Evidence for iC3 generation during cardiopulmonary bypass as the result of blood–gas interaction

M. PEKNA, L. NILSSON*, K. NILSSON-EKDAHL, U. R. NILSSON & B. NILSSON *Department of Clinical Immunology and Transfusion Medicine, and *Department of Thoracic Surgery, University Hospital, Uppsala, Sweden

SUMMARY

Earlier we have shown that iC3 is generated at the blood–gas interface *in vitro* and that the generation of this molecule is independent of complement activation and the composition of the gas. In order to investigate whether iC3 is also generated during cardiopulmonary bypass where blood comes into contact with oxygen bubbles, two bubble oxygenators were incubated at 37°C with human heparinized blood. A continuous increase in the level of iC3 was shown in the oxygen-perfused bubble oxygenator (up to 100 nmol/l after 180 min) in contrast to the unbubbled control. Similarly, in plasma drawn from patients undergoing cardiopulmonary bypass using either bubble or membrane oxygenators, the levels of iC3 were shown to increase continuously during the operation. Furthermore, this form of C3 was found to be susceptible to cleavage by factor I. The formation of iC3 at the blood–gas interface *in vivo* could be a mechanism by which gas bubbles induce clinical manifestations associated with complement activation, e.g. during cardiopulmonary bypass, adult respiratory distress syndrome and decompression sickness.

Keywords iC3 generation   blood–gas interface   cardiopulmonary bypass

INTRODUCTION

The activation of the complement system is a consistent phenomenon during cardiopulmonary bypass (CPB) surgery. The activation involves both the early [1–3] and the late phase [4,5] of the complement cascade and it is correlated with post-operative cardiac, renal, and pulmonary dysfunction [2], abnormal bleeding [2], and increased requirement for mechanical ventilation [3]. Despite the fact that several haematological consequences of CPB are minimized by the use of membrane oxygenators (MO), complement activation remains a risk factor also in MO patients [6–8].

The main activators of complement during CPB are factors related to the oxygenator [5] and the heparin–protamine complexes [9]. Although the exact mechanisms for the complement activation during the pump-oxygenator treatment remain unknown, both the contact of blood with the artificial surface and the blood–gas interaction have been shown to be involved [1].

The third component of complement (C3) plays a central role in the complement cascade (for review see [10]). One important property of this molecule is the presence of an internal thiolester bond [11]. Hydrolysis or similar modifica-

Correspondence: Marcela Pekna, Department of Clinical Immunology and Transfusion Medicine, University Hospital, S-751 85 Uppsala, Sweden.

tions of the internal thiolester bond in native C3 give rise to a form of C3, called iC3. In addition to the loss of the ability to attach covalently to biological targets, iC3 acquires 'C3b-like' properties. It is capable of binding factors B and H, and of forming the initial fluid-phase C3 convertase of the alternative pathway. It reacts with cellular C3 receptors [12] and it is also susceptible to cleavage and inactivation by factor I in conjunction with cofactors [13,14]. We have shown that the breakage of the thiolester bond is a prerequisite for the conformational change of iC3 induced by alkali denaturation and that the susceptibility to factor I cleavage correlates with the degree of this conformational change [15].

The blood oxygenation during CPB is accompanied by a significant denaturation of plasma proteins upon contact with surface polarizing forces at the blood–gas interface [16]. Earlier studies from our laboratory have demonstrated that the C3 molecule undergoes considerable conformational changes upon its adsorption to polystyrene [17], and it has also been shown that a similar modification occurs at the serum–gas interface in that iC3 is generated [18]. iC3 generation is independent of complement activation and its rate is unaffected by the composition of the gas, suggesting that the denaturation of C3 indeed occurred at the serum–gas interface. The produced iC3 or fragments thereof bound to human erythrocytes [18].

Here we report that the generation of iC3 takes place at the blood–gas interface not only *in vitro* but also during CPB using bubble or membrane oxygenators.

MATERIALS AND METHODS

Patients

Twenty patients (three females and 17 males) undergoing aortocoronary venous bypass were studied. The patients' average age was 56 (range 30–74) years. The study was approved by the Ethical Committee of the Medical Faculty.

Samples

Aliquots of blood were drawn into tubes containing EDTA (final concentration 10 mM), centrifuged and frozen immediately to −70°C before analysis.

Oxygen bubbling of human EDTA-blood

Oxygen bubbling was performed as described previously [18].

Bubble oxygenator experiment in vitro

Two bubble oxygenators, Bentley Bio-2TM (American Bentley, Inc., USA) containing 460 ml of heparinized whole human blood diluted with Ringer acetate up to 810 ml (final heparin concentration being 3·1 U/ml), were immersed in a 37°C water bath. The blood flow was adjusted to 1 *l*/min. While 1 *l*/min of pure oxygen was allowed to bubble through one of the oxygenators, the other one served as an unbubbled control. Aliquots were taken every 15 min.

Cardiopulmonary bypass

A Gambro heart-lung machine equipped with either Bentley 10 Plus bubble oxygenators (BO) (Bentley, American Bentley) (13 patients) or Cobe CML Excel flat sheet or Maxima hollow fibre MO (Maxima, Johnson & Johnson Cardiovascular, USA; Cobe CML Excel, COBE Laboratories, Inc., USA) (seven patients) was used. Before cardiopulmonary bypass, the system was primed with Ringer acetate solution. The average bypass period was 90 min and did not differ between the groups. Aliquots were taken every 30 min.

ELISA

The concentration of all parameters measured in CPB samples was corrected according to the haemodilution estimated by the assessment of C3c antigen concentration by radial immunodiffusion as described by Mancini *et al.* [19].

C3a and iC3/iC3 fragments. These two assays were performed as described previously [18]. In both assays microtitre plate-fixed MoAb 4SD17.3 against neoepitopes in C3a [20] served as a capture antibody. C3a and iC3/iC3 fragments were detected by biotinylated polyclonal anti-C3a antibody [18] and horseradish peroxidase (HRP)-conjugated anti-C3d antiserum (Dako A/S, Denmark), respectively.

TCC. The soluble terminal complement complexes C5b-9 (TCC) ELISA was the modification of the assay described by Mollnes [21]. After binding to microtitre plate-fixed anti-C9 MoAb MCaE11 specific for a neoantigen expressed in C9 (a kind gift from Dr T. E. Mollnes, Norway), TCC was detected by polyclonal anti-C5 antibodies (Dako) followed by HRP-conjugated anti-rabbit immunoglobulin (Dako). Zymosan-activated serum defined to contain 40 000 arbitrary units (AU) per ml was used as standard.

Affinity chromatography

MoAb 4SD17.3 against neoepitopes in C3a [20] was coupled to CNBr-Sepharose (Pharmacia Fine Chemicals AB, Sweden)

according to manufacturer's recommendations. The anti-neoC3a MoAb has previously been shown to react specifically either with the C3a fragment or neoantigenic determinants exposed on iC3/iC3 fragments containing the C3a domain, but not with native C3 or C3b [20]. Undiluted EDTA-plasma (50 μl) was incubated with the same volume of gel for 2 h at room temperature. The gel was washed four times with 0·1 M NaHCO₃, 0·5 M NaCl buffer pH 8·3, and analysed by SDS–PAGE under reducing conditions.

SDS–PAGE

SDS–PAGE was performed according to the method of Laemmli [22].

Western blot analysis

The Western blot was performed as described by [23] using MoAbs 7D9.2, 7D615.1 and 7D331.1 (against neoepitopes expressed in C3d, in the 20-kD and 40-kD fragments of C3c, respectively) [24], 7D323.1 (against a neoepitope in C3g) [25] and 7D169.7 (against epitopes in the β-chain of C3) [20]. The MoAbs were detected by HRP-conjugated rabbit anti-mouse immunoglobulin (Dako).

Statistical analysis

Statistical analysis of the data was performed using two-tailed unpaired or paired Student's t-test. The results are presented as mean ± s.e.m.

RESULTS

iC3 generation in a bubble oxygenator

Heparinized blood was circulated through two BO. Oxygen was bubbled through one of them while the other served as a control. iC3/iC3 fragments were continuously generated in the oxygen-perfused BO, reaching approximately 100 nmol/*l* after 180 min, while no change was observed in the unbubbled control (Fig. 1a). Figure 1b shows that the C3a levels were slightly higher in the oxygen-perfused BO during the whole experiment compared with the control (520 *versus* 390 nmol/*l* at the end of the incubation). After 180 min the levels of TCC reached approximately 4×10^5 AU/*l* in both BO (Fig. 1c).

iC3 generation during CPB using BO or MO

During CPB (Fig. 2a), there was a continuous generation of iC3/iC3 fragments both in the BO and the MO groups. After 90 min the initial levels increased by 53·4 ± 12·3 and 23·6 ± 4·2 nmol/*l*, respectively. In both groups the increase was statistically significant ($P < 0.02$) already after 30 min of pump-oxygenator treatment.

As shown in Fig. 2b, there was no apparent difference in the C3a levels when BO and MO were compared. In both groups, the C3a levels continuously increased until 30 min after CPB (by 236 ± 68 and 254 ± 54 nmol/*l*, respectively). In both groups the increase was statistically significant ($P < 0.01$) already after 30 min. A similar increase in the levels of TCC during CPB (by $2·29 \times 10^5 \pm 0·33 \times 10^5$ and $3·01 \times 10^5 \pm 0·81 \times 10^5$ AU/ml after 90 min, respectively) was observed (Fig. 2c). In both groups the increase was statistically significant ($P < 0.03$) already after 30 min. However, unlike C3a, the levels of TCC decreased after CPB.
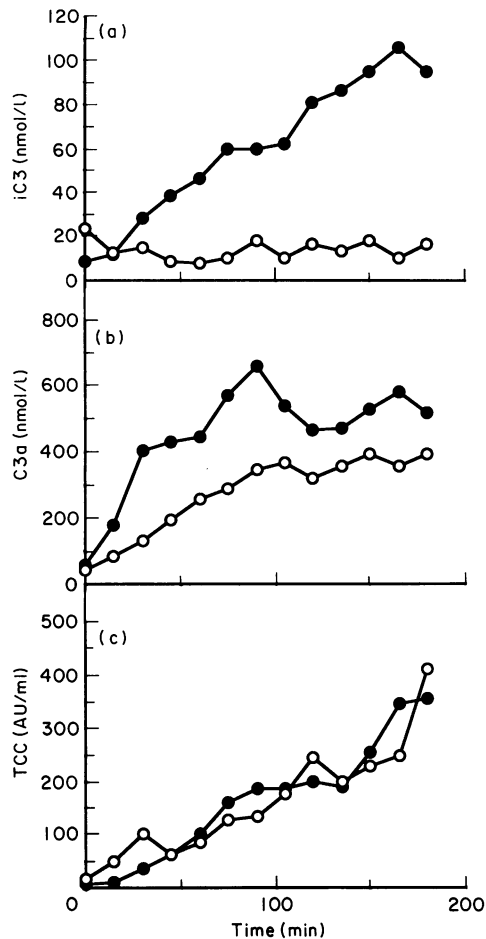
Fig. 1. Generation of (a) iC3/iC3 fragments, (b) C3a and (c) soluble terminal complement complexes C5b-9 (TCC) by oxygenation of heparinized human blood in an oxygen-perfused bubble oxygenator (●) and in an unbubbled control oxygenator (O). $n = 1$ for each of the oxygenators.

Fig. 2. The increase in the levels of (a) iC3/iC3 fragments, (b) C3a and (c) TCC during cardiopulmonary bypass (CPB) using bubble oxygenators ($n = 13$) (■) or membrane oxygenators ($n = 7$) (□). Data are presented as mean ± s.e.m. The initial levels of iC3/iC3 fragments were 51.3 ± 7 versus 22.6 ± 3.5 nmol/l; of C3a 45 ± 4 versus 86 ± 9 nmol/l and of TCC $1.14 \times 10^5 \pm 0.2 \times 10^5$ versus $0.8 \times 10^5 \pm 0.1 \times 10^5$ AU/l, respectively.

In neither of the groups were there statistically significant differences between the blood from the afferent and from the efferent tubings of the oxygenators with regard to any parameter studied.

iC3 fragments produced at the blood–gas interface

Figure 3 shows the iC3 fragments detected by Western blot analysis in EDTA-blood oxygen bubbled for 180 min. The intact α-chain (116 kD) was detected by the four MoAbs specific for the α-chain and a single 75-kD band by the MoAb specific for the β-chain. A 30-kD fragment was found to react with the MoAb against C3c(20 kD). A 76-kD fragment was detected by the MoAb anti-C3d, MoAb anti-C3g and the MoAb against the C3c(20 kD) fragment. A 41-kD fragment was shown to react only with MoAb anti-C3c(40 kD). These fragments were also produced by pump-oxygenator treatment of blood both in vitro and in vivo.

In blood samples from the BO in vitro (in Fig. 1), both a 41-kD and a 43-kD fragment were detected with the MoAb anti-C3c(40 kD) at the beginning of the experiment. During the incubation in the oxygen-perfused BO the 43-kD fragment declined concomitantly with the α-chain, while the intensity of the 41-kD fragment continuously increased. In contrast, the
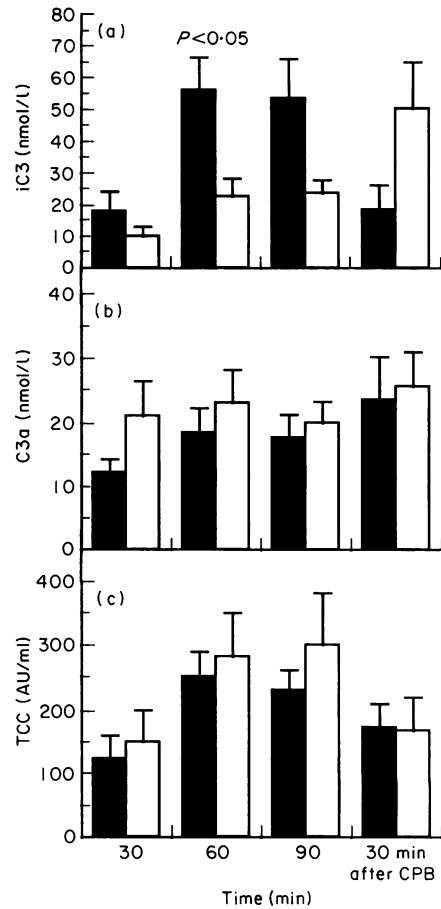
intensity of all the fragments decreased in the control BO (Fig. 4). The decrease is probably due to the inhibitory effect of C3a, which has the same binding ability to the anti-neoC3a Sepharose as iC3/iC3 fragments. Similar changes in the intensity of the two 40-kD fragments were demonstrated in oxygen-bubbled EDTA blood in vitro. No apparent change in the levels of these fragments was seen in the CPB samples.

DISCUSSION

In a previous study we have shown that contact between blood and gas leads to a conformational change of the C3 molecule which turns into iC3. This process is independent of the complement activation and of the composition of the gas [18]. In agreement with this, we here find a continuous increase in the levels of iC3/iC3 fragments in the oxygen-perfused BO in contrast to the unbubbled control. These results demonstrate that iC3 is generated in blood oxygenators and that the generation is dependent on the interaction between blood and gas. The higher C3a level in the oxygen-perfused BO than in the control suggests that iC3 is actively involved in the formation of the fluid-phase C3 convertase of the alternative pathway. However, the detection of C3a is also influenced by the levels of iC3/iC3 fragments which, to some extent, interfere with the
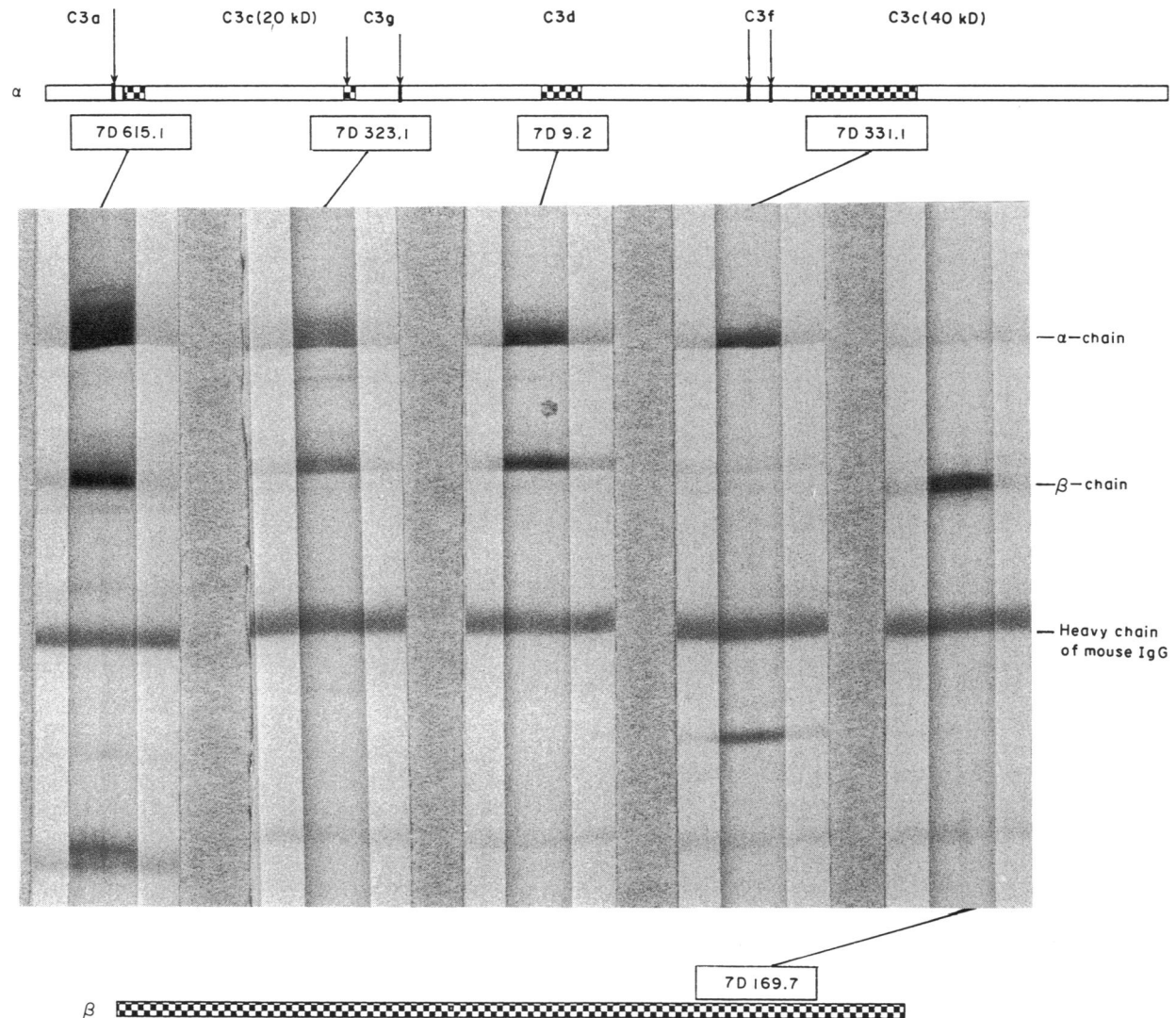
Fig. 3. iC3/iC3 fragments in EDTA-blood bubbled with oxygen for 180 min, were bound to Sepharose-conjugated anti-C3a (MoAb 4SD17.3) followed by SDS–PAGE and Western blot analysis. The figure shows the generated iC3 fragments as detected by different MoAbs and a graphical presentation of the locations in the iC3 molecule of the epitopes recognized by the MoAbs.

ELISA used for the C3a analysis [18]. The measurement of TCC in the oxygenators appears to be less useful in the assessment of the role of the gas in complement activation, since no significant difference was observed between the oxygen-perfused oxygenator and the control. This indicates that TCC was generated by the biomaterial.

In patients undergoing CPB, the BO gave rise to higher levels of iC3 than the MO. There was also a suggested difference between the BO and MO groups ($P = 0.052$), in that the levels of iC3/iC3 fragments tended to increase in the MO group after CPB in comparison with a decrease in the BO group. The reason for this discrepancy might be that blood collected from the MO for reinfusion after CPB contains higher levels of iC3 as compared with that of BO due to differences in oxygenator construction. In MO the blood is still in contact with oxygen

bubbles remaining on the membrane, even after the extracorporeal oxygenation is stopped.

The iC3 molecule generated at the blood–gas interface was found to be susceptible to cleavage by regulatory complement proteins as demonstrated by affinity chromatography on anti-neoC3a Sepharose followed by Western blot analysis. The intact α-chain (116 kD), and a 76-kD, a 41-kD, a 43-kD and a 30-kD fragment were detected by MoAbs specific for α-chain-derived fragments. These findings are consistent with the iC3 molecule being partially cleaved by factor I at its first and second cleavage sites [26] in a way similar to that of C3b [27]. The presence of C3c (20 kD) and C3g epitopes and the absence of C3c (40-kD) fragment epitopes in the 76-kD fragment of the α-chain rule out that this fragment is generated due to autolytic cleavage of the α-chain of C3 [28]. The spectrum of iC3/iC3 fragments in the
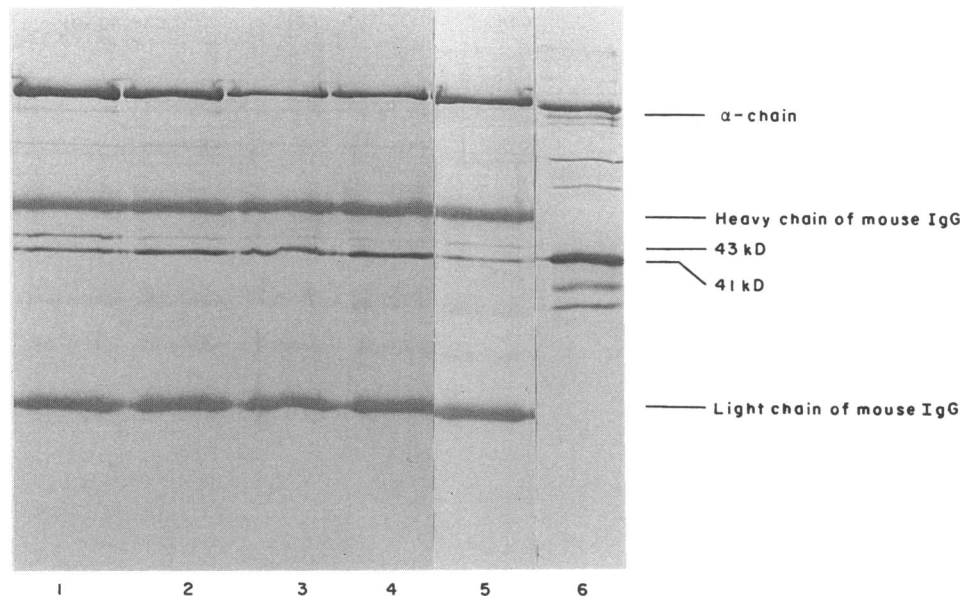
Fig. 4. Detection of the 41-kD and the 43-kD fragments of cleaved iC3 in heparinized blood circulated in the bubble oxygenator *in vitro*. The samples were analysed as in Fig. 3, and the iC3/iC3 fragments were detected by MoAb 7D331.1 directed against C3c (40 kD). The figure shows samples from the oxygen-perfused oxygenator at 0 min (lane 1), 60 min (lane 2), 135 min (lane 3) and 180 min (lane 4), from the control oxygenator at 180 min (lane 5), and methylamine-treated C3 cleaved with factors I and H [14] (not affinity-purified) (lane 6).

samples from CPB was the same as that in oxygen-bubbled EDTA-blood and in the bubble oxygenator *in vitro*. However, no change in the intensity of the 41-kD fragment during CPB could be seen, which may be due to the removal of iC3 fragments from the circulation.

The formation of iC3 in the patients' blood during CPB may be, at least partially, responsible for the deleterious effects of this procedure. After 90 min of extracorporeal blood oxygenation about 1% of the total amount (7000 nmol/*l*) of C3 was found to be converted into iC3. The continuous production of iC3 is likely to start an inflammatory reaction mediated not only by the assembly of active alternative pathway C3 convertases (iC3Bb) leading to C3a generation, but also by the interactions of iC3/iC3 fragments with complement receptors on leucocytes and cells of the fixed macrophage system. The production of iC3/iC3 fragments may therefore be a pathophysiological factor in the multiorgan failure seen after CPB.

This study indicates that iC3 can be generated at the blood–gas interface *in vivo*. Apart from CPB, this finding is likely to be relevant for other situations where blood is exposed to gas surfaces, e.g. in adult respiratory distress syndrome, in the lung under certain other pathological conditions and during episodes of decompression sickness. The latter has been studied on both humans and animals, and it has been proposed that complement activation plays a major role in the pathogenesis of decompression sickness, during which nitrogen bubbles are formed in the circulation [29,30].

ACKNOWLEDGMENTS

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