Inhibition of complement, neutrophil, and platelet activation by an anti-factor D monoclonal antibody in simulated cardiopulmonary bypass circuits

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Objectives: Patients undergoing cardiopulmonary bypass frequently manifest generalized systemic inflammation and occasionally manifest serious multiorgan failure. Inflammatory responses of bypass are triggered by contact of blood with artificial surfaces of the bypass circuits, surgical trauma, and ischemia-reperfusion injury. We studied the effects of specific inhibition of the alternative complement cascade by using an anti-factor D monoclonal antibody (166-32) in extracorporeal circulation of human whole blood used as a simulated model of cardiopulmonary bypass.

Methods: Five healthy blood donors were used in the study. Monoclonal antibody 166-32 was added to freshly collected, heparinized human blood recirculated in a pediatric cardiopulmonary bypass circuit at a final concentration of 18 µg/mL. An irrelevant monoclonal antibody was used as a negative control with the same donor blood in a parallel bypass circuit on the same day. Blood samples were collected at different time points during recirculation for measurement of activation of complement, neutrophils, and platelets by immunofluorocytometric methods and enzyme-linked immunosorbent assays.

Results: Monoclonal antibody 166-32 inhibited the alternative complement activation and the production of Bb, C3a, sC5b-9, and C5a. Upregulation of CD11b on neutrophils and CD62P on platelets was also significantly inhibited by monoclonal antibody 166-32. This is consistent with the inhibition of the release of neutrophil-specific myeloperoxidase and elastase and platelet thrombospondin. The production of proinflammatory cytokine interleukin 8 was also suppressed by the antibody.

Conclusions: The alternative complement cascade is predominantly activated during extracorporeal circulation. Anti-factor D monoclonal antibody 166-32 is effective in inhibiting the activation of complement, neutrophils, and platelets. Inhibition of the alternative complement pathway by targeting factor D could be useful in reducing systemic inflammation in patients undergoing cardiopulmonary bypass.

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Patients undergoing cardiopulmonary bypass (CPB) frequently manifest a generalized systemic inflammatory response syndrome, which may contribute to perioperative tissue injury and impaired hemostasis.¹⁻⁶ Serious inflammatory conditions could lead to delayed recovery and occasionally to multiorgan dysfunction.^{1,2,7} Complement activation has been implicated as the most important cause of the

Figure 1. Selective inhibition of the alternative complement activity in human whole blood by MAb 166-32 at 18 $\mu\text{g/mL}$ in the extracorporeal circulation studies before recirculation. MAb 166-32 did not inhibit the classical complement activity of the human plasma in the circuits. The negative control MAb G3-519 had no effects on either the alternative or the classical complement activity. Values are given as means $\pm$ SD from 5 paired experiments of the test and control circuits. Percentage of hemolysis was defined as indicated in the text. *P* values were determined by means of paired *t* tests between MAb 166-32- and MAb G3-519-treated circuits in the alternative complement hemolytic assays.

systemic inflammatory reaction.^{1,3,4,6,8,9} It is attributed to the interaction between blood and the artificial surfaces of extracorporeal circuits constituting CPB machines.^{10,11} Primary inflammatory substances are generated after activation of the complement system, including the anaphylatoxins C3a and C5a, the opsonin C3b, and the membrane attack complex C5b-9.¹² C3a and C5a are potent stimulators of neutrophils, monocytes, and platelets.¹³⁻¹⁶ Activation of these cells results in release of proinflammatory cytokines (eg, interleukin 1 [IL-1], IL-6, IL-8, and tumor necrosis factor α), oxidative free radicals, and proteases.¹⁷⁻²² C5a has been shown to upregulate adhesion molecules CD11b (α -integrin) and CD18 (β_2 -integrin) of Mac-1 in polymorphonuclear cells (PMNs; comprising mainly neutrophils) and to induce degranulation of PMNs to release proinflammatory enzymes.^{15,16,23-27} C5b-9 induces the expression of adhesion molecule P-selectin (CD62P) on platelets,²⁸ whereas both C5a and C5b-9 induce surface expression of P-selectin on endothelial cells.²⁹ These adhesion molecules are involved in the interaction among leukocytes, platelets, and endothelial cells. The expression of adhesion molecules on activated endothelial cells is responsible for sequestration of activated leukocytes, which then mediate tissue inflammation and injury.^{24,29,30} In addition, C3a and C5a stimulate chemotaxis of human mast cells and trigger the release of histamine, proteases, and leukotrienes, which

Figure 2. Bb and C4d production in the extracorporeal circuits treated with MAb 166-32 or the negative control MAb G3-519. Bb (A) and C4d (B) were measured by using a quantitative ELISA. Values are given as means $\pm$ SD from 5 paired experiments of the test and control circuits. MAb 166-32 completely inhibited the production of Bb, indicating complete suppression of the alternative complement pathway. *P* values were determined by 2-factor analysis of variance. There is no significant difference in the plasma levels of C4d between the test and control circuits as analyzed with 2-factor analysis of variance.

induce vascular permeability, vasoconstriction, and tissue inflammation.^{31,32}

Factor D is the rate-limiting enzyme important for the activation and amplification of the alternative complement pathway.^{33,34} Inasmuch as the concentration of factor D in human blood is among the lowest of all soluble complement components (approximately 2 $\mu\text{g/mL}$),³³ factor D is a potential target for inhibiting the activation of the alternative complement cascade associated with inflammatory responses. Previously, specific inhibition of the alternative complement pathway was demonstrated with monoclonal antibodies (MAbs) to human factor D with moderate activity in complement-mediated hemolytic assays.³⁵ Polyclonal rab-

bit antibodies to mouse factor D were shown to inhibit alternative complement activity in mice.³⁶ We have generated a potent and specific MAb to human factor D, designated 166-32. The antibody inhibits human factor D at a molar ratio of about 1:2 in complement-mediated hemolytic assays.³⁷ The antibody was shown to protect isolated rabbit hearts from human complement-mediated injury, as manifested by an overall maintenance of myocardial function after challenge with human plasma.³⁷

Recirculation of human whole blood in extracorporeal bypass circuits has been used extensively as a simulated model to study the activation of complement, leukocytes, and platelets in CPB.^{15,16,28} The results of our extracorporeal circulation studies demonstrate that complete inhibition of the alternative complement cascade by anti-factor D MAb 166-32 suppresses activation of neutrophils and platelets, as well as inhibition of proinflammatory cytokine IL-8 production.

Methods

Design and Preparation of Extracorporeal Circuits

In the study whole blood was obtained from 5 healthy volunteers after approval by the Human Investigation Committee of Baylor College of Medicine. Informed consent was obtained from each participant. Whole blood from the same donor was used in both test and negative control circuits on the same day to avoid any variations in complement activity in donor blood.

Extracorporeal circuits were assembled by using a hollow-fiber pediatric membrane oxygenator with an integrated heat exchanger module (D 901 LILLPUT 1; DIDECON, Mirandola, Italy), a pediatric venous reservoir with an integrated cardiectomy filter (D 752 Venomicard; DIDECON), a perfusion tubing set (Sorin Biomedical, Inc, Irvine, Calif), and a multistage roller pump (Stöckert Instruments GmbH, Munich, Germany). Oxygenator and circuitry were primed with lactated Ringer's solution (Baxter Healthcare Corp, Deerfield, Ill). The prime fluid was warmed to 32°C with a cooler-heater (Sarns/3M Health Care, Ann Arbor, Mich) and circulated at 500 mL/min, and the sweep gas flow was maintained at 0.25 L/min with 100% oxygen. The sweep gas was changed to a mixture of oxygen (95%) and carbon dioxide (5%) after the blood was added to the circuit. The pH, PCO₂, PO₂, and perfusate temperature were continuously monitored throughout the recirculation period. Sodium bicarbonate was added as required to maintain pH in the range of 7.25 to 7.40.

Extracorporeal Circuit Operation and Blood Sampling

Blood (250 mL) was drawn over the course of 5 to 10 minutes into a transfer pack (Haemo-Pak; Charteredmed, Inc, Lakewood, NJ) from healthy volunteers taking no medications. Before blood collection, porcine heparin (Elkins-Sinn, Cherry Hill, NJ) and anti-factor D MAb 166-32 (mouse immunoglobulin G₁) were added to a transfer pack at final blood concentrations of 5 U/mL and 18 µg/mL, respectively. This concentration of the antibody is equivalent to about 1.5 times the molar concentration of factor D in the blood. For the negative control, an isotype-matched irrelevant

Figure 3. C3a, sC5b-9, and C5a production in the extracorporeal circuits treated with MAb 166-32 or the negative control MAb G3-519. C3a (A), sC5b-9 (B), and C5a (C) were measured by means of a quantitative ELISA. MAb 166-32 inhibited the production of C3a, sC5b-9, and C5a in the extracorporeal circuits compared with that in the MAb G3-519 circuits. Values are given as means $\pm$ SD from 5 paired experiments of the test and control circuits. *P* values were determined by 2-factor analysis of variance.

Figure 4. Inhibition of CD11b expression on PMNs in the extracorporeal circuits treated with MAb 166-32 compared with the circuits treated with the negative control MAb G3-519. The expression level of CD11b was measured by means of an immunofluorocytometric method and is expressed as the percentage of mean channel fluorescence intensity relative to the value at time 0 (100%). Values are given as means $\pm$ SD from 5 paired experiments of the test and control circuits. *P* values were determined by 2-factor analysis of variance.

MAb (G3-519) was used at the same final concentration. MAb G3-519 (mouse immunoglobulin G₁) is specific to HIV-1 external envelope protein gp120. MAb G3-519 has previously been shown to have no effects on human complement activity.³⁷ The collected blood was then added to the reservoir through the prime port. Prime fluid was simultaneously withdrawn distal to the oxygenator outlet to yield a final circuit volume of 350 mL and a final hematocrit level of 25% to 28%. The pump flow rate was maintained at 500 mL/min, and the line pressure of the circuits was maintained at about 50 mm Hg throughout the entire experiment. Blood was recirculated with prime fluid, and complete mixing was accomplished within 3 minutes; a baseline sample was drawn and designated as time 0. In order to mimic the usual procedures of surgical operation under hypothermia, the circuit was then cooled to 27°C for 70 minutes, after which it was rewarmed to 37°C at 80 minutes. The temperature was maintained until 120 minutes, when the recirculation was terminated.

Blood samples were collected at 5, 10, 25, 40, 55, 70, 80, and 120 minutes during the recirculation. Plasma samples were prepared by immediate centrifugation at 2000g at 4°C. Aliquots of plasma were snap-frozen on dry ice and then stored at -80°C. The plasma samples were used for complement-mediated hemolytic assays and quantitation of neutrophil-specific myeloperoxidase and elastase, platelet thrombospondin, C5a, and IL-8. Aliquots of plasma for measurement of complement products C3a, C4d, sC5b-9, and Bb by means of enzyme-linked immunosorbent assay (ELISA) were immediately mixed with an equal volume of a specimen-stabilizing medium (Quidel, San Diego, Calif), snap-frozen on dry ice, and then stored at -80°C. Samples of whole blood were also collected for immunostaining of the activation cell-surface markers CD11b and CD62P on neutrophils and platelets, respectively. To prevent subsequent complement activation of the whole

A

B

Figure 5. Inhibition of myeloperoxidase (MPO) and elastase release from neutrophils in the extracorporeal circuits treated with MAb 166-32 compared with that in the circuits treated with the negative control MAb G3-519. Myeloperoxidase (A) and elastase- α_1 -antitrypsin inhibitor complex (B) were measured by a quantitative ELISA. Elastase released from activated neutrophils is quickly bound to α_1 -antitrypsin inhibitor in the blood. Values are given as means $\pm$ SD from 5 paired experiments of the test and control circuits. *P* values were determined by 2-factor analysis of variance.

blood samples during the staining procedure, we added 10 μ L of 1 mol/L ethylenediamine tetra-acetic acid to every milliliter of whole blood to give a final concentration of 10 mmol/L.

Complement-Mediated Hemolytic Assays

The alternative complement activity in the plasma samples from MAb 166-32-treated and MAb G3-519-treated circuits was measured with the use of rabbit red blood cells (RBCs). The plasma samples were treated with ethylene glycol-bis-(β -aminoethyl ether)-N,N,N',N'-tetra-acetic acid (EGTA) at a final concentration of 50 mmol/L to chelate Ca⁺⁺ for inhibition of the classical complement. Thirty microliters of rabbit RBC suspension (1.7×10^8 cells/mL) in gelatin/veronal-buffered saline solution (GVB/Mg-EGTA) containing 2 mmol/L MgCl₂ and 50 mmol/L EGTA were added to 100 μ L of human plasma (final plasma concentration,

77%). After incubation at 37°C for 30 minutes, the supernatants were collected, and optical density (OD) was read at 405 nm by using an ELISA plate reader. This OD reading was designated as OD_A. One hundred microliters of each plasma sample was mixed with 30 µL of GVB/Mg-EGTA to give the plasma color background (OD_B). For 100% hemolysis, 100 µL of water was mixed with 30 µL of rabbit RBC suspension. This OD reading was designated as OD₁₀₀. The percentage of hemolysis was determined by the following formula:

$$\text{Percentage hemolysis} = 100 \times (\text{OD}_A - \text{OD}_B) / \text{OD}_{100}$$

Spontaneous lysis of RBCs was negligible.

For classical pathway hemolysis, chicken RBCs (5×10^7 cells/mL) in GVB⁺⁺ containing 0.5 mmol/L MgCl₂ and 0.15 mmol/L CaCl₂ were sensitized with purified antichick RBC immunoglobulins at 8 µg/mL (InterCell Technologies, Hopewell, NJ) for 15 minutes at 4°C. The cells were then washed with GVB⁺⁺. The plasma samples were not treated with EGTA. The remaining procedures were identical to those for alternative pathway hemolysis described above.

Assays of Complement Activation Products

Plasma samples from the extracorporeal circuits were tested for the levels of Bb, C4d, C3a, and sC5b-9. Bb is a specific marker for the activation of the alternative complement pathway, whereas C4d is for the classical complement pathway. These substances were measured by means of commercially available quantitative ELISA kits (Quidel) according to the manufacturer's manuals.

Assays for Expression of CD11b on Neutrophils and CD62P on Platelets

The activation of neutrophils and platelets was quantitated by measuring the levels of the cell-surface expression of CD11b and CD62P on neutrophils and platelets, respectively. For CD11b labeling of neutrophils, 100 µL of whole blood collected from the circuits was immediately incubated with 20 µL of phycoerythrin-anti-CD11b antibody (Becton Dickinson, San Jose, Calif) for 10 minutes at room temperature. Then 1.4 mL of FACS Lysing solution (Becton Dickinson) was added for 10 minutes at room temperature to lyse RBCs and to fix leukocytes. The cells were then washed in phosphate-buffered saline solution and then fixed in 1% paraformaldehyde before analysis with an EPIC-XL flow cytometer (Coulter Corp, Miami, Fla). For double labeling to concomitantly identify the neutrophil population, fluorescein isothiocyanate (FITC)-anti-CD15 antibody (Becton Dickinson) was added for incubation together with phycoerythrin-anti-CD11b antibody.

For CD62P labeling of platelets, 40 µL of whole blood collected from the circuits was incubated with 20 µL of phycoerythrin-anti-CD62P antibody (Becton Dickinson) for 10 minutes at room temperature. Then the mixture was treated with FACS Lysing solution as described above. The platelets were washed in phosphate-buffered saline solution, fixed in 1% paraformaldehyde, and then analyzed as described above. For double labeling to concomitantly identify the platelet population, FITC-anti-CD41 antibody (Coulter) was added for incubation together with phycoerythrin-anti-CD62P antibody.

For flow cytometric measurement, PMN and platelet populations were identified by live gating on the basis of forward-scatter

Figure 6. Inhibition of CD62P expression on platelets in the extracorporeal circuits treated with MAb 166-32 compared with the circuits treated with the negative control MAb G3-519. The percentages of CD62P⁺ platelets (A) and the CD62P expression level in mean fluorescence intensity on platelets (B) are represented relative to the values at time 0 (100%). Values are given as means $\pm$ SD from 5 paired experiments of the test and control circuits. *P* values were determined by 2-factor analysis of variance.

versus side-scatter parameters and specific staining with FITC-anti-CD15 antibody and FITC-anti-CD41 antibody, respectively. The background staining was gated by means of isotype-matched labeled antibodies. The intensity of expression of CD11b and CD62P was represented by the mean fluorescence intensity.

Assay of C5a

C5a was measured quantitatively with an ELISA. Plasma samples were first treated with a precipitating reagent from a C5a radioimmunoassay kit (Amersham, Arlington Heights, Ill) to remove high-molecular-weight proteins, including C5. In the C5a ELISA purified rabbit anti-C5a antibodies (Calbiochem, La Jolla, Calif) were coated on wells of microtest plates to immobilize C5a in plasma samples. Purified sheep anti-human C5 antibodies (Biodesign, Kennebunk, Maine) were used to capture the immobilized C5a. The bound sheep antibodies were then detected by using horseradish peroxidase rabbit anti-sheep immunoglobulin G (Fc) antibodies (Jackson ImmunoResearch, West Grove, Pa). Peroxidase sub-

Figure 7. Inhibition of thrombospondin release from platelets in the extracorporeal circuits treated with MAb 166-32 compared with that in the circuits treated with the negative control MAb G3-519. Thrombospondin was measured by means of a quantitative ELISA. Values are given as means $\pm$ SD from 5 paired experiments of the test and control circuits. *P* values were determined by 2-factor analysis of variance.

strate solution was added for color development, and OD₄₅₀ was measured by means of an ELISA plate reader. Purified C5a (Advanced Research Technologies, San Diego, Calif) was used as the standard for calibration.

Assay of Neutrophil-Specific Myeloperoxidase

Activation of neutrophils was also measured with an ELISA kit (R&D Systems, Inc, Minneapolis, Minn) to quantitate the amount of neutrophil-specific myeloperoxidase in the plasma samples from the extracorporeal circuits. The assays were performed according to the manufacturer's manual.

Assay of Elastase- α_1 -Antitrypsin Inhibitor Complex

A quantitative ELISA was used to measure neutrophil-specific elastase- α_1 -antitrypsin inhibitor complex, as described previously.¹⁶ On activation, neutrophils release elastase, which is immediately complexed with α_1 -antitrypsin inhibitor in the blood.¹⁶ The assay used sheep anti-human neutrophil elastase polyclonal antibodies (Bioscience) to immobilize the complex and horseradish peroxidase-conjugated sheep anti- α_1 -antitrypsin inhibitor polyclonal antibodies (Bioscience) for detection. As a calibration standard, elastase- α_1 -antitrypsin inhibitor complex was made by mixing neutrophil-specific elastase (Elastin Products Company, Inc, Owensville, Mo) with α_1 -antitrypsin inhibitor (Calbiochem).

Assay of Platelet Thrombospondin

A quantitative ELISA was used to measure platelet thrombospondin, which is an α -granule glycoprotein involved in platelet aggregation and is released on activation. In the assay thrombospondin was captured by using a pair of noncompetitive antithrombospondin MABs, as described previously.³⁸ Monoclonal antithrombospondin antibody (clone P12, Coulter) was coated on wells of microtest plates to immobilize plasma thrombospondin. Biotinylated monoclonal antithrombospondin antibody (clone P10,

Coulter) was used to capture immobilized thrombospondin. Purified thrombospondin (Calbiochem) was used as a calibration standard in the assay.

IL-8 ELISA

IL-8 was measured by using a quantitative ELISA kit (R&D Systems).

Statistical Analysis

The data from 5 paired circuits were statistically analyzed and represented as means $\pm$ SD. The data of hemolytic assays were analyzed with paired *t* tests. A 2-factor analysis of variance in a randomized block design was used to analyze the data from the other assays.

Results

Selective Inhibition of the Alternative Complement Activity by MAb 166-32

In the extracorporeal circulation study, MAb 166-32 was added to heparinized human whole blood at a final molar concentration of 1.5 times that of factor D in the blood. An isotype-matched negative control MAB, G3-519, was also studied at the same concentration in a parallel circuit by using whole blood from the same donor on the same day. The alternative and classical complement activities in the blood treated with the antibodies before recirculation were measured by means of hemolytic assays with rabbit RBCs and sensitized chicken RBCs, respectively. In the alternative pathway hemolytic assays, plasma from MAB G3-519-treated circuits at a final concentration of 77% induced lysis of rabbit RBCs by 65%, whereas no hemolysis was observed with plasma from MAB 166-32-treated circuits (Figure 1). In contrast, neither MAB 166-32 nor MAB G3-519 inhibited the lysis of sensitized chicken RBCs through the classical complement pathway (Figure 1). Therefore, MAB 166-32 is a selective and potent inhibitor of the alternative complement pathway.

Complement Activation in Extracorporeal Circuits

The selective inhibition of the alternative complement pathway by MAB 166-32 is in agreement with the complete inhibition of the production of Bb throughout the entire duration of recirculation (Figure 2, A). Bb is the enzymatic product of factor B on proteolysis by factor D, and therefore it is a specific marker for the activation of the alternative complement pathway. On the other hand, there was an increase in the plasma concentration of C4d in both circuits treated with either MAB 166-32 or MAB G3-519 (Figure 2, B). C4d is a specific marker for activation of the classical complement pathway.

The plasma concentrations of C3a, sC5b-9, and C5a in the extracorporeal circuits during recirculation were also measured. MAB 166-32 strongly inhibited the production of these substances (Figure 3, A-C). The formation of sC5b-9

and C5a was completely inhibited during recirculation at hypothermic temperatures. At the end of 120 minutes of recirculation, MAb 166-32 inhibited the production of C3a, sC5b-9, and C5a by 67%, 77%, and 88%, respectively, compared with that of the negative control MAb G3-519. Because the alternative complement activity of the plasma in the test circuits was completely inhibited by MAb 166-32, the production of C3a, sC5b-9, and C5a in these circuits could be attributed to some activation of the classical pathway, as indicated by the elevation of plasma C4d levels (Figure 2, B).

Activation of Neutrophils in Extracorporeal Circuits

Activation of neutrophils as manifested by upregulation of surface expression of CD11b was measured by means of immunocytofluorometric analyses. The expression of CD11b on neutrophils in circuits treated with MAb 166-32 was significantly reduced compared with the negative control MAb G3-519 (Figure 4). At the end of 120 minutes of recirculation, MAb 166-32 inhibited CD11b expression on neutrophils by 57% compared with MAb G3-519.

The activation of neutrophils was also studied by measuring the levels of neutrophil-specific myeloperoxidase and elastase in plasma. The enzymes are stored in azurophilic primary granules of neutrophils and released on degranulation of activated neutrophils.²⁵⁻²⁷ Consistent with the inhibition of neutrophil activation as manifested by the upregulation of CD11b expression (Figure 4), the release of both myeloperoxidase and elastase was also significantly inhibited by MAb 166-32 in the extracorporeal circuits compared with in the MAb G3-519-treated circuits (Figure 5, A and B). At the end of 120 minutes of recirculation, MAb 166-32 inhibited the release of myeloperoxidase and elastase by 67% and 41%, respectively, compared with MAb G3-519.

Activation of Platelets in Extracorporeal Circuits

The effects of MAb 166-32 on platelet activation during recirculation in extracorporeal circuits were examined. The number of CD62P⁺ platelets and the level of CD62P expression were significantly reduced in MAb 166-32-treated circuits compared with in MAb G3-519-treated circuits (Figure 6, A and B). At the end of 120 minutes of recirculation, the number of CD62⁺ platelets and the level of CD62 expression were reduced by 45% and 54%, respectively, in MAb 166-32-treated circuits compared with in MAb G3-519-treated circuits. These results corroborate with the inhibition of thrombospondin release from activated platelets by MAb 166-32 treatment by 53% at the end of 120 minutes of recirculation compared with MAb G3-519 (Figure 7).

Production of IL-8 in Extracorporeal Circuits

To further examine the effects of complement inhibition on other inflammatory responses in whole blood during recir-

Figure 8. Inhibition of IL-8 production in the extracorporeal circuits treated with MAb 166-32 compared with that in the circuits treated with the negative control MAb G3-519. IL-8 was measured by a quantitative ELISA. Values are given as means $\pm$ SD from 5 paired experiments of the test and control circuits. P values were determined by 2-factor analysis of variance.

culation, we examined the production of the proinflammatory cytokine IL-8 in the test and control extracorporeal circuits. IL-8 is produced predominantly by activated neutrophils, monocytes-macrophages, and T cells. During the recirculation at hypothermic temperatures, the plasma levels of IL-8 did not increase, even in MAb G3-519-treated circuits. However, the plasma levels of IL-8 increased dramatically when the circuits were warmed to 37°C. At the end of 120 minutes of recirculation, IL-8 production in MAb 166-32-treated circuits was significantly reduced by 73% compared with MAb G3-519-treated circuits (Figure 8).

Discussion

The complement cascade is activated predominantly through the alternative or the classical pathway.¹² Activation of the alternative complement pathway is induced through the direct activation of C3, whereas the classical complement pathway is activated through the initial activation of C1. Recent studies show that complement can also be activated through the lectin pathway, which involves the initial binding of mannose-binding lectin and the subsequent activation of C2 and C4, which are common to the classical complement pathway.^{39,40} Interestingly, accumulating evidence indicates that the alternative complement pathway participates in the amplification of the activity of both the classical complement and the lectin pathways as a result of C3b deposition, which triggers the formation of the alternative C3/C5 convertases.^{40,41} Therefore, the alternative complement pathway may play a broad role in inflammation resulting from complement activation.

In CPB circuits the alternative complement pathway plays a predominant role in complement activation, result-

ing from the interaction of blood with the artificial surfaces of CPB circuits.^{1,6,11} However, natural human antibodies against dialysis membranes have been described, and it is possible that natural antibodies against components of the CPB circuits or membrane could be responsible for the C4d production in our study.⁴² Interestingly, the alternative complement pathway was shown to play a crucial role in the amplification of the classical complement pathway once it was initiated.⁴² In addition, activation of the classical complement pathway in CPB circuits could also be triggered by means of contact activation of the intrinsic coagulation pathway.^{10,43,44} As a result, factor XIIa, factor XIa, and kallikrein bind C1 esterase inhibitor, an endogenous inhibitor of activated C1. When the plasma concentration of C1 esterase inhibitor is diminished, C1 becomes more susceptible to activation, and thus the classical complement pathway is augmented. Inasmuch as the production of C5a and sC5b-9 was substantially inhibited compared with that of C3a in our study, the moderate activation of the classical pathway as indicated by the formation of C4d during hypothermic recirculation does not seem to effectively activate C5. Similarly, although protamine neutralization of heparin after CPB causes activation of the classical complement pathway,^{6,45} the activation of C5 by protamine-heparin complexes is found to be ineffective.^{6,8} Using anti-factor D MAb 166-32 to selectively inhibit the alternative complement pathway, we confirm the previous findings that the alternative complement pathway is predominantly activated in CPB circuits.^{1,6,8}

The results from our study show that anti-factor D MAb 166-32 is a potent and specific inhibitor of the alternative complement pathway. At a concentration (18 µg/mL) equivalent to 1.5 times the molar concentration of factor D in human blood, MAb 166-32 completely inhibits the alternative complement activity, as evidenced by the complete blockade of Bb formation in the CPB circuits. Inhibition of the alternative complement pathway by anti-factor D MAb 166-32 suppresses the production of C3a, sC5b-9, and C5a. These substances are known to activate neutrophils, monocytes, platelets, mast cells, and endothelial cells.^{13-16,28,29} Reduction of CD11 (α-integrin) expression on neutrophils prevents their binding to platelets and endothelial cells through the interaction with CD62P on these cells.^{46,47} Sequestration of activated neutrophils on endothelial cells can result in tissue inflammation and injury.^{24,29,30} It has been shown that CD11b expression on monocytes is increased by C3a but not C5a and sC5b-9 in CPB circuits.^{15,16} Therefore, the reduction of C3a production by MAb 166-32 in our study could be important for controlling monocyte adhesion. Although the exact roles of activated monocytes in the inflammatory response of CPB have yet to be delineated, it is likely that proinflammatory cytokine release and tissue factor-mediated thrombosis are

involved.^{13,48} Because CR3 (Mac-1; CD11/CD18) on leukocytes can bind to iC3b on complement-activated surfaces in CPB circuits,⁴⁹ this could contribute to the depletion of leukocytes commonly observed in patients undergoing CPB.^{15,44} Inhibition of CD11b upregulation by MAb 166-32 in our study could prevent the loss of leukocytes during extracorporeal circulation.

The results from our study also show that inhibition of the alternative complement pathway by anti-factor D MAb 166-32 suppresses the degranulation of neutrophils, as indicated by the reduced release of myeloperoxidase and elastase. Myeloperoxidase is responsible for production of hypochlorous acid, a potent oxidant that could cause tissue damage, whereas elastase could mediate tissue injury as a result of hydrolysis of elastin in extracellular matrices.²⁵⁻²⁷ The incomplete inhibition of neutrophil degranulation by anti-factor D MAb 166-32 in this study might be attributed to contact activation, which results in the production of kallikrein, a mediator that is known to activate neutrophils to release elastase.⁵⁰ Similarly, platelets could be activated by thrombin as a result of initiation of the intrinsic coagulation pathway after contact activation.⁵¹

IL-8 is closely associated with inflammatory diseases and CPB.¹⁹⁻²² Our results show that anti-factor D MAb reduces the release of IL-8. IL-8 causes chemotaxis of neutrophils, T cells, and basophils; degranulation of neutrophils; and adhesion of neutrophils to endothelial cells.³⁰ Therefore, the reduction of IL-8 production could reduce multiple inflammatory responses observed in CPB. Delayed increase of plasma IL-8 in our extracorporeal circulation study is consistent with the observation in operations involving CPB.^{19,21,22}

One limitation of the simulated extracorporeal circulation model of CPB used in this study is the inability to study the inflammatory responses of endothelial cells caused by complement activation. In this regard, both C5a and C5b-9 were shown to induce surface expression of P-selectin on endothelial cells.²⁹

Several complement inhibitors are being studied for potential applications in CPB. They include a recombinant soluble complement receptor 1 (sCR1),⁵² a humanized single-chain anti-C5 antibody (h5G1.1-scFv),⁵³ a recombinant fusion hybrid (CAB-2) of human membrane cofactor protein and human decay accelerating factor,¹⁶ and a 13-residue C3-binding cyclic peptide (Compstatin).⁵⁴ sCR1 and CAB-2 inhibit the classical and alternative complement pathways at the steps of C3 and C5 activation. Compstatin inhibits both complement pathways at the step of C3 activation, whereas h5G1.1-scFv does so only at the step of C5 activation. The results of our study strongly indicate that the alternative complement activation plays an important role in inducing a plethora of cellular and humoral inflammatory responses in CPB. Anti-factor D MAb 166-32 is effective in

inhibiting the activation of C3 and C5 and the other inflammatory responses in extracorporeal circulation. Therefore, complete inhibition of the alternative complement pathway by targeting factor D could be useful to reduce systemic inflammation in patients undergoing CPB.

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