

## Complement activation and depletion during LDL-apheresis by heparin-induced extracorporeal LDL-precipitation (HELP)

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Received 28 June 1990 and in revised form 30 October 1990

**Abstract.** The heparin-induced extracorporeal elimination of low density lipoproteins (LDL) is a well-established clinical procedure to markedly reduce cholesterol levels. The biocompatibility of this artificial filter system (HELP) was investigated by quantitation of representative complement proteins within the extracorporeal circuit using established ELISA procedures, based on monoclonal antibodies recognizing exclusively either native (C6, C7) or activated proteins (act.C3, C5a, TCC). HELP was found to be a self-limiting extracorporeal system with respect to complement activation, since act.C3 and TCC, generated mainly at the plasma filter, were partially adsorbed to the following HELP specific filters to concentrations which were lower than those obtained before the plasma filter. C5a, which increased 14.5-fold at the plasma filter was not eliminated by the following filters; however, elevated levels were not found in the patients at the end of apheresis and no leucocytopenia was observed.

**Keywords.** Complement, ELISA, extracorporeal plasma treatment, hypercholesterolaemia, LDL-apheresis, monoclonal antibody.

### Introduction

Heparin-induced extracorporeal LDL-precipitation (HELP) was developed to selectively remove LDL-cholesterol from the plasma of patients suffering from severe hypercholesterolaemia. It is based on the observation of Burstein & Scholnick [1] that lipoproteins can be differentially precipitated by heparin at acid pH in the absence of divalent cations. Seidel & Wieland [2,3] extended these findings to develop a specific

system for both the quantification of LDL-cholesterol in serum or plasma as well as the extracorporeal elimination of LDL from plasma based on their specific precipitation by heparin at pH 5–12. In addition to LDL, Armstrong *et al.* [4–6] demonstrated that a limited number of other plasma proteins, such as fibrinogen, the lipoprotein Lp (a) and the complement proteins C3 and C4 are extensively precipitated in the extracorporeal circuit of the HELP system. Although the efficiency and clinical safety of regular HELP treatment was recently reported by Eisenhauer *et al.* [7,8], until now no information has been available on the biocompatibility of this system with respect to complement activation. The fact that apparently no clinically visible side-effects were yet observed does not at all imply that this system is safe in terms of biocompatibility. In contrast, complement activation is a clinically relevant phenomenon induced by the filter materials used in the extracorporeal circuit and of crucial importance in defining the biocompatibility of dialysis membranes [9,11].

C3 is regarded as the central protein in the complement reaction sequence since it is essential to both the alternative and the classical pathways (Fig. 1) of the complement system [12]. The major cleavage product C3b is part of the C3-convertase of the alternative pathway as well as part of both pathways' C5-convertases. C3dg, a subsequent fragment during complement activation, is the major ligand for the CD21 molecule, the complement receptor 2 (CR2) on B-cells. The C3-fragments C3b, iC3b, C3dg, C3d are collectively termed activated C3 (act.C3).

C5a, generated by proteolytic action of the C5-convertases mediates the expression of a granulocyte adhesion-promoting surface glycoprotein on granulocytes thereby producing granulocytopenia during haemodialysis [13].

C5b, the other cleavage product of C5 forms together with C6, C7, C8 and C9 the terminal complement complex (TCC). During complement activation not only membrane-bound TCC but also fluid phase TCC is generated. In the fluid phase this complex also contains S-protein [14]. The membrane-bound form is

This work is dedicated to Prof. Fritz Scheler, Department of Internal Medicine, University of Göttingen, on the occasion of his 65th birthday.

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responsible for cell lysis [15] and in sublytic doses for a variety of non-lethal biological properties, including stimulation or release of inflammatory mediators from the target cell [16].

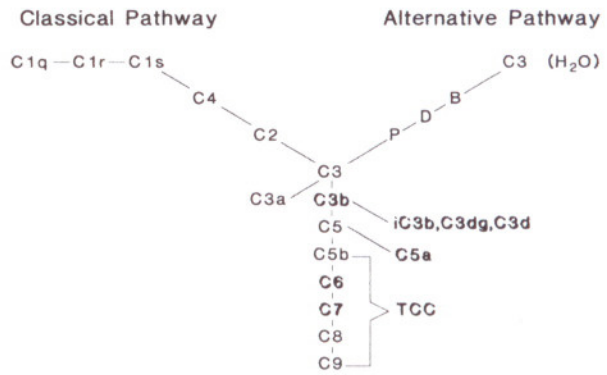
The three most important functions of the complement system, namely the interaction with other immune cells, the generation of anaphylatoxins and the lysis of cells are chiefly caused by C3-fragments, by the anaphylatoxin C5a and by the terminal complement complex TCC, respectively.

The quantitation of activated complement proteins is far more sensitive for the assessment of complement activation than is the measurement of the decrease in native complement proteins. We have, therefore, purified and characterized specific monoclonal antibodies (mAb) to establish sensitive enzyme-linked immunosorbent assays (ELISA) for the determination of the two central terminal components C6 and C7 in their native forms and for the complement protein fragments act.C3, C5a as well as for TCC. These ELISA have been used to investigate the effect of HELP treatment on complement activation and complement protein elimination to ascertain the biocompatibility of this system.

**Patients and methods**

*Patients*

Complement activation and elimination was studied in 24 patients (14 males and 10 females, age ranging from 27 to 65 years) who were undergoing regular HELP treatment at weekly intervals on account of coronary heart disease associated with severe hypercholesterolaemia. The LDL-cholesterol serum levels before the first HELP treatment ranged from 204 to 474 mg dl<sup>-1</sup> with a mean of 293 mg dl<sup>-1</sup> (SD 84, n=24) under



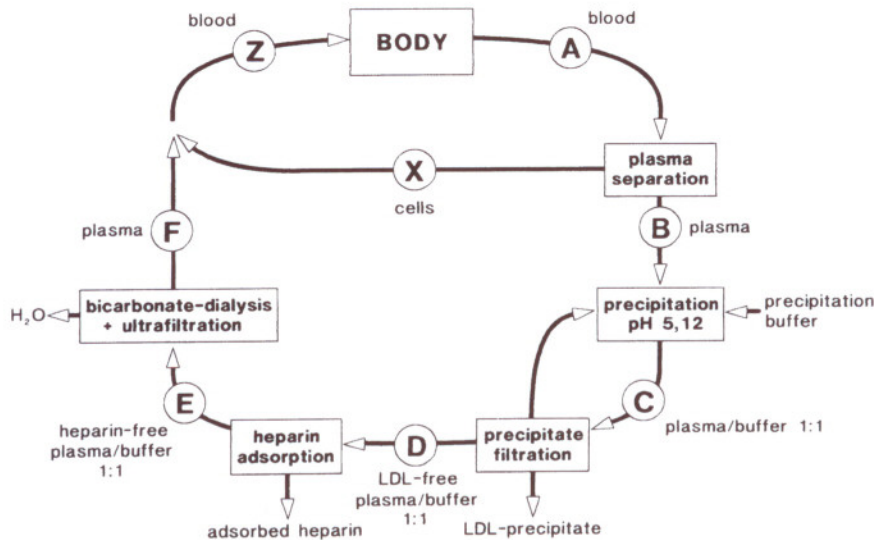
**Figure 1.** Complement cascade: the proteins investigated in this study are in bold type.

conventional diet and drug (cholestyramin plus fibrate analogues or nicotinic acid) therapy.

In 17 patients native and activated complement components were quantitated before systemic anticoagulation as well as directly after HELP treatment.

In a further seven patients, samples were withdrawn from the arterial access before contact with any of the filters (Fig. 2A) and at specific points in the extracorporeal circuit (Fig. 2 B-F). Samples were also obtained from the blood bypass (Fig. 2 X) and from the combined tube (Fig. 2 Z).

The blood was collected into a syringe containing dipotassium-EDTA to give a final concentration of 4 mmol. The plasma was separated from the blood cells by centrifugation at 1500 × g for 10 min at 4°C and was stored at -70°C. All samples were frozen as quickly as possible. Multiple freezing and thawing was avoided. Informed written consent was obtained from each patient after a full explanation of the purpose, nature and risks of HELP was given.



**Figure 2.** Extracorporeal circuit of the HELP system with the points (A, B, C, D, E, F, X and Z) at which the samples for complement quantitation were taken.



**Table 1.** Systemic complement concentrations before (SA) and after apheresis (EA) in comparison with the normal ranges obtained from healthy blood donors

	<i>n</i>	SA	EA	Normal range
act. C3 ( $\mu\text{g ml}^{-1}$ )	70	33.2 $\pm$ 14	31.7 $\pm$ 13	10–35
C5a (ng ml <sup>-1</sup> )	45	3.1 $\pm$ 1.6	2.8 $\pm$ 1.7	0.5–2.5
C6 ( $\mu\text{g ml}^{-1}$ )	31	70.8 $\pm$ 41	37.6 $\pm$ 20	20–80
C7 ( $\mu\text{g ml}^{-1}$ )	33	118.9 $\pm$ 59	93.4 $\pm$ 44	30–180
TCC (ng ml <sup>-1</sup> )	55	371.6 $\pm$ 176	276.1 $\pm$ 143	30–600

### Buffers

PBS: 9 mM phosphate buffer pH 7.2, containing 140 mM NaCl. PBS-Tween: PBS containing 0.05% (v/v) Tween 20 (Serva, Heidelberg, Germany).

### ELISA for the quantitation of act.C3, C5, C6, C7 and TCC

The generation, purification and characterization of monoclonal (mAb) and polyclonal antibodies (pAb) directed against act.C3, C5a, C6, C7 and TCC and the ELISA procedures for the quantitation of these complement proteins have been described in detail elsewhere [17–21]. Briefly, mAb directed against the complement proteins were adsorbed to microplate wells overnight at 4°C. The optimized amount of mAb was dissolved in 100  $\mu\text{l}$  coating buffer: 68  $\mu\text{l}$  0.2 M Na<sub>2</sub>CO<sub>3</sub>, 32  $\mu\text{l}$  0.2 M NaHCO<sub>3</sub>, pH 10.6. The microplate wells were washed twice with PBS-Tween between all the following steps. All incubations were carried out at 20°C for 1 h. After blocking with PBS, supplemented with 1% gelatine (GPBS) the wells were incubated with an optimized dilution of human plasma. The dilution in the circuit due to the addition of an equal volume of precipitation buffer (Fig. 2 C–E) was taken into consideration. Biotinylated mAb or pAb were then added to the wells after which peroxidase-labelled streptavidin (Amersham, Braunschweig, Germany) was added. Finally, 2,2' azino-di (3-ethylbenzthiazolinesulphonate) (ABTS) (Boehringer, Mannheim, Germany) was introduced and the absorption at 410 nm was recorded using an automatic ELISA plate reader (Dynatech, Alexandria, USA). The data were expressed as the increase in optical density per minute and compared to a dilution of a standard. All ELISA are based on mAb which are not only complement protein specific but also exclusively directed against the native or the activated form. mAb directed to the latter are neo-epitope specific and therefore avoid precipitation steps for the elimination of native complement proteins. Biotinylated rabbit anti-C3d IgG (Dako, Hamburg, Germany)—for the detection of mAb-bound C3 fragments in the act.C3 ELISA—was the only commercially available antibody used in the five ELISA. Standard ranges of all ELISA are compiled in Table 1. These were obtained

**Table 2.** Systemic complement concentrations obtained immediately before each apheresis

	<i>n</i>	Weeks of therapy				
		0	1	2	3	6
act. C3 ( $\mu\text{g ml}^{-1}$ )	4	54	49	49.4	55.2	50.2
C5a (ng ml <sup>-1</sup> )	2	3.5	4.1	2.7	3	2.6
C6 ( $\mu\text{g ml}^{-1}$ )	1	126	100	116	105.5	131
C7 ( $\mu\text{g ml}^{-1}$ )	1	84.2	124.4	79.6	106.8	79.6
TCC (ng ml <sup>-1</sup> )	2	403.1	414.7	309.4	383.1	400.9

by quantitating the complement components in over 50 plasma samples of laboratory personnel and healthy blood donors.

### Heparin-induced extracorporeal LDL-precipitation (HELP)

The HELP procedure is described in detail elsewhere [4,7,22]. Briefly, plasma is obtained by filtration of heparinized whole blood through a 0.55- $\mu\text{m}$  polypropylene filter and is then mixed continuously with an equal volume of a 0.2-mol sodium acetate buffer (pH 4.85) containing 100 i.u. ml<sup>-1</sup> of heparin (B. Braun-Melsungen AG, Melsungen, Germany). Precipitation occurs at a final pH of around 5.12. The suspension is cleared by a 0.4- $\mu\text{m}$  polycarbonate filter and excess heparin is completely adsorbed by an anion exchange filter (DEAE cellulose). Finally, physiological pH is restored by bicarbonate dialysis and excess fluid is removed by ultrafiltration before the plasma is mixed with the blood cells and returned to the patient.

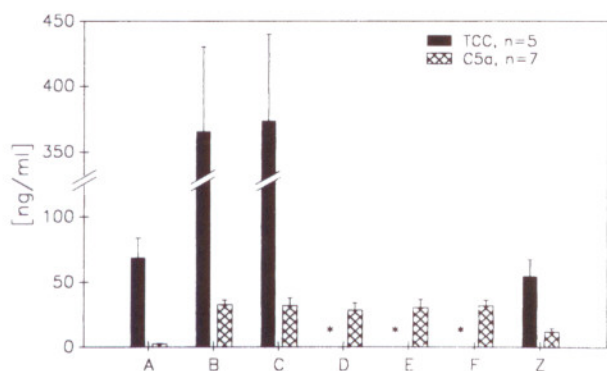
## Results

### Quantitation of complement proteins

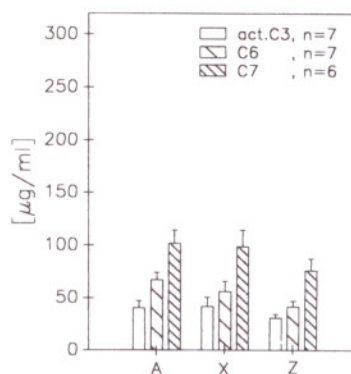
*Before apheresis.* At the start of apheresis (SA) mean concentrations of C7 and TCC were within the normal range while those for act.C3 and C6 were in the upper normal range compared to healthy blood donors (Table 1). The mean C5a concentration in the patients, however, was somewhat above the upper normal values. No major changes occurred in the values of any of these parameters over a period of 6 weeks of regular HELP treatment (Table 2).

*Within the extracorporeal circuit.* The plasma filter produced an increase in act.C3 (1.5-fold), C5a (14.5-fold) and TCC (5.3-fold) while native C6 (13%) and C7 (15%) were reduced (Figs 3 B and 4 B). C5a and TCC remained at these elevated levels after addition of the heparin-sodium-acetate buffer to accomplish precipitation (Fig. 3 C). Act.C3, however, was also generated (4.5-fold) at this stage, whereas C6 (22%) and C7 (28%) were further reduced (Fig. 4 C). It has to be considered that at this point all proteins retained by the subsequent polycarbonate filter had been reintroduced to the system by the recirculation hoop.

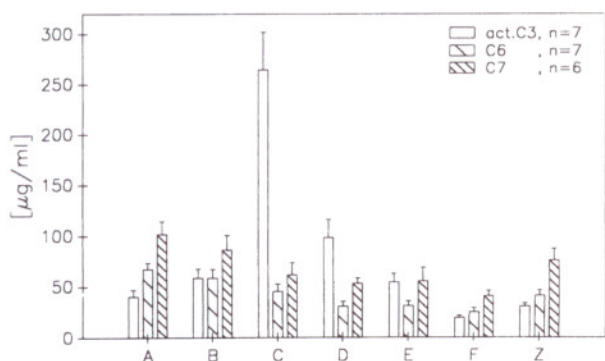




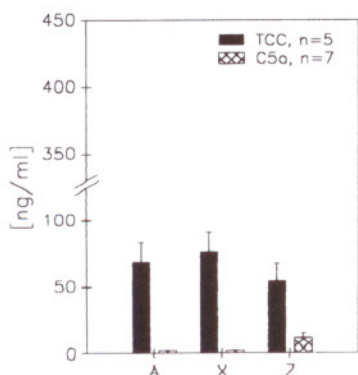
**Figure 3.** Mean concentrations of C5a and TCC measured at various points in the extracorporeal circuit of the HELP system. The points (A, B, C, D, E, F and Z) at which the samples were taken are illustrated in Fig. 2. At the points D, E and F, TCC concentrations below the detection limit of the TCC-ELISA ( $12 \text{ ng ml}^{-1}$ ) were obtained in all patients ( $n=5$ ), marked \*.



**Figure 6.** Mean concentrations of act.C3, C6 and C7 measured at various points in the extracorporeal circuit of the HELP system. The points (A, X and Z) at which the samples were taken are illustrated in Fig. 2.



**Figure 4.** Mean concentrations of act.C3, C6 and C7 measured at various points in the extracorporeal circuit of the HELP system. The points (A, B, C, D, E, F and Z) at which the samples were taken are illustrated in Fig. 2.



**Figure 5.** Mean concentrations of C5a and TCC measured at various points in the extracorporeal circuit of the HELP system. The points (A, X and Z) at which the samples were taken are illustrated in Fig. 2.

C5a passed the precipitation filter whereas TCC was totally eliminated (Fig. 3 D). Act.C3, C6 and C7 were partially eliminated (reduction of 63, 32 and 14%, respectively) (Fig. 4 D).

There was no major change in the C5a concentration during adsorption of excess heparin or during ultrafiltration and bicarbonate dialysis; TCC, eliminated during the preceding step, could no longer be detected (Fig. 3 E and F). Act.C3 showed some affinity for these filters under the given conditions since the remaining concentration was reduced by 50% and then 64%; C6 and C7 passed the heparin adsorber but decreased during the ultrafiltration and bicarbonate dialysis by 20 and 27%, respectively (Fig. 4 E and F).

If the concentrations before entry into the circuit (Figs 3 A and 4 A) are compared with those obtained after passage through all filters (Figs 3 F and 4 F) a marked reduction of C6 (63%) and C7 (60%) and a total elimination of TCC (100%) had occurred. Despite the generation of new act.C3 its value was also reduced after passing all filters (Fig. 4 F). C5a, which was generated at the plasma filter, was not affected by any of the following filters; a 14-fold increase (Fig. 3 F) compared to the concentration obtained before contact with any of the filters (Fig. 3 A) was found.

The flow in the apheresis circuit was  $25 \text{ ml min}^{-1}$  and in the blood bypass  $55 \text{ ml min}^{-1}$ . In the combined tube there was a flow of  $80 \text{ ml min}^{-1}$ . When we compare the concentrations in the blood bypass (Figs 5 X and 6 X) and after dialysis (Figs 3 F and 4 F) with those of the combined tube (Figs 3 Z and 4 Z) the averages of the measured concentrations were 7.6% lower on average than the concentrations calculated, considering the flow rates (calculation not shown).

The overall changes in complement protein concentrations quantitated after passage through all filters (Figs 3 F and 4 F) were reduced in the combined tubing (Figs 3 Z and 4 Z) after mixing with the blood cells when the complement concentrations (Figs 5 X and 6 X) were comparable to those measured before contact with the plasma filter (Figs 5 A and 6 A). In the



**Table 3.** Haematological parameters and albumin concentrations before (SA) and after apheresis (EA)

	<i>n</i>	SA	EA
Haemoglobin (g dl <sup>-1</sup> )	6	14.1	13.6
Leucocytes (× 10 <sup>3</sup> μl <sup>-1</sup> )	6	7.3	7.7
Erythrocytes (× 10 <sup>6</sup> μl <sup>-1</sup> )	6	4.6	4.4
Albumin (g dl <sup>-1</sup> )	7	4.7	4.1

**Table 4.** Haematological parameters and albumin concentrations within the extracorporeal circuit of the HELP system

	<i>n</i>	A	B	F	Z
Haemoglobin (g dl <sup>-1</sup> )	6	14.5	—	—	14.2
Leucocytes (× 10 <sup>3</sup> μl <sup>-1</sup> )	6	5.1	—	—	5.3
Erythrocytes (× 10 <sup>6</sup> μl <sup>-1</sup> )	6	4.7	—	—	4.6
Albumin (g dl <sup>-1</sup> )	7	—	4	3.1	—

tubing leaving the extracorporeal circuit (Figs 5 Z and 6 Z) C6, C7, act.C3 and TCC were 38, 25, 23 or 20% lower, respectively when compared to the prefilter concentrations whereas the C5a concentration was 5.2-fold higher (Figs 5 A and 6 A).

*After apheresis.* At the end of apheresis (Table 1 EA) there was no major change in the plasma concentrations of C5a (*n* = 45) and act.C3 (*n* = 70) compared to the values obtained before apheresis (Table 1 SA) despite the higher concentration of the former (*n* = 7) and the lower concentration of the latter (*n* = 7) in the combined tubing (Figs 5 Z and 6 Z). Plasma C6 (47%), C7 (22%) and TCC (26%) were found to be markedly reduced after apheresis (Table 1 EA) as compared to the concentrations obtained before the start of apheresis.

#### Haematological parameters

Haemoglobin and erythrocyte concentrations were reduced by 3.6 and 4.6%, respectively at the end of apheresis (Table 3). During the extracorporeal passage no leucocytopenia occurred (Table 3). The incoming (from the patient, Table 4 A) and outgoing (to the patient, Table 4 Z) tubings of the extracorporeal circuit contained comparable haemoglobin, erythrocyte and leucocyte concentrations.

#### Albumin

The albumin concentration was 12.8% lower at the end of apheresis (Table 3 EA) as compared to the start of apheresis (Table 3 SA). This was due to an elimination of albumin in the extracorporeal circuit, where the

albumin concentration was 22.5% lower after passage through all filters (Table 4 F) as compared to that obtained directly after the plasma filter (Table 4 B).

#### Discussion

HELP is a highly effective extracorporeal procedure for the elimination of low-density lipoproteins from plasma with an efficiency of around 100% [7]. A total exchange of 2500–3000 ml plasma results in a LDL-reduction to 30–40% of the pre-apheresis concentration. Although over 80% of total protein can be returned to the patient, the small loss of protein that occurs cannot be explained by the elimination of LDL alone. Thus, despite its high efficiency, HELP is not entirely specific and a small number of other proteins, namely fibrinogen, plasminogen, complement components C3, C4 and C1-inhibitor, are also eliminated [7]. Since the blood clotting system has been shown to be directly linked to the complement system [23] and since many extracorporeal systems with artificial filters are known to activate complement [17,24] it might also be presumed that the complement cascade will be affected by HELP. The present study was designed to investigate in detail the changes in the complement cascade induced by the various components of the HELP system.

The polypropylene plasma filter used for the separation of plasma and cells is the only filter of the HELP system which comes into contact with the blood cells. The filter activated complement as shown by the increased levels of C3 fragments (act.C3), C5a and TCC that were measured on the plasma side of the filter (Fig 3 B and Fig 4 B). Other apheresis and dialysis techniques also generate by-products of the classical and alternative pathway such as C3a [25,26], act.C3 [17,25], Ba [17], and C5a [25,26] as well as TCC [25].

The initial 14.5-fold increase in C5a during HELP was not altered by any of the following filters. The lack of a further increase might be explained by good biocompatibility of the subsequent filters or by a reduction in C5 convertase formation due to the precipitation of native C3 and C4 [7]. It might also be explained by the elimination of C5 due to the multiple binding properties of heparin (see below). Despite the marked generation of C5a, its plasma levels at the end of treatment were unchanged. Two explanations may be offered to explain these findings. First, the treated plasma containing activated complement proteins is mixed with the blood cells which are returned to the patient. Since C5a is not increased in the tubing containing the blood cells a reduction of the C5a levels will occur at this point. Second, a rapid binding of C5a to granulocytes and monocytes [27] cannot be excluded. We did not investigate whether HELP inhibited the binding of C5a to its cellular receptor or the biological sequelae of this binding [28].

The 5.3-fold elevated TCC concentrations measured after the plasma filter were dramatically reduced after the precipitation filter. Native C6 and C7 were also



reduced. In addition, C6 and C7 concentrations were decreased by bicarbonate dialysis and were also lower in the precipitation chamber. These reductions may be caused by molecular interactions of C6, C7 and TCC with S-protein, heparin and lipoproteins. Terminal complement proteins are able to bind to S-protein via a basic binding domain close to its COOH terminus [29]. This domain also represents the heparin binding site of S-protein which is involved in the functional role of S-protein as a heparin-neutralizing protein [30]. Barnes *et al.* [31] have shown that at low pH binding of S-protein to heparin is promoted. Not only S-protein but also C6 and C7 can complex with heparin, especially at low pH (5.4) [32]. Furthermore, heparin binds LDL at acid pH which is the key principle of HELP [1]. Lipoproteins in turn may bind to nascent TCC thereby inactivating it [33]. In addition, the molecular structure of the LDL-receptor revealed a high level of homology with C6, C7, C8 and C9 [34-37] at the apoprotein binding domains [38,39]. Since all proteins discussed retain the ability to bind to each other and since LDL and heparin are completely adsorbed by either the polycarbonate filter or the heparin adsorber column, terminal complement components and the TCC may have been partially eliminated as complexes together with these proteins. This hypothesis may be confirmed by a future project dealing with the detection of complement proteins on the surfaces of the filters and in the eluates of HELP.

The increase of act.C3 at the plasma filter is due to complement activation. However, at the precipitation step with heparin at low pH a further increase in act.C3 was detected. This may be explained either by the precipitation itself and/or by the previous contact of the plasma with the polycarbonate filter via the recirculation loop. In other extracorporeal systems the appearance of C3 degradation products in the extracorporeal circulation was much higher when heparin was used as an anticoagulant compared to citrate [40,41]. A heparin-specific mechanism was therefore suggested. It was shown that heparin prevents the generation of the C3 amplification convertase by binding to C3b and masking the binding site for B on C3b [42]. In the case of a complement activation this would result in an increase of C3 fragments without generation of C5a or TCC. The binding of C3b to heparin is possibly potentiated due to the low pH (5.12) where the latter is still present as a polyanion, since a low-affinity interaction site between C3b and B was shown to be modulated by polyelectrolytes [43].

At the end of apheresis haematological parameters, such as erythrocyte or haemoglobin concentrations were reduced by about 4% reflecting the dilution effect of the blood by the physiological salt solution used to fill the extracorporeal circuit prior to apheresis. No dramatic change in leucocyte concentration was observed in accordance with earlier findings [7]. This is in contrast to observations made with cardiopulmonary bypass by Salama *et al.* [44], who suggested that the observed haemolysis and leucopenia might be caused

by complement activation on the surface of blood cells, since TCC was not only detected in the fluid phase but also on lysed erythrocyte membranes and granulocytes at the end of the extracorporeal bypass.

Since complement activation in HELP occurred in the absence of blood cells and since fluid phase TCC has no lytic capacity, the additional trigger for diverse pathological reactions collectively described as post-perfusion syndrome [26] which in part may be caused by TCC [45] was avoided.

The data obtained from within the circuit also explain the data at the end of apheresis, where C6, C7 and TCC were significantly reduced. Nevertheless all concentrations returned to normal within 7 days. No permanent change in complement concentrations over a period of 6 weeks was observed. The only major biological effect which has to be considered is the increase in C5a, even if it is not detectable at the end of apheresis because of immediate binding of this anaphylatoxin to granulocytes and monocytes.

We conclude that HELP, an apheresis system for the treatment of severe hypercholesterolaemia, possesses good biocompatibility with respect to the sequelae of complement activation.

In addition, our data strongly suggest that the biocompatibility of extracorporeal systems should be assessed by quantitating all three main complement activation products, act.C3, C5a and TCC, not only before and after the treatment but also within the circuit and, if possible, in the absence of blood cells.

#### Acknowledgments

The authors are grateful to the staff of the HELP-LDL-apheresis Unit. This work was supported by the Bundesministerium für Forschung und Technologie (01 ZJ 8502) and the Deutsche Forschungsgemeinschaft (SFB 236/B6, SFB 330/A6, SFB 330/B11).

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