WE investigated the *in vitro* effect of different forms of acidosis (pH 7.0) on the formation of anaphylatoxins C3a and C5a. Metabolic acidosis due to addition of hydrochloric acid (10 $\mu\text{mol/ml}$ blood) or lactic acid (5.5 $\mu\text{mol/ml}$) to heparin blood ($N=12$) caused significant activation of C3a and C5a compared to control (both $p=0.002$). Respiratory acidosis activated C3a ($p=0.007$) and C5a ($p=0.003$) compared to normocapnic controls. Making blood samples with lactic acidosis hypocapnic resulted in a median pH of 7.37. In this respiratory compensated metabolic acidosis, C3a and C5a were not increased. These experiments show that acidosis itself and not lactate trigger for activation of complement components C3 and C5.

Key words: Acidosis, Alkalosis, Complement system, Complement activation, Anaphylatoxins

Acidosis activates complement system *in vitro*

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Introduction

Hypoxia and reperfusion cause complement activation in animal experiments and in clinical studies.^{1–4} It could be shown, that after cytological damage, contact with cellular components such as mitochondria, or excess of hydroxyl radicals, was responsible for the activation of the complement system.^{5,6} On the other hand, *in vitro* studies showed a complement activation by acidosis or hypoxia only.^{7–9} In a previous study, we found complement activation induced by lactic acid.¹⁰ The aim of the present *in vitro* study was to investigate: (1) whether lactate itself or metabolic acidosis is responsible for activation, and (2) whether respiratory acidosis is also able to activate the complement system.

Material and Methods

Blood samples (10 ml) from 12 healthy volunteers (6 male and 6 female, age: 28–40 years) were collected and 50 IE Heparin was added. Each sample was divided into eight portions and placed in polypropylen tubes.

To investigate the influence of respiratory as well as metabolic changes of pH on anaphylatoxin formation, the portions were equilibrated with different gas mixtures and supplemented with acids resulting in marked changes in pH, $p\text{CO}_2$ and base deficit (Table 1). Probes used as controls are No. 1–3. Four portions were equilibrated with different gas mixtures at 37°C

to achieve normocapnia and normoxemia in control portions (No. 3; Table 1), respiratory acidosis (No 6; Table 1) or alkalosis (No. 7,8; Table 1). For this purpose we modified the method of Siriwardhana *et al.*¹¹ A 6 ml polypropylen tube (6 ml) was filled 1.5 to 2.0 ml blood and the end of a polypropylen tube with an inner diameter of 3 mm was placed into the blood a few millimetres above the bottom of the tube. Gas mixtures (O_2 , N_2 , CO_2) composed from medical gases by flowmeters were put through the tube into the blood sample. Gas flow was adjusted to one bubble per second for 20 min. Foam caused by bubbling was continuously removed by a suction catheter placed above the blood level. After equilibration, the blood samples were transferred into different tubes for further processing.

For metabolic acidosis 10 μmol hydrochloric acid (No. 4; Sigma-Aldrich, Deisenhofen, Germany) or 5.5 μmol lactate (No: 5; Sigma-Aldrich, Deisenhofen, Germany) were added per ml of blood. To achieve compensated metabolic acidosis, the sample was equilibrated with a hypocapnic gas mixture for 20 min and then 5.5 μmol lactate per ml of blood was added (No. 8).

Blood gas analysis, potassium and lactate concentrations were measured by a Radiometer Copenhagen ABL 505 (Willich, Germany) using heparinised syringes after all samples were incubated at 37°C for 1 h. To stop complement activation after incubation, we added 1 mg EDTA dissolved in purified water to each sample. Plasma was separated by centrifugation at

Table 1. Sample characteristics with different forms of acidosis after gas equilibration, addition of acids and 1 h incubation time at 37°C (N=12). Values are given as medians with quartiles

No	Name	Treatment	Sample procedure	pH value	pCO ₂ (mmHg)	Base excess (mEq/L)	Potassium (mmol/L)	C3a (µg/L)	C5a (µg/L)
1	baseline value (without incubation)	bubbling: additive:	No No	7.36 7.35/7.36	50 46/51	0.0 0.0/0.0	4.1 4.0/4.3	128 89/144	0.13 0.10/0.42
2	incubation control	bubbling: additive:	No No	7.30 7.27/7.33	50 44/53	-3.0 -2.4/-4.4	3.5 3.4/3.7	1595 972/2070	1.4 0.9/2.6
3	bubble control	bubbling: additive:	78% N ₂ , 5% CO ₂ , 17% O ₂ , 20 min No	7.30 7.25/7.35	51 45/57	-2.2 -0.8/-3.0	4.2 3.9/4.4	1025 802/1257	1.4 0.8/2.8
4	HCl acidosis	bubbling: additive:	No 10 µmol HCl per 1 ml sample	7.01 7.00/7.05	78 71/85	-13.8 -12.4/-14.9	5.2 4.5/5.7	3360 2860/3850	21.8 17.8/35.2
5	lactic acidosis	bubbling: additive:	No 5.5 µmol lactate per 1 ml sample	6.98 6.90/7.00	57 50/76	-17.1 -16.2/-19.6	6.4 6.4/6.8	2390 1975/2670	4.4 3.2/6.6
6	respiratory acidosis	bubbling: additive:	81% N ₂ , 19% CO ₂ , 20 min No	7.01 6.97/7.04	137 123/158	-5.2 -3.9/-7.3	4.3 3.9/4.7	1625 1080/1868	3.3 1.8/4.2
7	respiratory alkalosis	bubbling: additive:	83% N ₂ , 17% O ₂ , 20 min No	7.54 7.49/7.57	20 17/23	-3.5 -2.4/-4.0	4.4 4.1/4.7	917 825/1425	1.4 0.8/2.8
8	respiratory alkalosis + lactate	bubbling: additive:	83% N ₂ , 17% O ₂ , 20 min 5.5 µmol lactate per 1 ml sample	7.37 7.32/7.38	28 24/34	-8.3 -7.3/-10.5	4.2 4.1/4.5	935 760/1150	1.8 1.3/5.4

4°C at 3000 g for 10 min and frozen immediately at -80°C.

Anaphylatoxins C3a and C5a were determined as previously described.¹² C3a enzyme immunoassay (EIA, Fa. Progen Biotechnik GmbH, Heidelberg, Germany) selectively detects C3a-desArg using monoclonal antibodies.¹³ C5a was determined with a specific sandwich EIA (Fa. Behring, Marburg, Germany).¹⁴

Statistical analysis

As most of the data were not normally distributed, results were shown as medians with quartiles. Differences between controls and study samples were assessed using the Wilcoxon test. Statistical significance was assumed at *p*<0.05. All calculations and tests were performed with the SPSS-PC software (Chicago, Illinois, USA).

Results

Influence of incubation and equilibration procedures

The incubation (No. 2) and equilibration (No. 3) procedure led to a measurable complement activation compared to the baseline value (No. 1; Table 1). Therefore the results were compared to controls of the same handling method but without pH change.

Influence of metabolic and respiratory acidosis

Addition of hydrochloric as well as lactic acid caused a marked metabolic acidosis. Concentrations of C3a, C5a, and potassium were higher after incubation in acidic blood compared to incubation of control samples (No. 4 and 5 versus 2; Table 1). The potassium concentration was lower in hydrochloric acidosis and the anaphylatoxin concentrations were higher than

those from lactic acidosis (No. 4 versus 5). The anaphylatoxins were also higher in the hypercapnic samples compared to the normocapnic controls (No. 6 versus 3; Table 1).

Influence of respiratory alkalosis

Respiratory alkalosis had no influence on anaphylatoxin concentration (No. 7 versus 3; Table 1).

Influence of respiratory compensated metabolic acidosis

Although measured median lactate (13.1 versus 11.6 mmol/l) was not different in the lactic samples (No. 5 versus 8; $p=0.75$), anaphylatoxins were higher in acidic samples compared to respiratory compensated normacidic samples (No. 5 versus 8; Table 1).

Discussion

The present *in vitro* study confirms the results of our previous study that acidosis activates complement systems in heparin blood. The study shows that the acidosis itself is the trigger for activation, because all three forms of acidosis (hydrochloric or lactic acid or carbon dioxide) lead to a significant increase of anaphylatoxin concentrations. Lactate did not lead to an activation, when acidosis was prevented by previous respiratory alkalosis (No. 8; Table 1).

Heparin was chosen as anticoagulant, because the complement may be markedly spontaneously activated in serum and both EDTA and sodium citrate possibly influence pH values and impair complement activation by calcium binding. Heparin however did not completely prevent spontaneous complement activation even after incubation for 1 h at 37°C and using a bubble oxygenator for gas equilibration. Therefore we could only compare the extent of complement activation by acidosis to the spontaneous activation that occurred during the sample preparation procedure. As pH levels were similar in lactic and hydrochloric acidosis, the activation of the complement system was stronger for hydrochloric acid. The effects of the two acids on the blood gas analysis were different. Whereas lactate led to a larger increase in base deficit, hydrochloric acid caused a stronger release of carbon dioxide. This difference may explain the stronger complement activation by hydrochloric acid. Respiratory acidosis following equilibration of blood samples with elevated carbon dioxide concentrations also led to an activation of the complement system compared to equilibration control. In contrast to acidosis, respiratory alkalosis did not have an influence on the complement system.

With the used *in vitro* setting, we could not clarify the mechanism of anaphylatoxin formation by acidosis. Complement activation in acidosis mediated by

membrane fragments of damaged erythrocytes⁶ is improbable, as we could show that activation also occurs in plasma without cell components.¹⁰ There are other arguments against membrane fragment-induced complement activation in acidosis, increased potassium values and a slight visible hemolysis occurred only for metabolic but not for respiratory acidosis, but both forms of acidosis activate the complement system. A probable mechanism is that acidification of plasma inactivates complement protease inhibitors, this inhibition then results in complement activation in the absence or decreased presence of functional inhibitors.

The activation of the complement system by acidosis may explain the elevation of anaphylatoxins in different diseases, such as perinatal asphyxia, myocardial infarction and shock.^{3,4,15-17} These processes probably participate in the pathogenesis of reperfusion injury.^{4,17-19}

Conclusion

We have shown evidence that acidosis activates complement factors C3 and C5. This is independent from the type of acidosis which occurs.

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