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Avoiding ambient air in test tubes during incubations of human whole-blood minimizes complement background activation

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ABSTRACT

Background: In vitro, the complement system can be studied in test tubes incubated with anticoagulated human whole-blood. Background activation of complement may mask inflammatory signals. Air bubbles are known to activate complement. We examined if removing ambient air from test tubes before incubation reduced background complement activation.

Methods: Blood from twelve donors was anticoagulated with the thrombin inhibitor lepirudin and incubated with either no air, ambient air or air bubbles in polypropylene tubes at 37 °C for 180 min on a roller mixer. After incubation, EDTA was added, plasma isolated and analyzed for seven complement activation products using ELISA. Results are presented as means with 95% confidence intervals.

Results: Blood incubated without air had significantly lower complement activation compared to blood incubated with ambient air; C4d 273 (192–364) vs. 379 (263–494) ng/mL ($p = 0.002$), C4bc 8.2 (4.1–13) vs. 12 (3.2–21) CAU/mL ($p = 0.01$), C3a 1351 (873–1838) vs. 2944 (2315–3572) ng/mL ($p = 0.0005$), C3bc 31 (17–46) vs. 68 (52–84) CAU/mL (p = 0.002), C3bBbP 134 (97–171) vs. 427 (358–506) CAU/mL ($p < 0.0001$), C5a 3.5 (1.9–5 0.2) vs. 15 (1.8–27)) ng/mL ($p = 0.003$), TCC 4.6 (2.8–6.3) vs. 9.9 (7.3–12) CAU/mL ($p = 0.006$). At the end of the experiment blood incubated with air bubbles had a higher complement activation than blood incubated with ambient air with an average 26 fold increase (range 1.6–59) from baseline of all activation products; C4d 551 (337–766) ng/mL, C4bc 21 (5.0–36) CAU/mL, C3a 3983 (3518–4448) ng/mL, C4bc 103 (86–121) CAU/mL, C3bBbP 626 (543–708) CAU/mL, C5a 10 (2.8–18) ng/mL and TCC 10 (6.0–14) CAU/mL.

Conclusion: Avoiding air in test tubes during whole-blood experiments reduced background complement activation substantially and represents an important improvement to the lepirudin whole-blood model. This could also apply to other in vitro models.

1. Introduction

In 2002 we published a unique whole-blood model to study the role of complement in the inflammatory response in a holistic manner (Mollnes et al. 2002). This was made possible by anticoagulating the blood with the thrombin-specific inhibitor lepirudin, which does not interfere with the complement system when collecting blood for in vitro experiments. Undesired background activation of complement and other systems of inflammation in experimental whole-blood models is a common problem during in vitro studies (Lappegård et al. 2004), potentially masking biologically relevant signals in the experiments. In 1980, Track and colleagues discovered that the C3 molecule contained an internal thioester bond. This esther was continuously hydrolyzed at a low degree in the fluid phase and obtained a similar structure to C3b, but C3a was not cleaved off (Tack et al. 1980). This molecule was named iC3b, "C3b-like C3" and C3(H_2O). The C3 hydrolysis concept has recently been reviewed (Fromell et al. 2020) and the role of C3(H_2O) is still under debate. Blood-gas interfaces, such as found on air bubbles in

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blood, have been shown to activate the complement system in vitro by the thioesther hydrolysis of C3 to C3(H_2O). Interestingly, this hydrolyzation is independent of what type of gas the blood interfaces with, e.g. oxygen and nitrogen have similar effects on the complement system (Ekdahl et al. 1992).

Despite knowledge of this C3 hydrolyzation, it is common laboratory practice to accept the presence of ambient air in test tubes during in vitro experiments. To our knowledge, the effect of ambient air inside test tubes on the complement system during incubations has not previously been examined.

Thus, we studied if eliminating ambient air from test tubes during whole-blood experiments would reduce unwanted background activation of complement. Additionally, we examined if increasing the blood-air interface area by incubation with air bubbles would increase complement activation.

2. Material and methods

Sixty 5 mL polypropylene Nunc 5 mL test tubes (Nalgene, Roskilde, DK) were prefilled with 80 µL lepirudin 2.5 mg/mL (Thermo Fisher Scientific, Roskilde, DK) and preserved at −20 °C. A vacuum of −19 mL air was applied to rethawed tubes using a syringe and a 23 G needle (Becton Dickinson, Franklin Lakes, NJ). Blood from twelve healthy human donors (five woman and seven men) aged between 40- and 50-years was drawn using a BD Vacutainer Eclipse blood collection needle (Becton Dickinson) into three lepirudin filled Nunc test tubes per donor, giving a final blood lepirudin concentration of 50 µg/mL. Blood from each donor was subsequently transferred to a 50 mL polypropylene conical Falcon tube (Corning, Tamaulipas, Mexico) preheated to 37 °C. The blood samples "Baseline", "Ambient air" and "Air bubbles" were prepared in the following way: On a block heater set to 37 °C, 858 µL blood and 142 µL PBS with Ca^{2+} and Mg^{2+} was transferred to three polypropylene 1.8 mL Nunc tubes. To the baseline sample 14.6 µL EDTA was added immediately to block further complement activation, and the tube kept on ice. The "No air" sample was prepared as follows: In a 5 mL Nunc tube 3 mL blood and 497 µL PBS with Ca^{2+} and Mg^{2+} was mixed and from here transferred to a 1.8 mL Nunc tube filling the tube and the tube lid completely. The tube was then carefully capped, ensuring no air was present inside (Fig. 1A). In the "Baseline" and "Ambient air" samples, approximately 3 mL ambient air was left between blood and tube cap (Fig. 1B). To the "Air bubbles" sample, ambient air was bubbled through the blood using a syringe and needle, resulting in a mixture of air bubbles and blood filling the tube completely (Fig. 1C).

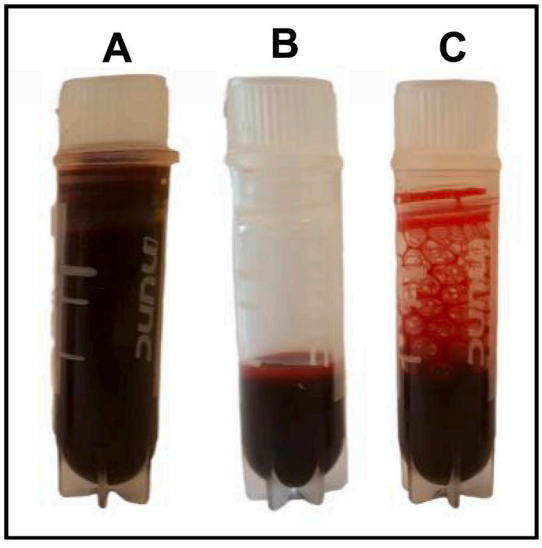

Fig. 1. Blood was incubated in 1.8 mL polypropylene tubes. A. Completely filled with blood, leaving no air inside the tube (the "No air" samples). B. Partly filled with blood leaving ambient air between blood sample and tube cap (the "Baseline" and "Ambient air" samples). C. Partly filled with blood followed by air bubbling, creating a blood-bubble mixture (the "Air bubbles" samples).

All tubes were capped and incubated on a Rock'n'Roller tube roller mixer (Labinco, Breda, NL) at 37 °C for 180 min. After incubation, 1 mL blood was transferred from the "No Air" tube to a new 1.8 mL Nunc tube. To this tube and the two other incubated tubes, 14.6 µL EDTA was added for a final concentration of 10 mM to block further complement activation. The samples were centrifuged at 3000*g* for 20 min at 4 °C, plasma transferred to polypropylene matrix tubes and frozen at −80 °C and later thawed for analysis of C3a, C3bc, C5a and TCC. Additionally, C4d, C4bc and C3bBbP was also analyzed in plasma from six of the donors.

Commercial ELISA kits were used for C4d (C4d fragment, Quidel Corp., Athens, OH), C3a (MicroVue C3a Plus EIA, Quidel) and C5a (C5a Human ELISA kit, HyCult Biotech, Uden, The Netherlands). In-house developed ELISA assays was performed as previously described (Bergseth et al. 2013) and were used for analysis of C4bc, C3bBbP, C3bc and TCC. ELISA plates were read using a Tecan Infinite M200 plate scanner (Tecan Group, Männedorf, Switzerland) and the Magellan 7.1 SP1 software (Tecan Group).

2.1. Statistics

Results were extracted and fold change between groups were calculated using Microsoft Excel for Mac ver. 16.16.9 (Microsoft Inc., Redmond, CA). Statistical analysis and charting of results were done using Prism for Mac ver. 8.4.2 (Graphpad Software, La Jolla, CA).

Missing baseline readouts due to lack of sample material were substituted with a random number between the lowest and the highest baseline result in the series. All results were stated as means with 95% confidence interval. As results did not follow a Gaussian distribution, data were log-transformed, and groups compared using the repeated measures ANOVA with Geisser-Greenhouse correction. Significances were calculated using the Fischer's LSD test. $p < 0.05$ was considered significant.

3. Results

After 180 min of incubation, all complement activation products were increased in all samples, except for C4d in blood incubated without air (Table 1 and Fig. 2). The average level of complement activation products were significantly lower in blood incubated without ambient air in the tubes compared to blood incubated with ambient air in tubes: C4d 273 (192–364) vs. 379 (263–494) ng/mL ($p = 0.002$), C4bc 8.2 (4.1–13) vs. 12 (3.2–21) CAU/mL ($p = 0.01$), C3a 1351 (873–1838) vs. 2944 (2315–3572) ng/mL ($p = 0.0005$), C3bc 31 (17–46) vs. 68 (52–84) CAU/mL (p = 0.002), C3bBbP 134 (97–171) vs. 427 (358–506) CAU/mL ($p < 0.0001$), C5a 3.5 (1.9–5.2) vs. 15

Table 1
The increase in seven complement activation products in lepirudin anticoagulated blood incubated with either no air, ambient air or air bubbles for 180 min.

Complement activation product[a]	Fold increase from baseline		
	No air	Ambient air	Air bubbles
C4d	0.8	1.1	1.6
C4bc	2.0	3.0	5.0
C3a	19	42	57
C3bc	7.4	16	24
C3bBbP	13	40	59
C5a	1.7	7.2	5.0
TCC	12	26	27

[a] C4d, C4bc and C3bBbP $n = 6$. C3a, C5a and TCC $n = 12$.

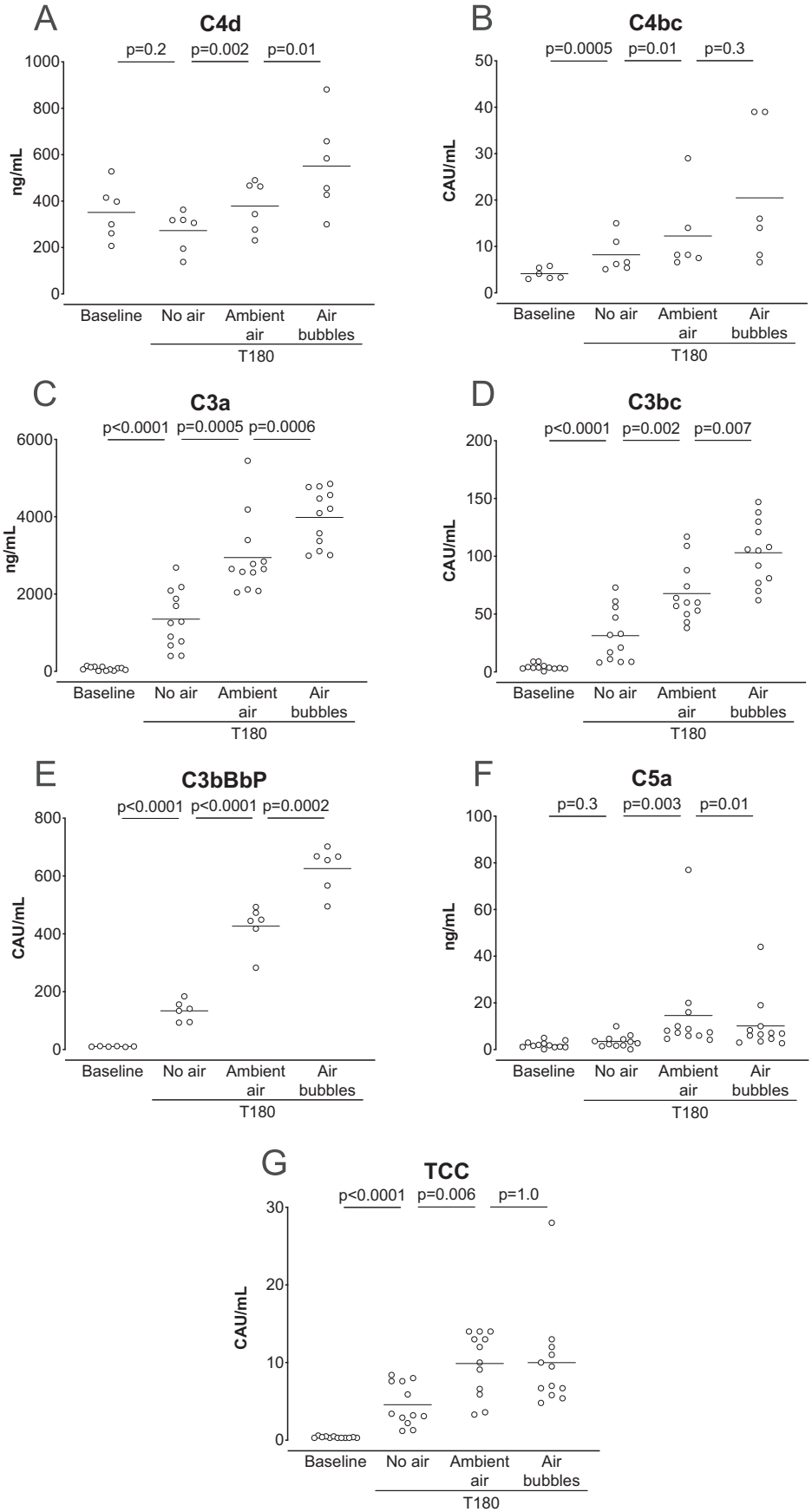

Fig. 2. Lepirudin anticoagulated human whole-blood from 12 donors incubated at 37 °C on a roller mixer in tubes with either no air, ambient air, or a mixture of air bubbles and blood. After 180 min the blood was analyzed for C4d (A), C4bc (B), C3a (C), C3bc (D), C3bBbP (E), C5a (F) and TCC (G). C4d, C3bc and C3bBbP were analyzed in six donors only. Individual readouts are shown as dots with horizontal line at mean. *p*-values were calculated on log-transformed data using repeated measures ANOVA with Geisser-Greenhouse correction and Fischer's LSD test.

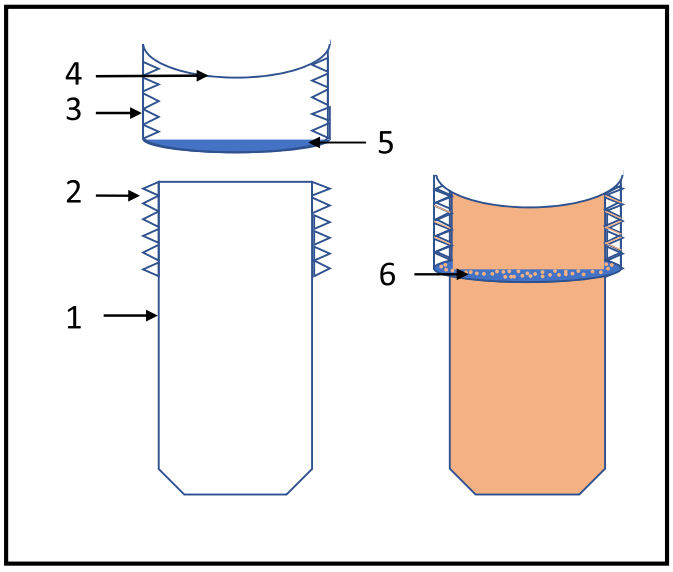

Fig. 3. Suggested design for a new test tube in non-reactive polypropylene plastic material (1) with threading on outside (2 and 3) and convex inside of the cap (4), ensuring air-tight closure of tube. Felt cap insert (5) for absorbing excess blood (red) displaced when capping tube (6). The tube can be produced in various sizes and diameters as needed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(1.8–27)) ng/mL (p = 0.003), TCC 4.6 (2.8–6.3) vs. 9.9 (7.3–12) CAU/mL (p = 0.006) (Fig. 2). Readouts from the individual donors are shown in supplement 1.

Seven of the 66 baseline readouts and one readout from the "No air" incubations were below lower detection limit and substituted with a random number between zero and the lowest detection limit. Due to limited amount of plasma, eight baseline readouts were substituted with a random number between the lowest and the highest baseline value in the series.

Blood incubated with air bubbles showed an average 26 fold increase from baseline (range 1.6–59) of all complement activation products. For C4d, C4bc, C3a, C3bc and C3bBbP the increase was higher than for blood incubated with ambient air only. For C5a and TCC there was no difference between incubation with bubbles or ambient air only (Table 1 and Fig. 2).

4. Discussion and conclusion

We have shown that removing ambient air from test tubes minimized background complement activation in incubated human whole-blood. We regard this as a substantial improvement of the original whole-blood model (Mollnes et al. 2002), as the very low background activation increases the sensitivity of the model, thus enabling us to decipher the complex inflammatory interplay in even greater detail. Notably, the effect of removing ambient air from the tubes was most pronounced for the activation of C3 and less pronounced for the activation of the C5-9 pathway. This is consistent with the previous findings that the alternative pathway and C3 is the main activating pathway at the surfaces between air and plasma (Ekdahl et al. 1992).

In line with this, we found that blood incubated with air bubbles had a higher complement C3 activation than blood incubated with ambient air only. However, the increases in C3 activation products observed in samples incubated with ambient air amounted to approximately 70% of the levels observed in samples incubated with air bubbles (Fig. 2). This difference in activation could be explained by a larger activating blood-air interface on the bubbles. However, we cannot exclude that other contributing factors, such as mechanical interaction of bubbles with the blood during roller mixer incubation could have contributed to the activation.

Our findings are highly relevant for most in vitro experiments using the whole-blood model, since unwanted background activation of complement by ambient air, which is normally present in experimental setups, may mask subtle but relevant biological findings. Hence, we suggest that in vitro blood experiments should preferably be conducted in completely air-free test tubes and the blood's exposure to ambient air should be minimized.

In order to work in the whole-blood model described, we collected blood in custom prepared lepirudin filled vacuum tubes. As a possible alternative, hirudin filled tubes (S-Monovette 1.6 mL Hirudin, Sarstedt, Nümbrecht, Germany) are commercially available.

It is possible that removal of ambient air from in vitro serum, plasma and whole-blood models also could reduce background activation when studying other biological systems, such as coagulation, cytokines and cell activation. Thus, we recommend that the effect of avoiding ambient air in other in vitro models on biological systems is elucidated further.

For clinical diagnostic purposes we suggest neither the use of our experimental whole-blood model nor the avoidance of ambient air in tubes for these purposes.

A major practical obstacle towards air-free sample handling is the lack of suitable test tubes. To our knowledge, no ordinary test tubes on the market are designed for this purpose. At present, avoiding air during sample handling is difficult, unhygienic due to difficulties filling tubes and tube caps completely, and potentially hazardous due to unavoidable blood spills during handling. Thus, new test tubes, as suggested in Fig. 3, needs to be designed to address this problem.

In conclusion, avoiding ambient air in test tubes during human whole-blood in vitro experiments reduce background complement activation substantially.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2020.112876.

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Declaration of Competing Interest

None of the authors declare any conflicts of interest.

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