Novel Anti-Factor D Monoclonal Antibody Inhibits Complement and Leukocyte Activation in a Baboon Model of Cardiopulmonary Bypass

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Background. Adverse outcomes after cardiopulmonary bypass (CPB) are often related to systemic inflammation triggered by complement and leukocyte activation. To determine how inhibition of the alternative complement pathway affects systemic inflammation and tissue injury, we studied a novel monoclonal antibody (Mab), anti-human factor D murine Mab 166-32, in baboons.

Methods. Fourteen baboons (mean weight, 15 kg) underwent hypothermic CPB. The treatment group (n = 7) received a single injection of anti-factor D Mab 166–32 (5 mg/kg), and the control group (n = 7) was given saline solution. After initiation of CPB, all animals were subjected to 20 minutes of core cooling (rectal temperature, 27°C), followed by 60 minutes of aortic cross-clamping, 25 minutes of rewarming, and 30 minutes of normothermic CPB. Blood samples were collected before CPB, during CPB, and 1, 2, 3, 6, and 18 hours after CPB. To measure neutrophil and monocyte activation, we performed flow cytometry for CD11b expression, ELISA for complement activation (Bb, C3a, C4d, and sC5b-9) and interleukin-6 (IL-6) production, and tissue injury studies for creatine kinase MB isoenzymes (CK-MB), creatine kinase (CK), and lactic dehydrogenase (LDH) levels.

Results. Anti-factor D Mab almost completely inhibited plasma Bb, C3a, and sC5b-9 production during CPB ($P < .001$). CD11b expression on neutrophils ($129 \pm 5\%$ vs. $210 \pm 42\%$; $P = .0006$) and on monocytes ($139 \pm 14\%$ vs. $245 \pm 43\%$; $P = .0002$) was also lower in the treatment group during CPB. The treated animals had a significantly smaller increase in plasma IL-6 concentrations than did the control animals (71 ± 27 pg/mL vs. 104 ± 54 pg/mL; $P = .0002$). CK-MB levels were also lower in the treatment group 6 hours after the end of CPB (204 ± 30 vs. 335 ± 59 IU/L; $P = .003$) and 18 hours after the end of CPB ($P < .05$). Creatine kinase levels (6 and 18 hours after the end of CPB) and LDH levels (3 and 6 hours after the end of CPB) showed patterns similar to those of CK-MB ($P < .05$).

Conclusions. The alternative complement pathway plays a major role in systemic inflammation during CPB. Inhibition of complement activation via the alternative pathway by anti-factor D Mab 166–32 significantly reduces leukocyte activation and tissue injury in our baboon model.

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A ccumulating evidence indicates that cardiac surgery induces systemic inflammatory responses, particularly when cardiopulmonary bypass (CPB) is used [1–5]. CPB induces complex inflammatory responses characterized by the activation of complement, leukocytes, platelets, and cytokine production. These systemic responses are attributed to several factors, including exposure of blood to nonphysiologic surfaces of the heart-lung circuit, ischemia-reperfusion of the involved tissues, surgical trauma and hypothermia [6–8]. Systemic inflammation causes many postoperative complications, including vital organ dysfunction that can lead to multiorgan failure and even death [2, 6–10]. It also results in prolonged hospitalization and increased hospital costs. The intensity of the inflammatory response appears to be directly correlated with the severity of CPB-related morbidity. Currently, there is no effective method for preventing this systemic inflammatory response syndrome in cardiac surgery patients, although some have attempted to ameliorate this with heparin-bonded circuits, leukocyte filters, or modified ultrafiltration.

The complement cascade is activated by 3 different pathways: the alternative, classical [11], and lectin [12] pathways. The alternative pathway plays a major role in complement activation because it amplifies the classical and lectin pathways [12–14]. Therefore, inhibition of the alternative complement pathway may help minimize

Doctors Fung and Lu disclose that they have a financial relationship with Tanox, Inc.

complement activation during CPB more than would inhibition of the classical or lectin pathways. Factor D is an ideal target for inhibiting the alternative complement pathway because factor D is a rate-limiting enzyme that is essential for activation and amplification of the alternative complement pathway. Compared with other soluble complement components, factor D has an extremely low concentration in the blood (about 2 μg/mL) [11].

Previously, we clearly documented that anti-factor D monoclonal antibody (Mab) 166-32 significantly inhibits the alternative complement pathway, including the production of Bb, C3a, sC5b-9, and C5a, and upregulation of CD11b on neutrophils and of CD62P on platelets; the release of neutrophil-specific elastase and myeloperoxidase; the production of pro-inflammatory cytokine interleukin (IL)-8; and the release of thrombospondin from platelets in a simulated CPB circuit using human blood [15]. The purpose of the present study was to investigate the effects of a novel Mab, anti-factor D Mab 166-32, on complement and leukocyte activation and tissue injury in a baboon model during and after CPB. We hypothesized that anti-factor D would inhibit complement and leukocyte activation and, therefore, reduce tissue injury.

Material and Methods

Fourteen healthy adult baboons (mean body weight, about 15 kg) were used in the study. All the animals received humane care according to the "Guide for the Care and Use of Laboratory Animals" of the National Research Council (National Academy Press, revised 1996). The baboons were pre-screened for negative serological reactivity with Mab 166-32 by ELISA.

Anesthesia and Surgery

Upon transfer from a squeeze cage to preoperative holding area, each baboon was sedated with an intramuscular injection of 10 mg/kg of ketamine hydrochloride. A 20 gauge angiocatheter was placed in the cephalic vein, and 5.0 mg of diazepam were administered intravenously. The oral and nasal airways were masked with 1.5% to 3.0% isoflurane for anesthesia induction. Intubation was achieved with a size 6 endotracheal tube. Anesthesia was maintained with 0.80% to 2.25% isoflurane, 100% O_2, and an inspiratory tidal volume of 13 mL/kg at a rate of 13 breaths per minute throughout the surgical procedure except during CPB. Pancuronium bromide (Pavulon), 0.1 mg/kg, was administered to achieve adequate muscle paralysis.

Carotid artery and internal jugular vein were exposed for continuous arterial pressure monitoring and recording and volume administration/antibody injection, respectively. After a median sternotomy, the pericardium was opened, and the ascending aorta and the right atrium were cannulated with a 14-French aortic cannula (Argyle, St. Louis, MO) and a 24 or 28-French single-stage venous cannula (Polystan A/S, Varlose, Denmark), respectively. Heparin (300 units/kg) was administered for anti-coagulation. The CPB circuit included a conventional non-pulsatile roller pump (Stöckert, Irvine, CA, USA), hollow-fiber membrane oxygenator (Capiox SX10; Terumo Corp., Tokyo, Japan), arterial filter (Terumo), and

pediatric tubing set (Baxter/Bentley, Irvine, CA). Lactated Ringer's solution was then used to prime the extracorporeal circuit. The priming volume for the whole circuit was approximately 600 ml. This low-prime circuit was used to avoid the need of donor blood. During CPB, the hematocrit was maintained at 24% to 26%. The pump flow rate was maintained at 100 ml/kg/min. During CPB, the mean arterial pressure was maintained at 50 - 60 mm Hg by adding isoflurane through the oxygenator as an inflow conduit. At the beginning of hypothermic CPB, a total dose of 200 cc of crystalloid cardioplegia (modofied Kirklin solution) was administered into the aortic root via a Conducer blood cardioplegia circuit (Terumo/Sarns, Somerset, NJ) for 5 minutes. At the end of CPB, protamine (1 mg/100 units of heparin) was administered for heparin neutralization. The heart rate and systemic arterial pressure were continuously monitored. At the end of the experiment, each animal was euthanized with an intravenous bolus of Beuthanasia-D (0.22 mg/kg).

Experimental Design and Blood Sampling

Of the 14 baboons, 7 animals (the treatment group) received a single injection of anti-factor D Mab 166-32 (5 mg/kg) (Tanox, Inc., Houston, TX), and the other 7 animals (the control group) were given saline solution. We chose this dosage of Mab 166-32 because we estimated that it would be sufficient to completely neutralize the factor D for about 7 hours, based on data from a separate study in which Mab 166-32 (1 mg/kg) completely inhibited factor D in rhesus monkeys for at least 1.5 hours. Mab 166-32 was found to inhibit baboon and monkey factor D as well as it inhibited human factor D.

After initiation of CPB, all animals were subjected to 20 minutes of core cooling (rectal temperature, 27°C), followed by 60 minutes of aortic cross-clamping, 25 minutes of rewarming, and 30 minutes of normothermic CPB. Eight of the experiments (4 in each group) were terminated 6 hours after the end of CPB; the remaining 6 experiments (3 in each group) were terminated 18 hours after the end of CPB.

Blood samples were taken from the animals for assays at different time points: Before injection of Mab 166-32 or saline (at 0 hour), after injection of Mab 166-32 or saline (at 45 minutes), before CPB (at 1 hour), 10 minutes in CPB at 37°C, 25 minutes in CPB at 27°C, 85 minutes in CPB at 27°C, 105 minutes in CPB at 37°C after re-warming, 135 minutes in CPB at 37°C prior to protamine administration (at 3.25 hours), 30 minutes off CPB at 37°C, 1 hour off CPB at 37°C, 2 hours off CPB at 37°C, 6 hours off CPB at 37°C (at 9.25 hours), and finally 18 hours off CPB at 37°C (at 21.25 hours).

Plasma and whole blood samples were used in different assays based on the procedures described earlier [15]:
(a) Plasma concentration of free Mab 166-32 was measured by ELISA using factor D as coating antigen.
(b) Functional activity of factor D in plasma was measured by two hemolytic assays: rabbit red blood cells for the alternative complement pathway and sensitized chicken red blood cells for the classical complement pathway.
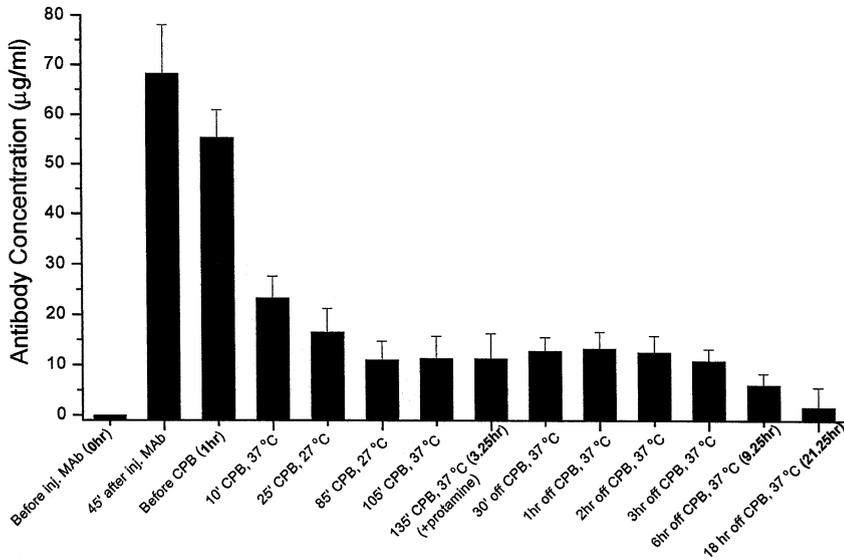(c) Plasma concentrations of complement Bb, C4d, and

Fig 1. Plasma concentration of free Mab 166-32 by ELISA using factor D as coating antigen (n = 7, mean ± SEM). (CPB = cardiopulmonary bypass; Inj = *injection of.)*

C3a were measured by ELISA (Quidel Corp., San Diego, CA), and sC5b-9 by ELISA [16].

(d) Expression of CD11b on neutrophils and monocytes was measured by immunofluorocytometric methods.

(e) Plasma concentrations of IL-6 was measured by ELISA (BioSource International, Inc., Camarillo, CA).

(f) Plasma concentration of lactic dehydrogenase (LDH), creatine kinase (CK), creatine kinase MB isoenzymes (CK-MB), creatinine, and blood urea nitrogen (BUN) were measured by standard blood chemistry assays.

Statistical Analysis

Two-sided ANOVA with repeated measures was used for statistical analysis between control and antibody groups. Pre-planned comparisons between control and treated groups within each experimental stage were tested using contrasts. No correction for multiple comparisons were made. A p value less than 0.05 was considered statisti-

cally significant. All results were expressed as mean ± standard error of mean (SEM).

Results

Pharmacokinetics of Mab 166-32

The plasma concentrations of the free antibody were measured by ELISA using human factor D as coating antigen (Fig 1). At 45 minutes after the antibody injection, the plasma concentration of free Mab 166-32 was 68.3 ± 9.9 μg/ml. The antibody concentration then decreased to 23.4 ± 4.4 μg/ml at 10 minutes after CPB, as a result of hemodilution upon the initiation of CPB. The antibody concentration remained at 10–13 μg/ml until 3 hours after CPB. The antibody concentration was reduced to 6.2 ± 2.3 μg/ml at 6 hours after CPB and to 1.7 ± 3.9 μg/ml at 18 hours after CPB.

Using an alternative complement hemolytic assay with rabbit red blood cells, the functional activity of factor D in the plasma samples from the Mab 166-32 treated animals was measured (Fig 2). The alternative complement he-
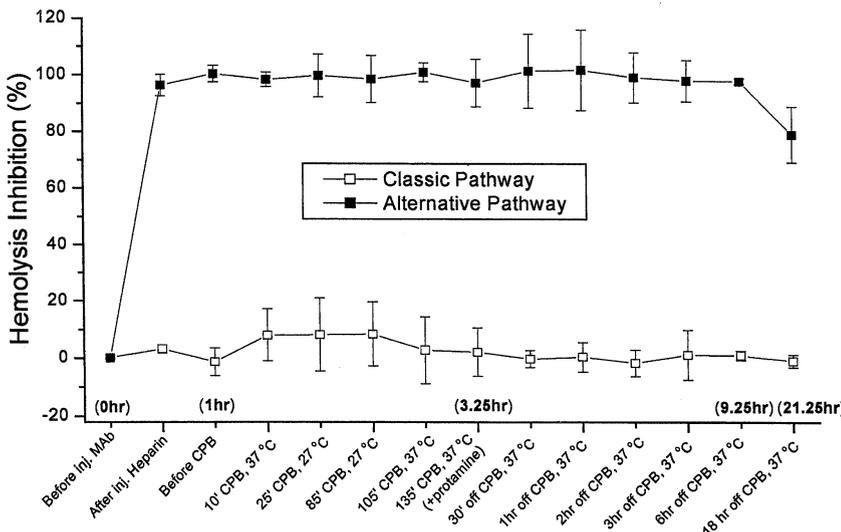

Fig 2. Functional activity of factor D in baboon plasma by two hemolytic assays: rabbit red blood cells for the alternative complement pathway and sensitized chicken red blood cells for the classical complement pathway (n = 7 in each group, mean ± SEM). (CPB = cardiopulmonary bypass; Inj = *injection of.)*
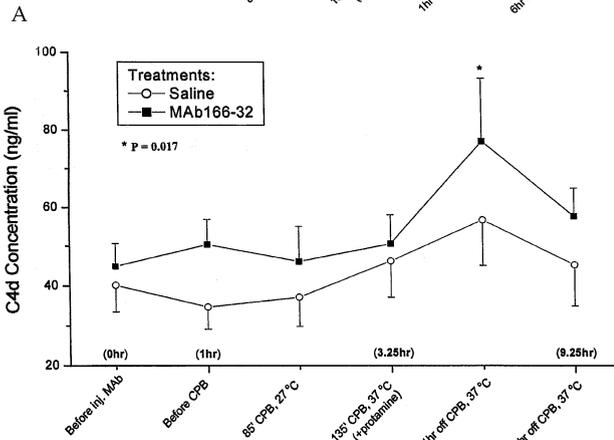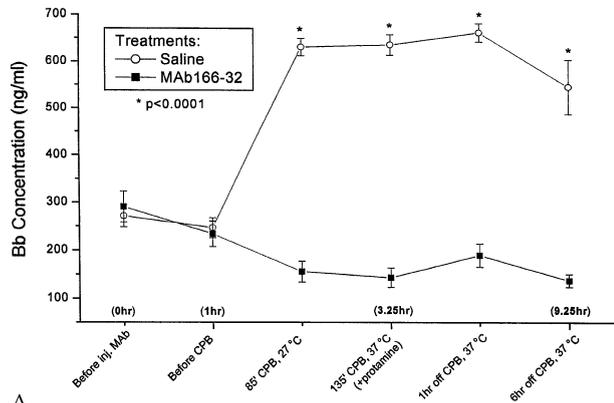
Fig 3. (A) Plasma Bb production (n = 7 in each group, mean ± SEM). (B) Plasma C4d production (n = 7 in each group, mean ± SEM). (CPB = cardiopulmonary bypass; Inj = injection of.)
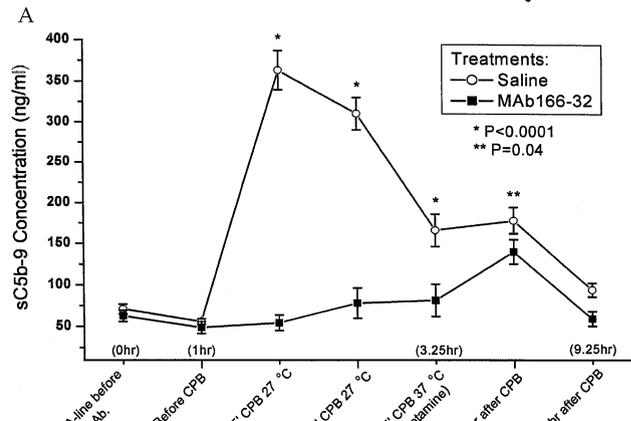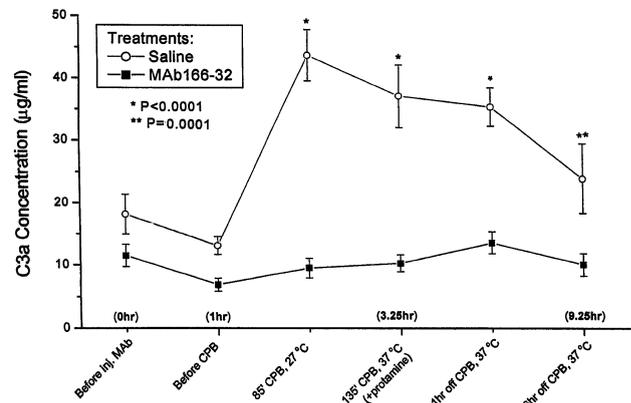

Fig 4. (A) Plasma C3a production (n = 7 in each group, mean ± SEM). (B) Plasma sC5b-9 production (n = 7 in each group, mean ± SEM). (CPB = cardiopulmonary bypass; Inj = injection of.)

molytic activity of the baboon plasma samples was completely inhibited at 6 hours after CPB. At 18 hours after CPB, the inhibition was reduced to 79.3 ± 10%. The data are consistent with the presence of free Mab 166-32 in the circulation until 18 hours after CPB (Fig 1). In hemolytic assays using sensitized chicken red blood cells to measure the classical complement activity, the corresponding plasma samples from the Mab 166-32 treated animals did not show any reduction in the classical complement activity by the antibody (Fig 2). These results confirm that Mab 166-32 is a specific inhibitor of the alternative complement pathway.

Activation of Complement, Leukocytes and IL-6

The specificity of Mab 166-32 in inhibiting the alternative complement pathway was also demonstrated by the complete inhibition of Bb formation (Fig 3A). Bb is the activation product of factor B upon proteolytic cleavage by factor D. The increase in Bb formation in the control animals is attributed to the activation of the alternative complement pathway during CPB. The reduction of plasma Bb concentration below the baseline in the Mab 166-32 treated animals could be due to the inhibition of the physiological activation of the alternative complement pathway.

Activation of the classical complement pathway was determined by measuring C4d, which is a specific marker

for the activation of C4 in the classical complement pathway. In the study, the plasma levels of C4d in both the Mab 166-32 treated and control animals were relatively stable with reference to the baseline (Fig 3B). However, there was a significant increase of C4d in both animal groups after neutralization of heparin with protamine at the end of the CPB as compared with the baseline, indicating the activation of the classical pathway as reported earlier in other studies [13–15, 17].

There was a dramatic increase of plasma C3a levels in the control animals (Fig 4A). In contrast, animals treated with Mab 166-32 showed almost complete inhibition of C3a production. A slight increase of C3a level is observed in the Mab 166-32 treated animals after neutralization of heparin with protamine. Anti-factor D Mab significantly inhibited sC5b-9 production (Fig 4B). Together, the results from Figs 3 and 4 support the notion that complement activation during CPB is predominantly mediated by the alternative complement pathway.

Activation of neutrophils and monocytes in the baboons was examined by measuring CD11b (α-integrin) expression using immunofluorocytometric methods. In the control animals, CD11b expression on neutrophils increased rapidly and reached maximum at about 85 minutes after the start of CPB (209 ± 42.9% of the baseline) (Fig 5). It then declined slowly back to around the baseline. In contrast, the increase of CD11b expres-
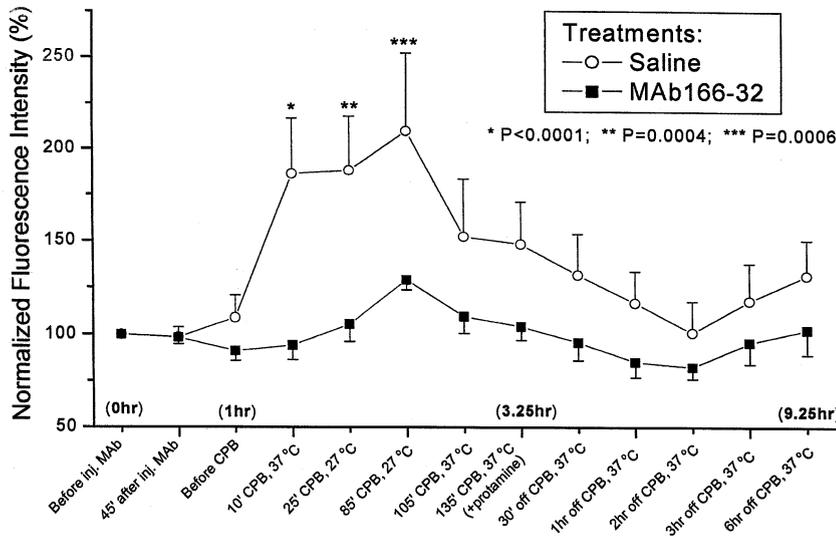
Fig 5. CD11b expression on neutrophils (n = 7 in each group, mean ± SEM). (CPB = cardiopulmonary bypass; Inj = injection of.)

sion on neutrophils in the Mab 166-32 treated animals ws much delayed and smaller in magnitude (Fig 5). The maximum level of CD11b expression was 129.3% ± 5.5% of the baseline in the antibody group. Similarly, the upregulation of CD11b expression on monocytes in Mab 166-32 treated animals was also reduced as compared to the control animals (Fig 6).

The effect of Mab 166-32 treatment on proinflammatory cytokine IL-6 was also examined. Baboons treated with Mab 166-32 had a significantly smaller increase in plasma IL-6 concentration as compared to the control animals (Fig 7).

The effects of Mab 166-32 treatment on tissue injury were also studied. Specifically for myocardial injury, the increase of plasma CK-MB levels at 6 and 18 hours after CPB was significantly lower in the Mab 166-32 treated animals as compared to the control animals (Fig 8). Similarly, the plasma CK levels were lower in the Mab 166-32 treated animals as compared to the control animals at 6 hours (299% ± 47% vs. 470% ± 101% $P = 0.01$) and 18 hours after CPB (420% ± 70% vs. 762% ± 358%, $P = 0.0004$). The increase of plasma LDH levels was also significantly lower in the Mab 166-32 treated animals as

compared to the control animals at 3 hours (120% ± 80% vs. 186% ± 17, $P = 0.0002$) and 6 hours after CPB (161% ± 51% vs. 202% ± 46%, $P = 0.03$). No plasma samples for the LDH measurement were collected at 18 hours after CPB.

Eighteen hours after CPB, we also examined the renal function of the baboons. A significant reduction in the increase of normalized plasma creatinine levels was found in the treated baboons as compared to the control animals (116 ± 13% vs. 141 ± 15%; $P = .01$). However, the absolute numbers (1.1 ± 0.2 mg/dL) were identical in both groups. Throughout the experiments, there was no significant intergroup difference in the BUN levels.

Comment

The triggering mechanism of systemic inflammatory syndrome in CPB is associated with complement activation [3, 7, 13, 17–19], although contact activation is also involved [18–20]. This activation leads to multiple humoral and cellular inflammatory responses.

The complement cascade can be activated directly by either the alternative or the classical pathway [11], and indirectly by the lectin pathway [12]. In the alternative
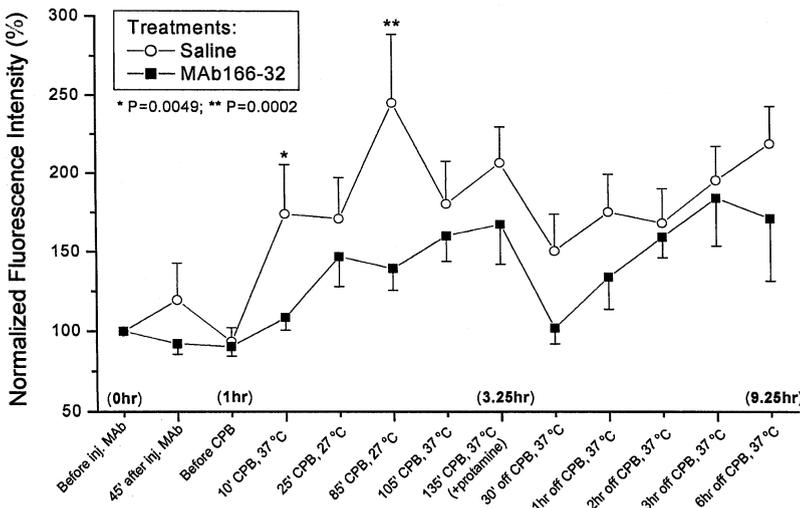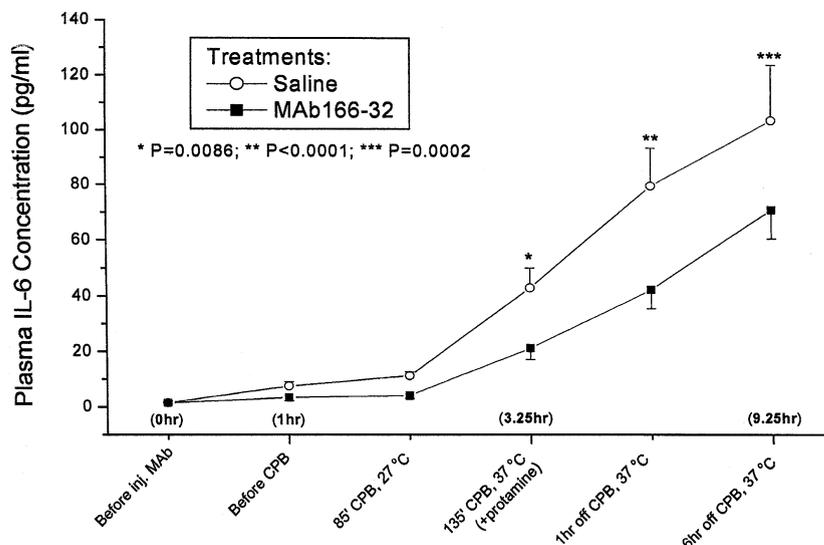

Fig 6. CD11b expression on monocytes (n = 7 in each group, mean ± SEM). (CPB = cardiopulmonary bypass; Inj = injection of.)

Fig 7. Plasma interleukin 6 (IL-6) levels (n = 7 in each group, mean ± SEM). (CPB = cardiopulmonary bypass; Inj = injection of.)

pathway, C3 is activated by exposure of blood to the artificial surfaces of the CPB circuit or by contact of C3 with complex polysaccharides and endotoxin. In the classical pathway, C1 is activated usually by antibody-antigen complexes. The classical pathway can also be activated by the administration of protamine to neutralize heparin immediately after weaning from CPB [13, 17].

Contact activation causes activation of factor XII, which is involved in the generation of plasmin [18–20]. Plasmin can activate C1 and C3. Complement activation generates a number of active split products, including the anaphylatoxins C3a and C5a. In turn, these substances stimulate the release of histamine, proteases and leukotrienes from mast cells and basophils, resulting in enhanced vascular permeability, vasoconstriction, and tissue inflammation [18, 20]. In addition, C3a and C5a are potent stimulators of neutrophils and monocytes, respectively [1, 21, 22]. The activation of these cells can result in platelet-leukocyte aggregation and release of cytotoxic substances from neutrophils, such as oxidative free radical and proteases.

During CPB, the alternative complement pathway is

found to be the major mechanism of complement activation [12–15]. In a prospective clinical study, cardiac, pulmonary, and renal dysfunction was significantly related to heightened C3a levels in the blood after CPB [2]. Both C5a and C5b-9 activate endothelial cells to express adhesion molecules essential for sequestration of activated leukocytes which then mediate tissue inflammation and injury [23, 24]. In our baboon model, we have demonstrated that the alternative complement pathway is the major mechanism of complement activation, and anti-factor D Mab significantly inhibited Bb, C3a, and sC5b-9 production.

During CPB, neutrophil activation can be triggered by a number of mediators, including C3a and C5a [1, 21, 22]. Neutrophil adherence to endothelial cells is the first step of tissue injury. Neutrophil-adhesive glycoprotein CD11b is primarily responsible for endothelial binding [25]. Neutrophil-endothelial adhesion is a major event leading to inflammation and reperfusion injury. Several studies have shown that CD11b expression levels on neutrophils are significantly increased after clinical or experimental cardiac surgery [1, 24, 25]. CD11b has also been shown to mediate lung injury and myocardial ischemia-reperfusion injury [23–25]. In our baboon model, the CD11b upregulation on neutrophils and monocytes was inhibited in the antibody treatment group as compared to the control group. The results are consistent with the fact that the CK, CK-MB, and LDH levels were significantly lower in the antibody treatment group after CPB. Collectively, anti-factor D Mab 166-32 was shown to reduce tissue injury due caused by systemic inflammation.

Interleukin-6 is produced by monocytes, macrophages, endothelial cells, and smooth muscle cells in response to their stimulation by TNFα, endotoxin, and particularly IL-1 [26, 27]. IL-6 is responsible for the generalized systemic inflammatory reaction known as the acute-phase response [26]. This reaction includes tachycardia and leukocytosis, as well as decreased synthesis of albumin and increased production of acute-phase proteins by hepatocytes. There is a significant correlation between the plasma levels of IL-6 and tissue injury [26–29]. In our
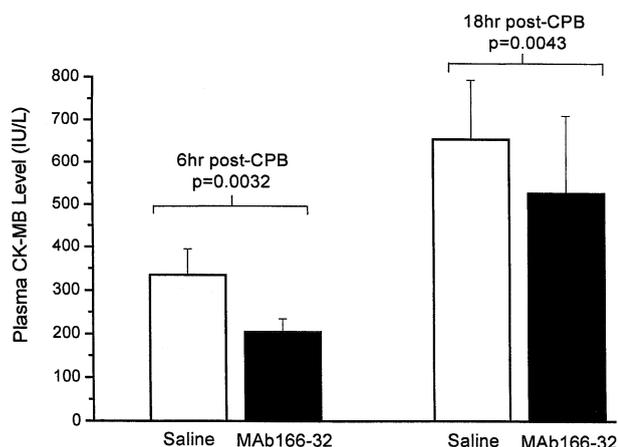

Fig 8. Plasma creatinine kinase isozymes (CK-MB) levels (n = 7 in each group, mean ± SEM). (CPB = cardiopulmonary bypass.)

baboon model, the increase of plasma IL-6 concentrations was reduced in the antibody treatment group as compared to the control group.

The data from our extracorporeal circulation study and the baboon study clearly demonstrate that the alternative complement pathway plays a predominant role in the inflammation and tissue injury in CPB [15]. Therefore specific inhibition of the alternative complement pathway instead of complete inhibition of all the complement pathways could be adequate and desirable. We anticipate that CPB patients will be treated with the anti-factor D Mab for a short duration (24–36 hours) during and immediately after the surgery. Factor D has a fast turnover rate in human (about 60% per hour). Therefore, once an appropriate dose of the antibody is used up in the neutralization of factor D, the alternative complement activity will resume quickly in 1–2 hours. Furthermore, a recent report by Biesma and associates have clearly shown that increased susceptibility for infections in individuals with partial factor D deficiency is unlikely [30].

Baboons were chosen as the animal model because Mab 166-32 does not react with factor D from non-primate animals (such as dogs and pigs) that might otherwise have been considered for this study. Himamatsu and associates have already shown that baboon blood has high immunologic cross-reactivity with most assays for human plasma proteins and cellular elements [31]. Clinical parameters of baboons compared favorably with normal clinical values established for humans [32].

Conclusions

Anti-factor D Mab 166-32 inhibits activation of the alternative complement pathway in a baboon model of CPB, thereby reducing the activation of neutrophils and monocytes, as well as the production of IL-6. Treatment with Mab 166-32 confers protection against myocardial injury. The alternative complement pathway may play a predominant role in the inflammation and tissue injury caused by extracorporeal circulation, surgical trauma, and ischemia/reperfusion. Therefore, Mab 166-32 could be useful for the treatment of systemic inflammatory response syndromes in CPB.

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INVITED COMMENTARY

The complement system, an integral component of innate immunity, is the first line of immunological defense against foreign pathogens. Although it is designed to target pathogens and is tightly regulated by a series of complement inhibitors, excessive activation and failure in the intrinsic regulation of the complement system result in tissue injury [1]. This injury is directly mediated by the membrane attack complex and indirectly by the anaphylatoxins (C3a, C4a, and C5a) through their effects on neutrophils, eosinophils, and mast cells.

Association of complement activation with inflammatory diseases has been recognized since the 1960s. However, it was not clear until recently whether complement activation in various pathologic conditions is coincidental or is truly responsible for the inflammation and tissue damage that are observed. Recent data from animal models of diseases produced using complement-deficient, knockout, and transgenic animals clearly indicate that complement activation is indeed a major source of tissue damage in many pathologic conditions [2]. These include various autoimmune diseases, neurodegenerative diseases, and immune complex diseases, and bioincompatibility situations such as dialysis, cardiopulmonary bypass (CPB), and xenotransplantation, to name a few. Thus, there is a clear need for developing complement inhibitors.

A central question in complement-targeted therapies is which protein(s) should be targeted. This question remained unanswered in many pathologic conditions because of a lack of information regarding the pathway(s) or anaphylatoxin(s) responsible for complement-related tissue damage. Under these circumstances, blocking all three pathways of complement by blocking the activation at the C3 level would be advisable. However, discerning the role of individual pathway(s) or anaphylatoxin(s) would allow the design of inhibitors that would permit partial functioning of the system, a desirable feature [2]. Undar and colleagues, in their article, used anti-factor D monoclonal antibodies (Mabs) to determine the role of the alternative pathway (AP) of complement activation in systemic inflammation and tissue injury during CPB. Their data, produced using a baboon model of CPB, clearly indicate that the AP is a major player in inducing systemic inflammation and tissue injury in this setting. Thus, anti-factor D Mabs could be developed for treating the systemic inflammatory response, which in turn could reduce organ failure subsequent to CPB.

At present, complement inhibitors, and specifically anti-human C5 Mabs (5G1.1 and 5G1.1-scFv; www.alex-ioninc.com), are being evaluated in phase I and phase II clinical trials in various clinical conditions, including CPB. The data obtained thus far are very encouraging. Although these Mabs have shown promise and will be useful in reducing the clinical morbidity in several diseases, recombinant protein therapies are not cost-effective. Thus, current emphasis is being placed on the development of small-molecule inhibitors of complement. Promising small-molecule inhibitors, which are currently under development, include a C3-specific peptide inhibitor, Compstatin [3–5], and newly designed C5aR antagonists [6, 7].

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