $C6^-$ recipients ($n = 5$) to separate the effects of systemic and local C6 production. Lungs transplanted from $C6^+$ donors to $C6^-$ recipients had increased alveolar macrophages and capillary injury. C6 production by lung allografts was demonstrated at the mRNA and protein levels. These results demonstrate that MAC causes vascular injury in lung allografts and that the location of injury is dependent on the source of C6. *The Journal of Immunology,* **2002, 169: 4620–4627.**

Complement can be a major mediator of adaptive humoral immune responses as well as innate immune responses. An increasing number of clinical and experimental studies of renal and cardiac allografts have linked complement activation to alloantigen-independent and -dependent responses. Complement activation occurs during the perioperative period (1–4), acute rejection (5–17), and chronic graft dysfunction (18–21). Split products of early complement components influence the localization, activation, and effector functions of platelets, granulocytes, monocytes, and lymphocytes, while membrane attack complex (MAC),[3] which is formed by the terminal components of complement (C5b-C9), can lead to rapid activation and destruction of target cells (1, 4, 22).

Animals with genetic deficiencies provide a means of separating the in vivo activity of individual complement components. We have demonstrated that a deficiency of C6, which prevents assembly of the MAC, can inhibit acute rejection of cardiac allografts in rats. This effect of C6 is especially prominent for allografts from PVG.R8 donors to PVG.1U recipients, which differ only at MHC class I Ags (5–7).

In contrast, the early components of complement, particularly C5a, have been reported to cause acute pulmonary distress in nontransplanted lungs (23–25). C5a is a powerful chemotactic and activating factor for neutrophils, macrophages, eosinophils, and mast cells. In addition, C5a induces the release of chemokines and cytokines from pulmonary macrophages and epithelial cells (26, 27). C5a has also been shown to activate endothelial cells of pulmonary vessels, resulting in surface expression of P-selectin (24, 28).

Although C5a has been demonstrated to be required for the full progression of certain inflammatory conditions in the lung, MAC can contribute to pulmonary injury (25). MAC can activate endothelial cells to cause the expression of P-selectin on endothelial cells and interact synergistically with TNF-α to promote enhanced expression of E-selectin and ICAM-1, which, in turn, would enhance leukocyte recruitment (29–31). MAC can also activate macrophages to release the proinflammatory cytokines TNF-α and IL-1. These findings underscore the diverse roles of complement activation products in the inflammatory cascade that leads to tissue injury.

In this study we investigated whether C6 and MAC contribute to acute rejection of MHC class I Ag-incompatible PVG.R8 lung allografts in PVG.1U rats. This strain combination was chosen for study because we and others have shown that cardiac allografts between these strains stimulate a vigorous Ab-mediated rejection (5, 32). Moreover, alloantibody responses to MHC class I Ags have been reported to be associated with lung allograft rejection clinically (33, 34).

An additional variable in lung allografts is the large number of pulmonary macrophages and extensive mucosal immune system (35–37). We have found that peritoneal macrophages are a significant source of C6 (7, 38). Therefore, we also investigated whether donor lung can be a source of C6 and contribute to pulmonary injury.

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[3] Abbreviations used in this paper: MAC, membrane attack complex; BALT, bronchus-associated lymphoid tissue; CT, competitive templates; PCNA, proliferating cell nuclear Ag; vWf, von Willebrand factor; WT, wild type.

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Materials and Methods

Animals

The derivation of PVG congenic rat strains with C6 deficiency has been described previously (6). The 9- to 12-wk-old male and female PVG.R8 (RTl.A^{a}B^u) and PVG.1U (RT1.A^{u}B^u) rats used in these study are mismatched at MHC class I Ags. Donor and recipient rats were always the same gender. C6 levels in the sera were analyzed by a sandwich ELISA, and MHC phenotypes of these congenic rats were confirmed by flow cytometry as described previously (6, 7). All animals received humane care in compliance with the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals prepared and formulated by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication 86-23, revised 1985).

Orthotopic lung transplantation

Donor left lungs were transplanted orthotopically into the left hemithorax of recipients using a modified technique of Marck et al. (39), and the pulmonary artery and vein were anastomosed using the cuff technique of Reis et al. (40). The mean graft ischemic time was 45 ± 10 min. A single dose of 10,000 U penicillin G was administrated i.m. immediately after surgery, and 10 mg/day gentamicin was administered i.m. from days 0–4 after transplantation.

Experimental design

Lung allografts were performed in four groups of rats (Table I). PVG.R8 (RTl.A^{a}B^u) rats always served as the donor and PVG.1U (RT1.A^{u}B^u) rats always were the recipients. The C6 status was varied as follows: C6-sufficient (C6$^+$) donors to C6$^+$ recipients (n = 5), C6-deficient (C6$^-$) donors to C6$^-$ recipients (n = 10), C6$^+$ donors to C6$^-$ recipients (n = 5), and C6$^-$ donors to C6$^+$ recipients (n = 5). All recipient rats were sacrificed 7 days after transplantation.

Histological evaluation of tissue section

At the time of sacrifice, the recipient rat was anesthetized and placed on a ventilator. The transplanted lung was exposed for gross evaluation. Then, two cross clamps were placed to isolated upper and lower poles of the transplants. The upper pole was excised and immediately frozen in liquid nitrogen for subsequent isolation of mRNA. The lower pole was excised and inflated with 7.5% gelatin (gelatin type A from porcine skin; Sigma, St. Louis, MO) and embedded in O.C.T compound (Miles, Elkhart, IN) for subsequent frozen sections. The central portion of the transplants was fixed by perfusion with 60% methanol with 10% acetic acid or 10% formalin before embedding in paraffin and sectioning at 7 μm. Paraffin sections were stained with H&E. The slides were evaluated by a pathologist blinded to the individual groups and were graded for the percentage of alveolar area with hemorrhage (scale of 0–4: a score of 0 = 0% area, 1 = 1–25% area, 2 = 26–50%, 3 = 51–75% area, and 4 = 76–100% area) and for acute cellular rejection based on the classification approved by International Society Heart and Lung Transplantation (41).

The paraffin sections were also stained by standard immunoperoxidase techniques using avidin-biotinylated enzyme complex for four markers: 1) T lymphocytes expressing CD8 Ag were detected with a purified mouse anti-rat CD8 mAb (OX-8; BD PharMingen, San Diego, CA); 2) macrophage infiltration was evaluated with a purified mouse mAb to ED1 (Serotec, Raleigh, NC) that recognizes a lysosomal membrane Ag (CD68) in rat macrophages (42); 3) proliferating cells were identified with PC10 (Novocastra, Newcastle upon Tyne, U.K.), an IgG2a mouse mAb to rat proliferating cell nuclear Ag (PCNA); and 4) von Willebrand factor (vWf) was localized with a polyclonal rabbit Ab to human vWf (DAKO, Glostrup, Denmark) that cross-reacts with the rat homologue.

IgG, IgM, and C3d were detected by immunofluorescent stains on frozen sections of grafts. Frozen 6-μm sections were prepared and mounted on gelatin-coated glass microscope slides. After acetone fixation, sections were stained for IgM deposition with cyanine-3-conjugated goat anti-rat IgM (Jackson ImmunoResearch, West Grove, PA) and for IgG deposition with FITC-conjugated F(ab′)$_2$ of goat anti-rat IgG (Jackson ImmunoResearch). C3d deposition was detected with FITC-conjugated rabbit anti-human C3d (DAKO).

Table I. *Experimental donor and recipient combinations*

Donor	Recipient	No. of Experimental Rat Pairs
PVG.R8 C6$^+$	PVG.1U C6$^+$	5 (male, 3; female, 2)
PVG.R8 C6$^-$	PVG.1U C6$^-$	10 (male, 6; female, 4)
PVG.R8 C6$^+$	PVG.1U C6$^-$	5 (male, 3; female, 2)
PVG.R8 C6$^-$	PVG.1U C6$^+$	5 (male, 3; female, 2)

Serum samples

Rat blood samples were collected by tail bleeding before surgery and at the time of sacrifice. Blood was allowed to clot for 30 min at 37°C and then for 1 h at 4°C. Serum was separated by centrifugation and was stored at −80°C until use.

Alloantibody assay

Alloantibodies were measured by flow cytometry on single-cell suspensions of cervical lymph nodes from PVG.R8 rats as described previously (43, 44). Briefly, the cells were incubated with 50 μl diluted sera (1/4, 1/16, 1/64, and 1/256). The washed cells were reacted with 50 μl PBS containing 0.2% BSA and 0.02% NaN_3 (PBA) containing a mixture of FITC-conjugated F(ab′)$_2$ of goat anti-rat IgG and PE-conjugated F(ab′)$_2$ of goat anti-rat μ-chain of rat IgM (Jackson ImmunoResearch). The cells were analyzed using a FACScan flow cytometer (BD Biosciences, Mountain View, CA).

C6 titers in sera

Circulating C6 levels were measured by ELISA as described previously (7). Briefly, the ELISA for detection of C6 was performed using mouse anti-rat C6 mAb 3G11 (a gift from Dr. W. Couser, University of Washington, Seattle, WA) to coat 96-well plates. After blocking the uncoated portions of the wells with PBA, serial dilutions of rat serum samples were incubated in the wells for 1 h. Following three washes with PBA, bound rat C6 was detected with a polyclonal goat anti-human C6 Ab (Calbiochem, La Jolla, CA) that cross-reacts with rat C6, followed by sequential incubation with biotin-conjugated donkey anti-goat IgG Ab (Jackson ImmunoResearch), horseradish-streptavidin (Zymed, South San Francisco, CA), and the substrate *o*-phenylenediamine (Sigma).

Analysis of C6 mRNA by competitive template RT-PCR

Total RNA was isolated from snap-frozen transplanted lungs, cervical lymph nodes, parathymic lymph nodes, and PBL that were homogenized in TRIzol (Life Technologies, Grand Island, NY) using standard procedures as described previously (7).

DNase treatment of RNA samples and reverse transcription reactions were performed as described previously (7). Briefly, RNA samples (2 μg) were treated with DNase I (amplification grade; Life Technologies) and reverse transcribed into cDNA in reaction mixtures containing 0.5 μl RNasin (40 U/ml; Promega, Madison, WI), 2.5 μl 10 mM dNTP (Pharmacia Biotech, Piscataway, NJ), 1 μg oligo(dT)$_{12–18}$ (Promega), 400 U (Moloney murine leukemia virus) reverse transcriptase (Bethesda Research Laboratory, Bethesda, MD), 10 μl 5× reverse transcriptase buffer, and diethylpyrocarbonate-water to a final volume of 50 μl. The RT reactions were conducted at 37°C for 90 min, heat-inactivated at 65°C for 10 min, and cooled for 3 min. PCRs were set up by using 5 μl cDNA (equivalent to 50 ng total RNA), 5 μl 10× amplification buffer, 3 μl 25 mM $MgCl_2$, 3 μl 2.5 mM dNTP, 1 μl of 10 mM of each primer, 0.25 μl of 5 U/μl *Taq* DNA polymerase (Promega), and dH_2O to a final volume of 50 μl. This mixture was overlaid with 100 μl light mineral oil (Sigma). The following sense and antisense oligonucleotide primer were used: rat C6 (direction 5′ to 3′; GGGGCAAGTATGACCTTCTC and TGGGACCGTTTTTCACAGT) and β-actin (CTATCGGCAATGAGCGGTC and CTTAGGAGTTGGG GGTGGCT). In preliminary experiments, optimal cycling conditions were established that allowed amplification of each cDNA in the linear range (6). Cycle numbers were 35 cycles at 57°C for C6 and 30 cycles at 63°C for β-actin. The PCR amplification was designed for initial denaturation of cDNA at 94°C for 2 min, and then cDNA was amplified in a predetermined number of cycles, each consisting of 1 min at 94°C, 1 min at annealing temperature, and 1 min for extension at 72°C. The final cycle extension was increased by an additional 7 min at 72°C. PCRs were performed in a Hybaid OmniGene thermocycler (Hybaid, Woodbridge, NJ).

Competitive templates (CT) for rat C6 and β-actin were designed to contain the same cDNA sequence as the gene of interest, except for deletion of 90–100 bp within the competitor DNA. Using CT as internal standards in RT-PCR allows the amplification of both wild-type (WT) cDNA and CT in the same reaction with the gene-specific primers. The individual products were separated and analyzed on the basis of size. To determine the amount of cDNA present in each sample, the same amplification technique was used first to measure the expression of the housekeeping gene β-actin. Samples of WT cDNA equivalent to 50 ng total RNA from each individual

RT reaction product were adjusted to contain equal concentrations of cDNA, based on the expression of β-actin in the sample. For each gene, 5 μl of the normalized RT product was coamplified with a constant amount of the gene-specific CT. The relative amounts of WT cDNA in the various samples were determined by calculating their respective sample intensity ratios of WT cDNA/CT DNA. Finally, all samples were normalized against the respective β-actin WT cDNA/CT DNA ratio. This normalization controls for the quantity of cDNA loaded in all samples. PCR products were separated on 2% agarose gels. The intensity of ethidium bromide luminescence was measured with a CCD image sensor using EagleSight 3.0 software (Stratagene, La Jolla, CA). This software provides basic analysis of the relative densities of gel images, which represent two-dimensional arrays of pixels. Gel images were further analyzed and semiquantified using the NIH Image 1.54 program.

Statistical analysis

Histological scores, alloantibody quantities, and C6 levels in sera were expressed as the mean ± SD. Histological differences were compared by Mann-Whitney *U* test. Differences between groups were considered statistically significant when $p < 0.05$.

Results

C6 deficiency extends the survival of MHC class I-incompatible PVG.R8 lung allografts in PVG.1U recipients

$C6^+$ lungs transplanted into $C6^+$ recipients ($n = 5$) were rejected completely by 7 days after transplantation, with grossly evident diffuse alveolar hemorrhage (Fig. 1*B*). In contrast, $C6^-$ lungs transplanted into $C6^-$ recipients ($n = 10$) had good vascular perfusion and air inflation beyond day 7, at which time five transplants were harvested for histology (Fig. 1*A*). The five remaining $C6^-$ lung transplants survived between 13 and 17 days in $C6^-$ recipients. These data indicate that C6 is critical for lung graft rejection in this strain combination.

C6 sufficiency results in vascular injury and alveolar hemorrhage

Intense vascular injury was the characteristic feature of acutely rejected lung allografts from $C6^+$ donors to $C6^+$ recipients. Extensive endothelial injury in arteries and arterioles was accompanied by profuse release of vWf that entrapped platelets in a thick layer along the distended vessel wall (Fig. 1*D*). Intravascular vWf and platelet aggregates extended into the alveolar capillaries expanding and occluding their lumens. In addition, diffuse perivascular and peribronchial infiltrates of macrophages ($ED1^+$) and lymphocytes (predominantly $CD8^+$) extended into the interstitial spaces. The alveolar spaces were filled with edema or hemorrhage. All the alveoli contained scattered lymphocytes and plasma cells

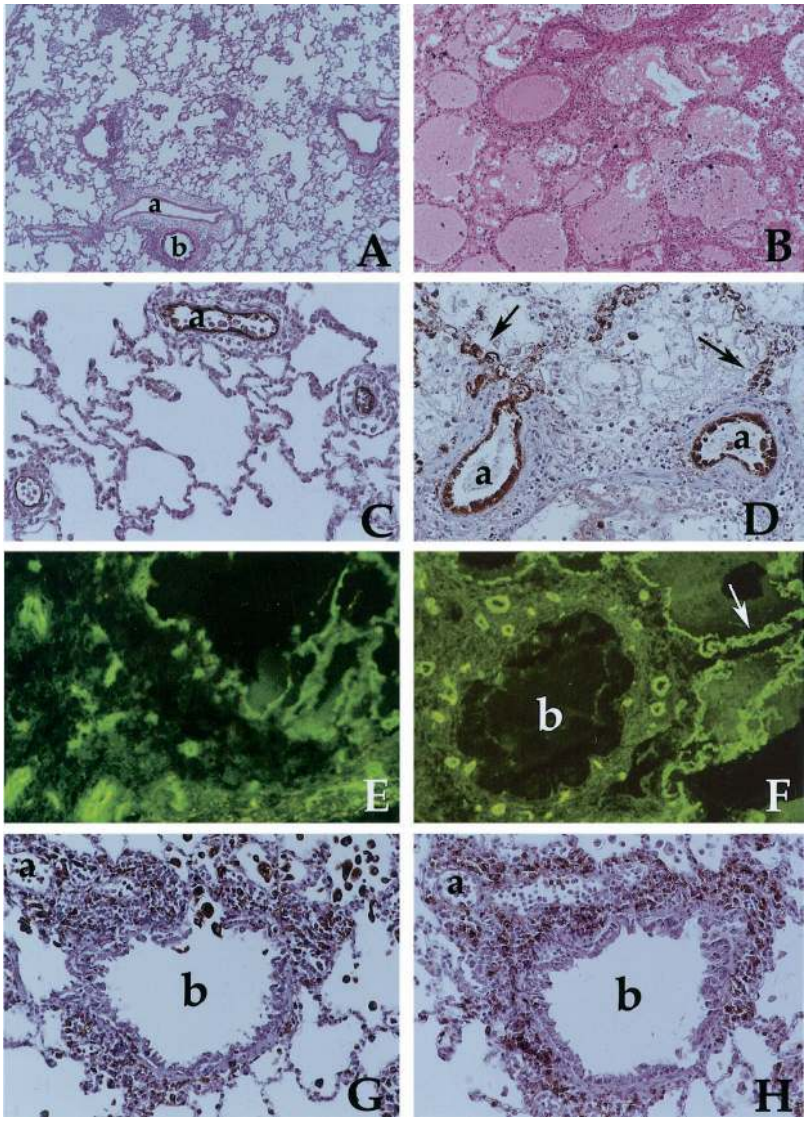

FIGURE 1. Histology of representative PVG.R8 lung allografts harvested 7 days after transplantation to PVG.1U recipients (*A* and *B*; magnification, ×40). Although perivascular and peribronchiolar mononuclear cells infiltration were severe in $C6^-$ allografts to $C6^-$ recipients, there was no alveolar edema or hemorrhage (*A*). Lung allografts from $C6^+$ donors to $C6^+$ recipients were rejected completely with an intense perivascular and peribronchiolar mononuclear cell infiltration accompanied by endothelial damage and diffuse alveolar edema and hemorrhage (*B*). Immunoperoxidase stain for vWf (*C* and *D*; magnification, ×100) demonstrated that vascular endothelial cells were quiescent, with minimal vWf release, in the $C6^-$ to $C6^-$ group (*C*). Copious intravascular release of vWf with platelet aggregation was evident in the $C6^+$ to $C6^+$ group (*D*). Intravascular vWf and platelet aggregation extended into alveolar capillaries (arrows). Immunofluorescent stains of frozen sections of the lung allografts show that C3d deposited on the endothelium of vessels in lung allografts in both $C6^-$ to $C6^-$ (*E*) and $C6^+$ to $C6^+$ (*F*) groups. C3d deposition can be observed in alveolar capillaries in lung allografts of $C6^+$ to $C6^+$ (arrow, *F*). Immunoperoxidase stains of $C6^-$ to $C6^-$ transplants demonstrate extensive perivascular and peribronchiolar $ED1^+$ macrophage infiltrates (*G*) and $CD8^+$ T lymphocyte infiltrates (*H*). a, arterioles; b, bronchioles.

as well as numerous macrophages. These macrophages were activated, as evidenced by frequent mitotic figures or binucleated cells with large amounts of cytoplasm containing phagocytic vesicles.

C6 deficiency impedes vascular injury and hemorrhage, but not cellular infiltrates

The major difference between lung allografts from $C6^+$ donors to $C6^+$ recipients and allografts from $C6^-$ donors to $C6^-$ recipients was the preservation of vascular integrity. Although marginated neutrophils and mononuclear leukocytes adhered to vascular endothelial cells in $C6^-$ lungs, minimal vWf was released, and no platelet aggregation was evident (Fig. 1*C*). Circumferential infiltrates of macrophages ($ED1^+$) and lymphocytes ($CD8^+$) surrounded most arteries and arterioles, and these infiltrates extended around adjacent bronchioles (Fig. 1, *G* and *H*). In contrast to $C6^+$ transplants, the alveolar septa and air spaces were generally free of infiltrates or injury, and hemorrhage was not present.

As a result, all the $C6^-$ to $C6^-$ lung allografts were graded as A2 to A3. These grades are defined by moderate (A2) to dense (A3) perivascular mononuclear infiltrates that were evident at low magnification with frequent concurrent bronchiolitis. The finding of mononuclear cell infiltrates and hemorrhage in air spaces increased the grade to severe acute rejection (A4) for all the $C6^+$ lung allografts in $C6^+$ recipients (Fig. 2*A*). When the degrees of mononuclear cell infiltrates and hemorrhage in air spaces were scored separately, the differences between lung allografts from $C6^-$ to $C6^-$ and from $C6^+$ to $C6^+$ were striking (Fig. 2).

These results demonstrate that in the absence of MAC, significant perivascular and peribronchiolar infiltrates of mononuclear cells accumulate, but vascular integrity is maintained. In contrast, MAC causes significant pulmonary vascular injury to lung allografts, as evidenced by damage to vascular endothelial cells.

C6 deficiency does not prevent the production of alloantibodies and deposition of IgM, IgG, and C3d in lung allografts

Lung allografts mismatched by MHC class I Ag elicited strong alloantibody responses. Both C6-sufficient and -deficient recipients had high levels of circulating IgM and moderate levels of IgG by 7 days after transplantation (Fig. 3). These circulating levels of alloantibody correlated with intense arterial deposits of IgM and C3d, with lesser amounts of IgG in the lung allografts in both the $C6^+$ to $C6^+$ group and the $C6^-$ to $C6^-$ group (Fig. 1, *E* and *F*). Thus, alloantibodies elicited by the transplants had bound to the target Ags and activated the early components of the complement cascade in both the $C6^+$ to $C6^+$ group and the $C6^-$ to $C6^-$ group.

Local and systemic C6 contribute to acute rejection of lung allografts

Evidence that C6 produced by donor cells in the lung can cause tissue damage was gathered in two sets of reciprocal experiments. In the first experiment the lung was eliminated as a source of C6 ($C6^-$ donors to $C6^+$ recipients), and in the second experiment the lung was the sole source of C6 ($C6^+$ donors to $C6^-$ recipients).

$C6^+$ recipients rejected only two of five lung allografts from $C6^-$ donors within 7 days. The two rejected lung allografts had extensive vascular injury and hemorrhage. The three functioning

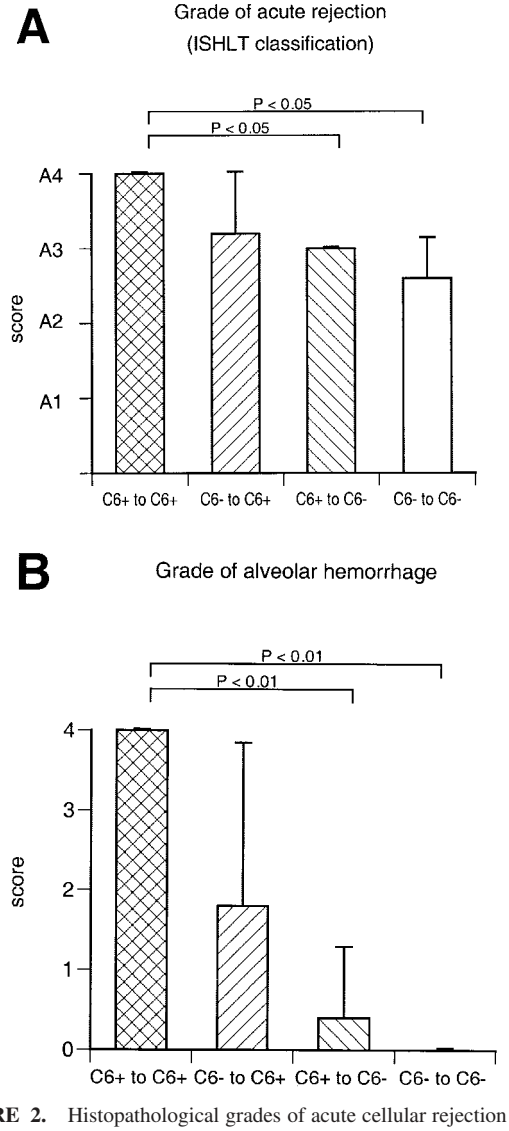

FIGURE 2. Histopathological grades of acute cellular rejection (*A*) and alveolar hemorrhage (*B*) 7 days after transplantation in the four groups of rats: $C6^+$ to $C6^+$ ($n = 5$), $C6^-$ to $C6^+$ ($n = 5$), $C6^+$ to $C6^-$ ($n = 5$), and $C6^-$ to $C6^-$ ($n = 5$) groups. All values in these graphs are expressed as the mean ± SD. Significant differences by Mann-Whitney *U* test are indicated.

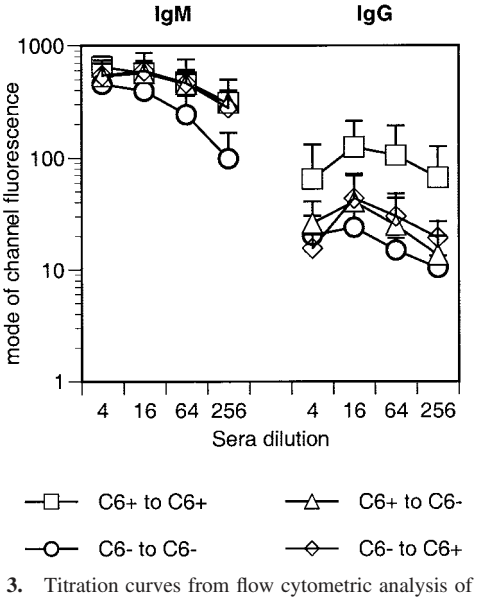

FIGURE 3. Titration curves from flow cytometric analysis of PVG.1U alloantibody responses elicited by PVG.R8 lung allografts 7 days after transplantation. Data from the four experimental groups are shown. Each point represents an average of five animals, with SD shown as vertical bars. High levels of IgM and moderate levels of IgG were produced in all groups.

lung allografts had little alveolar edema or hemorrhage 7 days after transplantation when the recipients were sacrificed in this group, but the arteries were congested with mononuclear cells, many of which were ED1-positive macrophages. Notably, these lungs had few macrophages or lymphocytes in the alveoli (Fig. 4*C*), and vWf staining was faint in the alveolar septal capillaries (Fig. 4*D*).

In contrast, all five of the lungs allografted from $C6^+$ donors to $C6^-$ recipients were functioning 7 days after transplantation, but they contained numerous macrophages in their alveoli, many of which were adherent to the septa and mitotically active (Fig. 4*A*). Stains for PCNA confirmed that 5–10% of the cells in the air spaces were proliferating (see inset in Fig. 4*A*). In addition, mononuclear cells and neutrophils expanded the alveolar capillaries and interstitial spaces, and vWf was detected in the alveolar septal capillaries (see inset in Fig. 4*B*). Neither of these features was present in the absence of C6 (Fig. 1*C*).

Local C6 production by lung allografts

Although the liver is the major source of C6 in the plasma, we have demonstrated that macrophages are a significant source of local C6 production (7). The lung is rich in macrophages, including those in the alveoli and BALT. Moreover, by 7 days after transplantation, macrophages constituted a large percentage of the infiltrates in the peribronchial, perivascular, and interstitial spaces of lung allografts. In addition, alveolar epithelial cells also produce early components of complement (45–47). Therefore, we investigated whether lung allografts can be a source of C6.

Sera from $C6^+$ recipients of lungs from $C6^+$ donors contained normal C6 levels (Fig. 5). No detectable C6 was found in sera from $C6^-$ recipients of lungs from $C6^-$ donors. The levels of C6 in sera from $C6^+$ recipients of lungs from $C6^-$ donors was almost equivalent to normal, confirming that lungs are not the primary source of circulating C6. However, circulating levels of C6 were partially reconstituted in $C6^-$ recipients by lung allografts from $C6^+$ donors (Fig. 5). The relatively low levels of C6 would not be expected to fully reflect the level of C6 in the lung compartments.

Semiquantitative competitive template PCR demonstrated mRNA for C6 in lungs transplanted from $C6^+$ donors to $C6^-$ recipients (Fig. 6). These results indicate that donor lung and leukocytes had produced C6 in the recipient by 7 days after transplantation.

Discussion

Animals with genetic deficiencies provide the most compelling evidence for the pathogenic and physiological function of individual complement components. With the increasing evidence that transplanted organs can be stimulated to produce many complement components in response to ischemia-reperfusion and nonspecific inflammation as well as during rejection (48–51), it is necessary that both donor and recipient be complement deficient to fully evaluate the effects of a complement component on allograft rejection. Before our production of congenic PVG rat strains with a C6 deficiency (6, 7), the effects of a C6 deficiency in the donor and recipient could not be evaluated in organ allografts.

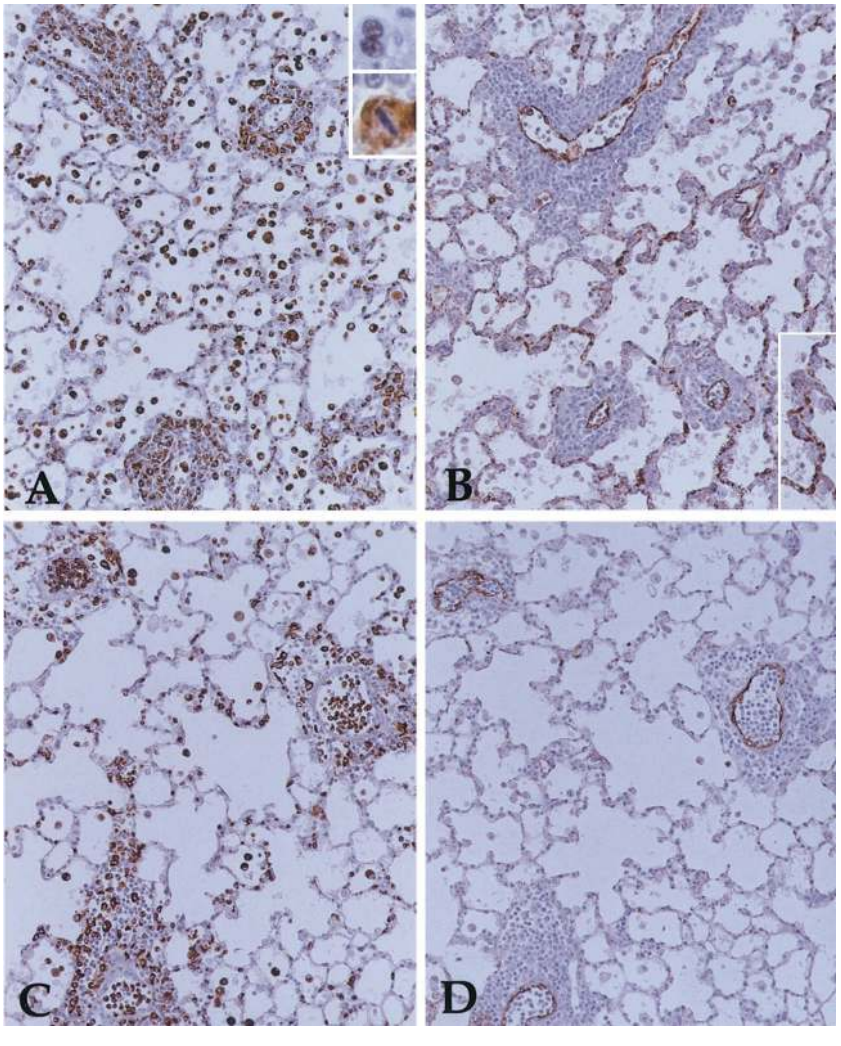

FIGURE 4. Immunoperoxidase stains of representative lung allografts harvested 7 days after transplantation from $C6^+$ to $C6^-$ and $C6^-$ to $C6^+$ groups (*A–D*; magnification, ×100). Immunoperoxidase stain for ED1 demonstrated numerous macrophages in the alveoli of $C6^+$ PVG.R8 lung allografts in $C6^-$ PVG.1U recipients, many of which were adherent to the septa and mitotically active (insets; mitotic figure in $ED1^+$ cell and PCNA stained binuclear cell; *A*). In contrast, relatively few macrophages were present in the alveoli of $C6^-$ lung allografts in $C6^+$ recipients, but intravascular accumulations of macrophages were prominent (*C*). Immunoperoxidase detected vWf in the dilated capillaries of the alveolar septa of $C6^+$ lung allografts in $C6^-$ recipients (inset, higher magnification; *B*), but not of $C6^-$ lung allografts in $C6^+$ recipients (*D*).

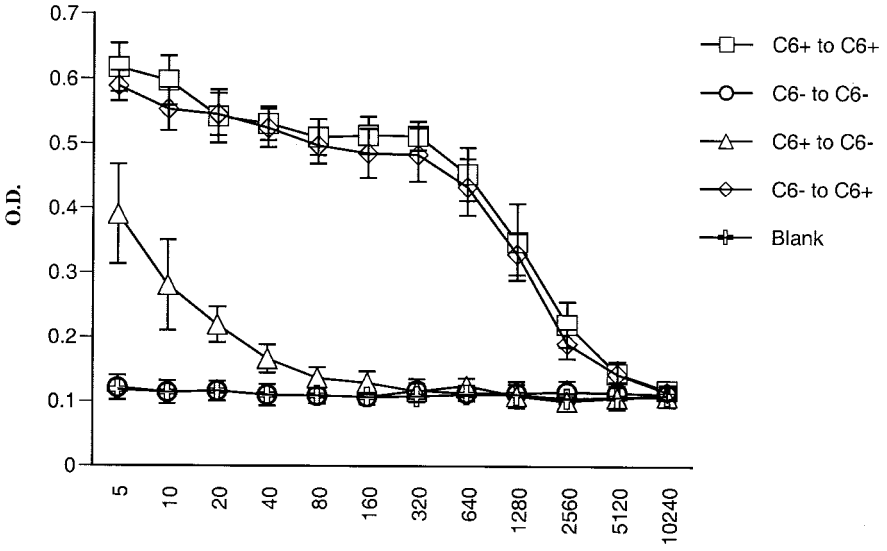

FIGURE 5. Titration curves in ELISA for C6 in sera from the four experimental groups. C6 levels were partially reconstituted in $C6^-$ recipients with $C6^+$ donor lung allografts. Each point represents the average of five animals, with SD shown as vertical bars.

Evidence of the C6 deficiency is based on quantitative measures and functional assays. C6 cannot be detected by ELISA (see Fig. 5) or Western blots of sera from C6-deficient rats (38). Functionally, purified human C6 restores the hemolytic activity to serum from C6-deficient rats in vitro (52) and in vivo (53). The C6-deficient rat has fully functional levels of C3 and C5 because human serum deficient for C3 or C5 restores hemolytic activity to C6-deficient rat serum in vitro (52). A complete deficiency of the C6 protein is further evidenced by our finding that C6-deficient rats recognize rat C6 as a foreign protein and produce a vigorous Ab response to C6 (54).

Our finding that these animals make vigorous IgM and IgG alloantibody responses to lung allografts in the present study agrees with our previous reports that Ab production to skin and heart allografts is normal in C6-deficient rats (5, 6). Similarly, the vigorous infiltration of lung allografts in C6-deficient recipients by macrophages and T lymphocytes in this study is in agreement with the fact that C6-deficient and -sufficient recipients reject skin allografts normally in vivo and have equivalent mixed lymphocyte responses in vitro (5, 20, 55).

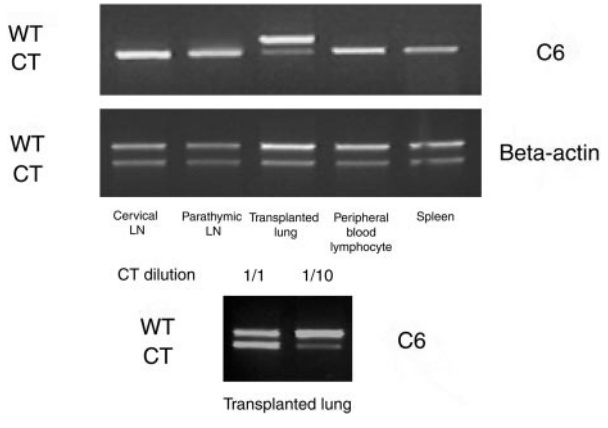

FIGURE 6. Competitive template RT-PCR analyses performed on tissues from a $C6^-$ recipient of a $C6^+$ donor lung allograft. Little C6 mRNA was detected in parathymic lymph nodes, cervical lymph nodes, spleen, and peripheral blood cells from the $C6^-$ recipient; Significant C6 mRNA was detected in the transplanted lung from the $C6^+$ donor. Expression of C6 and β-actin control mRNA was semiquantitated by the competitive template using the NIH Image 1.54 program.

The role of C6 and MAC in lung allograft rejection is of particular interest because the lung is known to be especially susceptible to complement-mediated injury. Pulmonary edema results from systemic activation of complement with cobra venom in experimental models or in clinical procedures such as cardiopulmonary bypass (23, 56). This injury has been attributed to biological effects of C5a and MAC. C5aR has been localized by immunohistology on bronchial and alveolar epithelial cells as well as vascular smooth muscle and endothelial cells of human lung tissue (26, 28, 57, 58). The potential for tissue activation through the C5aR has been verified for endothelial cells in culture. Foreman and colleagues (28) found that C5a could activate endothelial cells directly through C5aR and that this activation was augmented in the presence of TNF-α. Using C5aR knockout mice, Hopken et al. (59) have demonstrated a dominant role for the C5aR in the lung, but only a synergistic role together with other inflammatory mediators in immune complex-mediated peritonitis and skin injury.

The C6-deficient rats provide a means for separating the effects of MAC from those of C5a in lung allografts. In the absence of C6, lung allografts developed dense mononuclear cell infiltrates that were confined to the perivascular and peribronchiolar spaces where macrophages ($ED1^+$) and T cells ($CD8^+$) formed concentric layers that encircled the arteries and bronchioles. Cellular infiltrates have also been noted to remain limited to the perivascular compartment in experimental autoimmune encephalitis in C6-deficient rats (53). Although neutrophils and mononuclear cells accumulated intravascularly and marginated in the arteries, the vascular endothelial cell layer remained intact. The alveoli were free of exudates or hemorrhage, and the septa remained delicate and pristine. In these transplants vWf was confined to Weibel-Palade storage granules in endothelial cells of arteries. Scant vWf was detected in the alveolar septal capillaries.

In marked contrast, the availability of C6 to form MAC was accompanied by profuse release of vWf in arteries that entrapped layers of platelets and leukocytes. This is a characteristic feature of cardiac allograft rejection in these animals (7). Even sublytic quantities of isolated C5b-C9 cause the release of vWf from endothelial cells in vitro (29). Our in vivo observations suggest that MAC is pivotal in the activation and aggregation of platelets. vWf is one of the most potent activators of platelets that can be released by MAC. The vWf released by MAC is stored in Weibel-Palade bodies as a high m.w. multimer that mediates platelet adhesion in high

shear environments (60). Once activated, platelets themselves release additional vWf from their α storage granules.

We found that the source of C6 determined the focus of vWf release and the distribution of infiltrates. When the lung allograft was the sole source of C6, vWf release was concentrated in the alveolar capillaries. In contrast, when the recipient was the sole source of C6, vWf was released in the arteries.

We have demonstrated previously that macrophages can be a source of significant amounts of C6, and that macrophage production of C6 increases during cardiac allograft rejection (7). When the lung allograft was the sole source of C6, numerous activated macrophages were present in the alveoli. These macrophages and adjacent pulmonary epithelial cells were proliferating, as evidenced by frequent mitotic figures and positive staining for PCNA (Fig. 4). This finding is consistent with recent reports that deposition of sublytic amounts of MAC can induce DNA synthesis in rat mesangial cells in vitro (61). Niculescu and colleagues (62, 63) have reported that MAC stimulates mitosis in endothelial cells, B lymphocytes, and smooth muscle cells through phosphatidylinositol 3-kinase-induced STAT3 phosphorylation.

Our finding that local production of MAC is associated with the proliferation of alveolar macrophages and pulmonary epithelial cells provides a mechanism by which an immunologically stimulated lung can expand its capacity to produce complement locally. In contrast, when the allograft recipient was the sole source of C6, there was a paucity of activated macrophages in the alveoli. Instead, activated macrophages were a prominent component of the cells that expanded the congested pulmonary arteries in C6-competent recipients.

Both pulmonary macrophages and epithelial cells are potential sources of complement production. Although C6 production by pulmonary epithelial cells has not been assessed, bronchial epithelial and type II pneumocyte cell lines have been found to produce C3 and C5 (45–47). Complement synthesis by these cells is upregulated in response to IL-1and TNF-α. This may be particularly important to bronchial inflammation in acute and chronic rejection of lung allografts. In fact, local C3 production has been demonstrated to modulate the rejection of renal allografts (64).

Because vascular endothelial cells are the primary interface between donor tissues and recipient immune mediators, endothelial cells are the primary targets of acute rejection. Indeed, all of the International Society of Heart and Lung Transplantation grades of acute rejection include a vascular component of inflammation (65). In the present study the absence of C6 and the lack of MAC formation were demonstrated to decrease vascular injury.

In summary, C6 deficiency impedes vascular injury and alveolar hemorrhage, but not perivascular and peribronchiolar mononuclear cell infiltration or alloantibody production. When the allografted lung was the sole source of C6, pulmonary macrophages and epithelial cells proliferated in the alveoli in association with significant local C6 production. Thus, the source of C6 production determined the location of pulmonary injury.

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