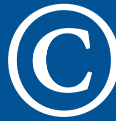

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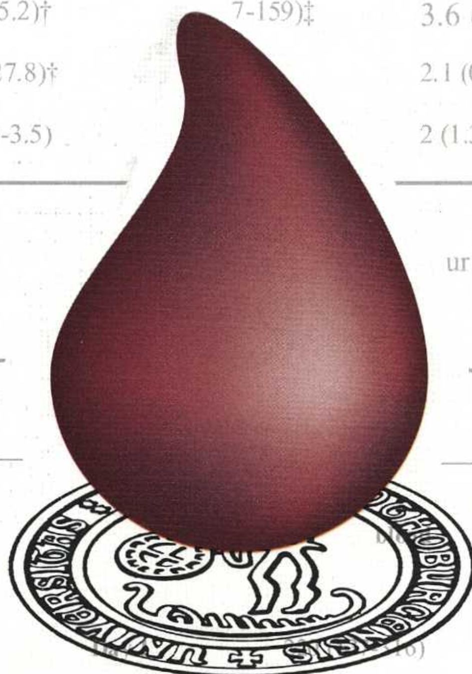
PREOPERATIVE DEPOSIT OF AUTOLOGOUS BLOOD

Effects on inflammatory mediators



Monica Hyllner

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PREOPERATIVE DEPOSIT OF AUTOLOGOUS BLOOD

Effects on inflammatory mediators

AKADEMISK AVHANDLING

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av

Monica Hyllner

leg. läkare

Fakultetsopponent: Docent Hans Hjelmqvist, Huddinge Universitetssjukhus, Stockholm

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I. Hyllner M, Arnestad JP, Bengtson JP, Rydberg L & Bengtsson A.
Complement activation during storage of whole blood, red cells, plasma, and buffy coat. Transfusion 1997; 37: 264-268

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ABSTRACT

Blood contains complex cascade systems and substances that can be activated during the processing of blood components and storage. Allogeneic blood, i.e. blood from someone else, is normally separated into components before storage and transfusion, while autologous blood (the patient's own blood) often is used as whole blood. Allogeneic transfusions are associated with a variety of risks and preoperative autologous blood donation (PABD) has therefore become an established alternative. For patients with cancer, the immunosuppressive effect of allogeneic blood may be detrimental, but PABD is difficult because of the urgency of surgery. Normally, PABD begins 4-6 weeks before the scheduled operation and blood is tapped weekly. The additional use of recombinant erythropoietin (rHuEPO) therapy increases the volume of tapped autologous blood before surgery. However, other studies indicate that rHuEPO therapy suppresses postoperative endogenous erythropoietin (EPO) production and stimulates inflammatory mediator release. The aim of the present thesis was to investigate the effects on perioperative erythropoiesis, and the inflammatory mediator release during the predeposit and storage of autologous blood.

In the present study, blood from healthy blood donors was collected and stored as whole blood or as separate components. Complement activation and release of pro-inflammatory cytokines were followed during the storage time. In addition, the effect of prestorage leucocyte filtration on inflammatory mediators was studied. Women undergoing radical hysterectomy were scheduled to predeposit three units of autologous blood during two weeks before surgery, with or without rHuEPO therapy. Erythropoiesis and the immune response were investigated during the pre- and postoperative follow-up.

The results demonstrate that complement is activated during storage of whole blood and plasma, and the cytokine IL-8 is released during storage of whole blood. Prestorage filtration of plasma activates the complement cascade but does not influence cytokine generation. Clearly, it was possible for women to predeposit three units of blood in only two weeks prior to surgery. A haemoglobin level below the 100 g/l donation limit can be prevented in one patient out of seven, by treating women with rHuEPO. The use of rHuEPO increases the postoperative endogenous EPO response but does not influence the cytokine release. The present thesis suggests that PABD can be offered to female patients undergoing cancer surgery, and that autologous blood can be transfused as whole blood.

Key words: allogeneic, autologous, complement, cytokines, blood storage, erythropoiesis, hysterectomy, plasma, prestorage leucocyte filtration, rHuEPO, whole blood.

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Effects on inflammatory mediators

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"Blood is still the best thing possible to have in our veins"

Woody Allen

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PUBLICATIONS AND MANUSCRIPTS

This thesis is based on the following publications and manuscripts, which are referred to in the text by their Roman numerals.

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ABBREVIATIONS

Allogeneic transfusion	transfusion of blood from someone else
Autologous transfusion	reinfusion of the patient's own blood
$C1_{inh}$	C1-esterase inhibitor
C3a	complement anaphylatoxin C3a
C5a	complement anaphylatoxin C5a
C5b-9(m)	membrane-bound terminal C5b-9 complement complex
CPDA	citrate-phosphate-dextrose-adenine
desArg	des Arginine
ELISA	enzyme-linked immunosorbent assay
EPO	erythropoietin
FNHTRs	febrile nonhaemolytic transfusion reactions
IL	interleukin
IU	international units
PABD	preoperative autologous blood donation
rHuEPO	recombinant human erythropoietin
SAGM	saline-adenine-glucose-mannitol
SC5b-9	soluble terminal C5b-9 complement complex
s-EPO	serum erythropoietin
TCC	terminal complement complex
TNF-α	tumour necrosis factor-alpha

INTRODUCTION

"There is a shortage of blood. Donate blood." This request from the Blood Bank is often repeated in the radio and newspapers when the demand of allogeneic blood exceeds the supply. Allogeneic blood is collected from someone else than the patient and used for transfusion. Autologous blood, on the other hand, is collected from the patient himself. Each unit of allogeneic whole blood is normally separated into several components before storage, while autologous blood often is stored as whole blood. Allogeneic blood transfusion has been in use for almost a century but is still associated with different well recognised inherent and unavoidable risks such as infectious disease transmission, adverse immunologic reactions and immunosuppression. The immunosuppressive effect is associated with an increase in postoperative infection rates as well as cancer recurrence and shortened survival (Blumberg *et al.*, 1990; Chang *et al.*, 2000; Van de Watering *et al.*, 2001). To avoid risks and to decrease the demand for allogeneic blood, several alternatives have been developed. These alternatives include preoperative autologous blood donation (PABD), recombinant human erythropoietin (rHuEPO) therapy, haemodilution, intra- and postoperative blood salvage or a combination of techniques.

Blood contains several complex cascade systems and biologically active substances that can be activated during the processing of blood components and subsequent storage (Silliman *et al.*, 1994; Edvardsen *et al.*, 2001). With a transfusion, cellular components of blood and coagulation factors are transferred as desired and required. However, inflammatory mediators that may be responsible for adverse transfusion reactions are also transferred. The clinical significance of inflammatory mediators in transfused blood products remains unclear. It is speculated that they may have an additive and detrimental effect on the complex activated immune network in severe conditions, especially following massive transfusion (Kristiansson *et al.*, 1996). To reduce adverse reactions, an increasing number of blood banks are introducing reduction of leucocytes in allogeneic blood before storage. Prestorage leucocyte filtration diminishes the accumulation of leucocyte-derived cytokines during storage but does not eliminate transfusion reactions (Chalandon *et al.*, 1999; Ibojie *et al.*, 2002). Other biologic response modifiers that become activated and accumulated during storage of blood products may play a role in the remaining reactions.

Predeposit of autologous blood is probably the most effective and most frequently used form of autologous blood replacement. Collection of autologous blood is usually performed once a week and starts 4-6 weeks before the scheduled operation. In cancer surgery, immunosuppression from allogeneic blood transfusions may be detrimental with increased

cancer recurrence and shortened survival. There is also limited time available for an autologous program; so as not to delay the cancer operation, collection has to be performed more rapidly. The additional use of rHuEPO therapy increases the volume of autologous blood that can be donated before elective surgery (Mercuriali *et al.*, 1993). However, other studies indicate that rHuEPO therapy suppresses postoperative endogenous erythropoietin (EPO) production and stimulates inflammatory mediator release (Tasaki *et al.*, 1992; Takemasa *et al.*, 2000). If so, this would be a clinically important disadvantage of rHuEPO therapy as regards postoperative haemoglobin recovery.

BACKGROUND

Transfusion medicine history

The English physician William Harvey discovered the circulation of blood in 1628 and in 1665, the physicians Richard Lower of Cornwall and Edmund King performed the first successful blood transfusion from one dog to another. Jean Baptiste Denis, professor of surgery in Paris, transfused blood from a lamb to a dying boy in 1667. The first successful human allogeneic blood transfusion took place in 1818. James Blundell, a British obstetrician, performed a transfusion of human blood for the treatment of postpartum haemorrhage. Great advances in transfusion medicine came in 1900 when Karl Landsteiner, an Austrian physician, discovered the first three human blood groups and later in 1939/40 the Rh blood groups. A major initial problem of storing blood was coagulation. In 1914, Louis Agote and Albert Hustin, independently, added sodium citrate allowing longer preservation of blood and in 1915, Richard Weil demonstrated the refrigerated storage of such anticoagulated blood. Francis Peyton Rous and JR Turner of the Rockefeller Institute in 1916 introduced citrate-glucose that permitted storage of blood for a longer term. Oswald Robertson, an American army officer, is credited with creating the blood depots on a battlefield during World War I. Bernard Fantus originated the term “blood bank” and established the first hospital blood bank in 1937 at the Cook County Hospital in Chicago.

Storage of blood

Long-term storage of blood and blood components is a routine procedure necessary to meet the various needs of modern medicine. A whole blood donation of 450 ml takes about 10-20 minutes. Each unit of allogeneic blood is normally separated into several components by centrifugation and the use of a triple-bag system. The red cells and plasma are separated from the buffy coat and transferred to the bags in a closed system. The buffy coat mainly

consists of leucocytes and platelets from which the platelet concentrate is obtained. Whole blood may be stored for a maximum of 35 days, and red cells and plasma for 42 days. The storage time is determined by the post-transfusion survival of the red blood cells. These products are kept refrigerated at a temperature of +2-6 °C. The optimal storage temperature for platelets is +18-22 °C, which limits the storage time to 5 days. The blood is tested for blood type, Rh type and unexpected red blood cell antibodies. Screening tests are also performed to exclude donor infection with hepatitis viruses B and C, human immunodeficiency viruses (HIV) 1 and 2, human T-lymphotropic viruses (HTLV) I and II and syphilis.

Allogeneic transfusion risks and alternatives

Allogeneic blood transfusion is associated with risks such as adverse immunologic reactions, immunosuppression and infectious disease transmission. The immunologic reactions include allergic reactions, haemolytic and febrile nonhaemolytic transfusion reactions (FNHTRs). Less common but life-threatening reactions are transfusion-associated graft-versus-host disease and transfusion-associated acute lung injury (Silliman *et al.*, 2003; Yassura *et al.*, 2000). Other effects of blood transfusion referred to as transfusion-associated immunomodulation are believed to be mediated via immunosuppression (Blajchman, 2002). The underlying mechanism has not been determined and the role of allogeneic blood transfusion remains controversial. In association with total hip replacement surgery, lower levels of IL-6 and IL-8 were found in patients transfused with allogeneic blood compared with patients receiving autologous whole blood (Åvall *et al.*, 1997, 2002). This may be explained by suppressed cellular immune function in the allogeneic group. Leucocytes may be linked to immunosuppressive effects and leucoreduction of transfused blood significantly reduces the prevalence of postoperative infections, although it has not been shown to affect long-term survival and/or cancer recurrence (Jensen *et al.*, 1996; Van de Watering *et al.*, 2001). Leucodepletion results in a significantly decreased mortality in cardiac surgery when more than three units of blood are transfused (Van de Watering *et al.*, 1998). The immunosuppressive effect of blood transfusion may be beneficial in organ transplantation and in disorders such as inflammatory bowel disease. Allogeneic blood transfusion is thought to protect renal allografts from rejection and to reduce the recurrence rate of Crohn's disease (Opelz & Terasaki, 1978; Peters *et al.*, 1989). For patients with cancer and trauma, immunosuppression may on the other hand be detrimental. There are studies indicating an increase in postoperative infection rates as well as cancer recurrence and shortened survival

after allogeneic blood transfusion compared with patients without transfusion or transfused with autologous blood (Blumberg *et al.*, 1990; Carson *et al.*, 1999; Van de Watering 2001). One single unit of transfused allogeneic blood has been associated with an increased risk of postoperative infections (Vignali, 1996). However, other investigators who explicitly consider potential confounding factors have not been able to provide data to support an association between allogeneic transfusion and complications (Vamvakas *et al.*, 1996; McAlister *et al.*, 1998).

The risk of acquiring blood-borne disease from allogeneic transfusion has been minimised and the risk-benefit of allogeneic blood has clearly changed. The introduction of blood screening technology using nucleic acid amplification tests to detect viruses promises to reduce the risks even further. Many transfusion complications are linked to donor leucocytes. An increasing number of blood banks, also in Sweden, have therefore introduced universal leucoreduction by prestorage filtration of blood components in order to avoid leucocyte-associated reactions.

Alternatives to allogeneic blood transfusion include PABD, rHuEPO therapy, haemodilution, intra-and postoperative blood salvage or a combination of these techniques. Preoperative blood collection for autologous transfusion has become an established alternative to traditional allogeneic blood transfusion. Most transfusion complications of allogeneic blood are eliminated since the patient serves as his own blood donor and therefore receives the safest blood. A limiting factor for the volume of blood donated may be that autologous blood donors have been reported to develop mild anaemia with only a moderate increase in erythropoiesis and EPO production (Kickler & Spivak 1988; Lorentz *et al.*, 1991). The additional use of rHuEPO in preoperative autologous blood collection programs has been shown to make the collection of blood more efficient (Goodnough *et al.*, 1989; Mercuriali *et al.*, 1993). The volume of blood donated can be increased and the patient maintains a higher haematocrit at the time of operation.

Preoperative autologous blood donation

The first documented use of autologous blood is dated to 1921 when Dr FC Grant, University Hospital in Philadelphia, performed a successful autologous predeposit phlebotomy in a patient undergoing an elective operation for a cerebellar tumour (Grant, 1921). PABD is applicable to elective procedures where the expected blood loss is large and transfusion likely. Surgical procedures where PABD may be indicated are e.g. coronary artery bypass graft, radical prostatectomy and orthopaedic procedures such as total hip and knee

replacement. A guideline for the amount of units required is the blood order on the operating list. Donor regulations and requirements are more flexible than for allogeneic donors. Criteria such as age and past or present medical problems do not necessarily preclude autologous donation if the patient is a candidate for elective surgical procedures. Any patient with a haemoglobin value less than 100-110 g/l, active infection or bacteraemia, or unstable vital signs is however rejected (Thomas *et al.*, 1996). Patients usually begin donations 4-6 weeks before the scheduled operation, are tapped every 3-7 days, take oral iron supplementation and deposit the last unit 72 hours before operation (Toy & Kerr, 1996). The occurrence of adverse effects in autologous donors is no greater than for allogeneic donors. The blood is collected into citrate-phosphate-dextrose-adenine (CPDA)-1 whole blood bags (450 ml) and stored up to 35 days at 4 °C. Autologous blood requires special labelling and segregated storage compared with allogeneic blood. The "leap-frog" technique may be used if surgery is postponed, infusing the oldest unit to the patient to allow a fresh one to be tapped. The technique has also been suggested for PABD programs.

Erythropoietin

History and physiology

Carnot and Deflandre (1906) first formulated the hypothesis of erythropoiesis control by a humoral factor "hémopoïétine" in 1906. The name "erythropoietin" was introduced in 1948 and the molecule was isolated and purified from human urine in 1977 (Bonsdorff & Jalavisto, 1948; Miyake, 1977). EPO is a glycoprotein hormone produced by the kidneys (90 %) and the liver (10 %), and is released into the circulation in response to tissue hypoxia. The hormone originates from peritubular interstitial cells in the cortex of the kidneys (Lacombe *et al.*, 1988). The EPO concentration in plasma increases exponentially with decreasing haemoglobin concentration (anaemia), or when the pO_2 in the inspiratory gas is lowered (hypoxaemia) (Jelkmann, 1992). The hormone is the primary regulator of human erythropoiesis. In the bone marrow, EPO binds to and activates specific receptors on the surface of erythroid progenitor cells, which then differentiate into functional erythrocytes. These specific cell surface receptors belong to the cytokine receptor superfamily (Zhu & D'Andrea, 1994). About 1 % of circulating red blood cells is usually renewed each day ($2\text{-}3 \times 10^{11}$/day). The rate of erythropoiesis can increase up to tenfold following blood loss or exposure to high altitude (Schobersberger *et al.*, 1998). Acute anaemia induces an increase in EPO production by the mouse kidney within 1-2 hours and the maximum EPO production occurs after 4 hours (Bondurant & Koury, 1986).

Recombinant human erythropoietin

EPO consists of two fractions, α and β, with similar biological activity, molecular mass and amino acid composition but different electrophoretic mobility and carbohydrate composition. The synthesised recombinant product (epoetin α and β) became available in 1985. Results suggest that epoetin can activate a broad spectrum of progenitor cells (Stockenhuber *et al.*, 1990; Abraham *et al.*, 1992). The pharmacokinetics vary depending on the route of administration. Brief peaks in plasma levels and a short half-life of 6-8 hours characterise intravenous administration (Flaharty *et al.*, 1990). The subcutaneous route results in lower but more sustained plasma levels and the mean half-life for elimination of 100 IU/kg rHuEPO is 18 hours (Hughes *et al.*, 1989). A hyperglycosylated recombinant analogue of EPO called novel erythropoiesis-stimulating factor (NESP, darbepoetin) has been produced for therapeutic use (Fisher, 2003). Darbepoetin has a higher carbohydrate content (52 % vs. 40 %) than epoetin resulting in about 3-fold longer half-life after intravenous administration. The amino acid sequence differs from that of human EPO at five positions. Darbepoetin can maintain haemoglobin levels just as effectively as rHuEPO at less frequent dosing in patients with chronic kidney disease (Allon *et al.*, 2002).

The serum erythropoietin (s-EPO) levels are expressed in international units (IU). The concentrations are similar in men and women despite the difference in haemoglobin levels and EPO levels are independent of age (Cotes, 1982). Published values of the mean s-EPO concentration are about 15 IU/l with a range of 6-32 in non-anaemic individuals.

Therapy

There are several indications for rHuEPO therapy. The anaemia associated with chronic renal disease can be corrected with epoetin therapy and quality of life can be improved. The majority of dialysis patients need a weekly dose of < 200 U/kg, generally given 3 times a week, and the preferred route of administration is the subcutaneous (Bergström, 1993). Subcutaneous compared with intravenous administration results in a more favourable plasma concentration profile and makes administration more convenient, with the possibility of self-administration at home (Salmonson *et al.*, 1990). Therapy is also useful in other types of anaemia, such as in rheumatoid arthritis, malignancies or chemotherapy. RHuEPO given to patients with a normal haematocrit before elective surgery may lead to more efficient autologous blood donation and reduced dependence on allogeneic transfusions (Mercuriali *et al.*, 1993; Rauh *et al.*, 2002).

The most common cause of blunted response to rHuEPO therapy is iron deficiency and patients should be given iron supplementation during rHuEPO therapy (Crosby, 2002). An important adverse effect of rHuEPO therapy in uremic patients is arterial hypertension. This is explained by the increase in haematocrit, which leads to an increase in blood viscosity and peripheral vascular resistance (Nonnast-Daniel *et al.*, 1989). Other reported side effects are thrombocytosis, epileptiform seizures, iron deficiency, allergic reactions and clotting of the vascular access (Bennett, 1991).

Inflammatory mediators

The Complement System

History

The immune system is a complex, highly regulated process that protects an individual from injury. The injury or challenge may be of endogenous origin, e.g. host tumour or aged cells, or exogenous e.g. microorganisms, transplant cells, toxins or allergens. Two types of immunity, innate and adaptive, work together to defend the human body against diverse challenges. The innate immunity includes a cellular response with phagocytosis, and a humoral that leads to the activation of complement. The term *complement* was originally proposed by Ehrlich in 1899 to describe how serum complements and amplifies the action of antibodies so as to cause bacterial lysis. Later studies have demonstrated that complement also serves as an independent immune system.

Nomenclature and activation

The complement system consists of more than 30 different proteins that proceed in a cascade sequence of activation (Figure 1). The "a" fragment is a smaller, released peptide that can promote the local inflammatory response, whereas the "b" fragment is the remaining, larger component that binds with the target and continues the cascade. The individual complement components are numbered numerically in the order they were described: C1, C4, C2, C3, C5, C6, C7, C8 and C9. The activation is organised into two main pathways, the classical and the alternative (Liszewski & Atkinson, 1993). The lectin pathway is initiated by carbohydrates in the cell wall of certain microorganisms and activates complement through the classical pathway (Ikeda, 1987). The classical pathway is usually activated by IgG or IgM antibodies bound to antigens on the surface of a microorganism. Five proteins that participate in this pathway only include the complex of C1 (C1q, C1r and C1s), C4 and C2. The alternative pathway is the first line of defence against invading microorganisms with the

ability to recognise immediately and to respond to foreign membranes/elements. Proteins unique to the alternative pathway are symbolised by letters e.g. factor B, factor D and factor P. Both pathways lead to the proteolytic cleavage of the central C3 protein with the assembly of membrane attack complexes and the recruitment of various white blood cells. The activated C3b binds to the membrane of a microbial cell and initiates the local assembly of the late complement components to large membrane attack complexes, C5b-9, that form transmembrane channels and induce lysis of the invading cell (Müller-Eberhard, 1986). Immune adherence is the binding of C3b to immune complexes or opsonized particles. This facilitates the removal of immune complexes in the liver and spleen and phagocytosis of the microbial cell. The cleavage of C3, C4 and C5 results in the generation of biologically active components C3a, C4a and C5a. C5a is the most potent, followed by C3a and distantly by C4a (Hugli, 1984). The anaphylatoxins C3a and C5a are complement split products that act as mediators of inflammatory reactions. They cause tissue damage, smooth muscle contraction, increased vascular permeability and release histamine from mast cells and basophils (Hugli, 1979; Hugli & Marceau, 1985).

Control mechanisms

The body does not leave a reaction uncontrolled. Regulatory proteins control complement activation to protect the host against self-damage. Soluble serum proteins (e.g. C1-esterase inhibitor ($C1_{inh}$), ß1H (H), C3b Inactivator (I), clusterin, vitronectin) and integral membrane proteins (e.g. DAF, CD59) act as inhibitors or inactivators of specific reactions or products in the complement cascade. The plasma enzyme carboxypeptidase controls the effect of C3a, C4a and C5a by removing a carboxy-terminal arginine (*desArg*). The anaphylactic properties are hereby lost, but some of the chemotactic effect of C5a remains. Many of the activated components in the cascade are unstable and lose activity within milliseconds if not combined with their target.

Synthesis

The proteins are mainly produced in the liver. The exception is C1, which is synthesised in the epithelial cells of the intestine. Activated macrophages and monocytes can synthesise limited quantities of most complement components at the site of inflammation.

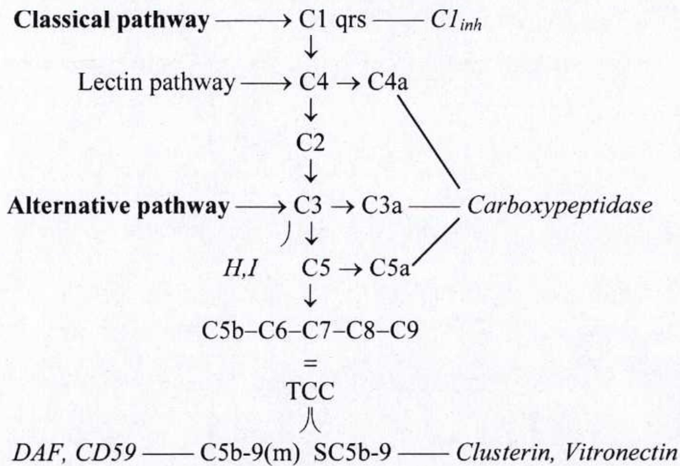


***Figure 1**. A schematic illustration of the complement cascade and activation pathways. The regulatory proteins are printed in italics.*

Clinical aspects

Complement activation follows trauma and ischaemia, and correlates with the severity of the injury. The formation of anaphylatoxins C3a and C5a and terminal C5b-9 complement complex is important to defend the human body against diverse challenges. However, excessive activation of the cascade system may be deleterious. Extensive formation of anaphylatoxins and terminal C5b-9 complement complex may damage remote organs such as the lungs, liver or the kidney, and organ dysfunction may develop if the inflammatory response is not limited to the site of injury (Figure 2). Clinical studies of multiple organ failure and sepsis have demonstrated an association between high anaphylatoxin levels and mortality (Hecke *et al.*, 1997).

Activation by foreign surfaces

When blood comes in contact with foreign materials, e.g. dialysis membranes or a heart-lung machine, the complement system is activated (Hakim *et al.*, 1984; Cavarocchi, 1986). Exposure of plasma to a plastic surface, e.g. blood-bag, has the potential to activate the complement cascade (Sevast'ianov & Tseytlina, 1984). Modification of the artificial surface by coating with heparin reduces activation of inflammatory systems (Videm *et al.*, 1999). The degree of complement activation is an indicator of the biocompatibility of different artificial materials.

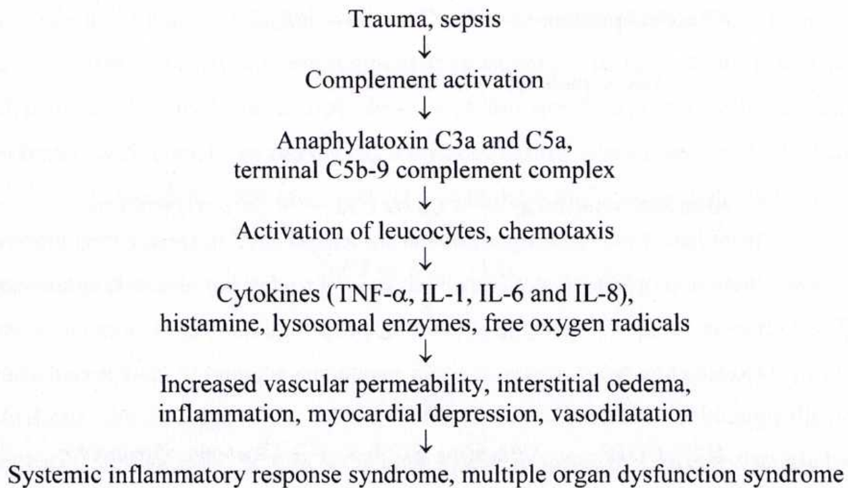


Figure 2. *The inflammation cascade.*

The cytokine network

Cytokines are a heterogeneous group of protein molecules mainly produced and secreted by leucocytes. They are extremely potent and act at picogram per liter concentrations in a complex network. They are involved in both immunity and inflammation, and regulate the amplitude and duration of the inflammatory response by binding to specific cell surface receptors (Balkwill & Burke, 1989). There are four major groups of cytokines, colony-stimulating factor (CSF), interferon (IFN), tumour necrosis factor (TNF), and interleukins (IL). They transmit messages between cells to regulate cell growth, differentiation and activity.

Clinical aspects

Release of cytokines occurs in association with different diseases (Damas *et al.*, 1997). Clinical studies of multiple organ failure and sepsis have demonstrated an association between high interleukin levels and mortality (Marty *et al.*, 1994; Meduri *et al.*, 1995). The pro-inflammatory cytokines TNF-α, IL-1β and IL-6 are involved in the acute inflammatory response and they all have potent pyrogenic activity, whereas IL-8 is a neutrophil-chemotactic and neutrophil–activating factor (Bellomo, 1992). The febrile response is mediated by prostaglandin E_2 production in the hypothalamus (Stitt, 1986).

Tumour necrosis factor

TNF exists as two polypeptides, α and β, with similar but not identical effects. TNF-α is pro-inflammatory and produced by macrophages, monocytes and lymphocytes in response to lipopolysaccharide, viruses, tumour cells, complement and toxins. Anaphylatoxins act synergistically with lipopolysaccharides leading to higher IL-1 and TNF release by monocytes and macrophages (Cavaillon *et al.*, 1990). Experimental studies show multiple effects, such as induction of tumour cell necrosis and endothelial cell proliferation (Sugarman *et al.*, 1985; Saegusa *et al.*, 1990). Symptoms of TNF-α release are fever, lactic acidosis, hyperglycaemia, stress hormone release, capillary leak and haemodynamic effects seen in septic shock (Dinarello *et al.*, 1986). TNF-α is proposed to be a key mediator of organ injury during sepsis (Bellomo, 1992).

Interleukin-6

IL-6 is a glycoprotein produced by various types of cells, such as activated T and B cells, monocytes and fibroblasts (Kishimoto, 1989). The half-life is approximately 1 hour. Viral infections, IL-1, TNF-α, interferon gamma, and lipopolysaccharide induce the production (Van Snick 1990). IL-6 plays a major role in host defence systems by regulating antibody production and acute phase protein synthesis (Lotz *et al.*, 1989). IL-6 impairs cardiac function and is a potent vasodilator of skeletal muscle resistance vessels (Finkel *et al.*, 1993; Minghini *et al.*, 1998).

Interleukin-8

IL-8 is a polypeptide produced by a variety of cells in response to stimulation by IL-1, TNF-α and lipopolysaccharide. IL-8 is known as a chemotactic and inflammatory cytokine inducing neutrophil chemotaxis and enzyme release (Van Dervort & Danner, 1990; Kunkel *et al.*, 1991). High concentrations have been demonstrated in septic shock patients. Clinical sepsis studies have demonstrated an association between high IL-8 levels and a fatal prognosis (Damas *et al.*, 1997).

AIMS OF THE INVESTIGATIONS

The overall objective of the present thesis was to investigate the effects on perioperative erythropoiesis, and the inflammatory mediator release during the predeposit and storage of autologous blood. Several basic aims were established:

- Complement activation during storage of blood components.
- Cytokine release during storage of whole blood and plasma.
- The effect of prestorage leucocyte filtration on inflammatory mediator release.
- The effect of preoperative rHuEPO therapy on endogenous EPO response and cytokine release.
- Whether an intensive autologous blood donation schedule increases EPO levels.
- Whether preoperative rHuEPO therapy enables autologous blood collection in a short period of time.

PATIENTS AND METHODS

Patients

The Human Ethics Committee of the University of Göteborg approved the studies and all patients gave their informed consent. Blood from 12 volunteers and 24 blood donors was collected and stored under ordinary blood bank conditions at 4 °C for 35 days (**I-II**). Forty-one women with cervical carcinoma scheduled for radical hysterectomy and pelvic lymphadenectomy were randomised to preoperative autologous blood donation with or without rHuEPO therapy (**III-IV**). All patients were scheduled to deposit three units of whole blood during two weeks prior to operation. At each visit, blood was collected if the capillary haemoglobin level was ≥ 100 g/l. All patients received the same type of general anaesthesia during operation and all patients were transfused with one to three units of autologous whole blood. Transfusion was given intraoperatively when the haemoglobin level decreased to less than 85 g/l or if the patient showed clinical signs of hypovolaemia. Peripheral venous blood samples were obtained before blood collection at the preoperative visits. In the rHuEPO group, samples were also taken 10 minutes after intravenous erythropoietin administration. Furthermore, samples were taken preoperatively the day before operation or the same day, one hour after operation, on day 1 after operation, on day 5 and finally at five weeks after operation. Analyses were performed of blood concentrations of folates, haemoglobin, leucocytes, platelets, erythrocyte volume fraction, and reticulocytes. Serum concentrations of erythropoietin, folates, vitamin B_{12}, bilirubin, ferritin, haptoglobin, iron and total iron binding capacity were also measured.

Blood collection and storage

Paper I. Whole blood was collected into CPDA-1 blood bags (BB*SCD456P, Terumo, Tokyo, Japan) and stored as whole blood, or collected into a triple blood bag system (Optipac, R1693, Baxter, La Chatre, France) and separated into red cells in saline-adenine-glucose-mannitol (SAGM) medium, plasma and buffy-coat. All units were stored for 5 weeks under ordinary blood bank conditions. Samples were collected, by sterile technique, on storage days 1, 2, 3, 7, 14, 21, 28 and finally day 35. The complement components $C1_{inh}$, C3, C4, C5, C3a, C5a and SC5b-9 were determined.

Paper II. Eight out of 24 blood units collected from healthy blood donors (450 ml) were collected into Baxter CPDA-1 single bag KGR 6113B and stored as whole blood. Eight units were collected into a triple bag system, Baxter CPD-SAGMAN Optipac KGR 7322B, and separated into plasma, red cells and buffy coat. The final 8 units were collected as whole

blood into Baxter CPD-SAGMAN Optipac KGR 7487B, and separated into plasma and red cells after leucocyte filtration with filter ASAHI RZ 2000. Baxter S.A., Maurepas, France, manufactured all bags. Samples were collected weekly during the storage time for analyses of potassium, leucocytes, free plasma haemoglobin, C3a, SC5b-9, IL-6, IL-8 and TNF-α. Blood cultures were taken from the bags (**I-II**) at the end of storage and they were all negative.

The autologous whole blood units (**III-IV**) were collected into CPDA-1 bags containing 327 mg citrate, 251 mg phosphate and 27.5 mg adenine per 100 ml (Baxter, Deerfield, Illinois, USA).

Erythropoietin therapy

The women in the rHuEPO group received 10.000 IU (150 IU per kg) of rHuEPO every day for ten days from the first donation visit (**III-IV**). At the three visits for blood donation, a nurse administered rHuEPO intravenously after phlebotomy. The other seven days the patients themselves administered the rHuEPO subcutaneously. No rHuEPO was given postoperatively.

Erythropoietin determinations

Paper IV. Samples were drawn into tubes for serum with no additives. The samples were immediately centrifuged for 10 minutes at 4000 rpm to remove the cells. The remaining plasma was then frozen within 30 minutes and stored at -80 ºC until analysis. S-EPO was measured with a commercially available enzyme-linked immunosorbent assay (ELISA), (Roche Molecular Biochemicals, Mannheim, Germany). The reference value is 9.9 ± 2.9 IU/l. All tests were duplicated.

Complement and cytokine determinations

All samples were drawn into tubes containing 0.054 ml of 0.34 M ethylenediamine tetraacetic acid per 4.5 ml of blood. The tubes were then immediately centrifuged for 10 minutes at 4000 rpm to remove the cells. The supernatant was frozen in separate tubes within 30 minutes and stored at -80 °C until assay. All analyses were performed in duplicate.

Paper I. The concentrations of the complement components $C1_{inh}$, C3, C4, and C5 was determined with rocket immunoelectrophoresis technique (antibodies from Behring, Behringwerk AG, Marburg, Germany). ELISA was used to analyse the anaphylatoxin

$C3a/C3a_{desArg}$ (Progen Biotecnik, Heidelberg, Germany), and $C5a/C5a_{desArg}$ and SC5b-9 (Behring, Behringwerk AG, Marburg, Germany).

Paper II. C3a and SC5b-9 were analysed with a commercially available ELISA (Quidel, San Diego, CA, USA). The assay does not distinguish between C3a and $C3a_{desArg}$. The cytokines were analysed with an ELISA (Endogen, Woburn, MA, USA). The detection limits were 1 pg/ml for IL-6 and 2 pg/ml for TNF-α and IL-8.

Paper IV. IL-6 and IL-8 were analysed with an ELISA (R&D Systems Europe Ltd., Abingdon, UK).

Statistical methods

Papers I-IV: The values are given as medians and 25-75 % percentiles or ranges of the values. The Mann-Whitney test, two-tailed, was used for all comparisons between the two groups. Assessment of changes within groups was performed by Friedman's test, which can be considered a nonparametric method of ANOVA for repeated measures. If significant changes were found, comparisons within groups were performed by using Wilcoxon's test for pair comparisons, two-tailed. Differences were considered significant at $p<0.05$.

In **paper II,** a linear regression analysis was performed for each bag and each laboratory variable, with time as the independent variable and the different laboratory variables as the dependent one. Within each blood component group we tested for each variable whether there was a significant change over time by applying Wilcoxon's test for paired comparisons to the sample of regression coefficients. Comparisons between plasma and filtered plasma were performed by Mann-Whitney's test with respect to the regression coefficients and with respect to the level at day 21, calculated by the regression function of each bag. Day 21 was chosen as the centre of the studied time period. By performing the analysis this way, we limited the number of comparisons in order to reduce the risk of significances by chance. Two-tailed tests were used.

In **paper III**, the left tail (below 125 g/l) of haemoglobin distribution was assumed to coincide with a normal distribution. The maximum likelihood estimate of the mean and SD of that distribution was determined by a special program (Anders Odén, Kungälv, Sweden).

The correlation coefficient between EPO and IL-6 in **paper IV** was calculated by simple linear correlation (Pearson's method).

RESULTS

Complement activation during storage of blood components

Complement concentrations in whole blood, plasma and buffy coat increased during storage (**I**). High concentrations of C3a were found in plasma and buffy coat after 14 days of storage (Figure 3). The concentrations of anaphylatoxins C3a and C5a in whole blood increased significantly during storage (Figure 4). Increased C5a levels were observed already after 7 days of storage and C3a after 21 days of storage. Anaphylatoxin C5a was significantly increased in buffy coat after 7 days. Concentrations of C3a, C5a, SC5b-9, $C1_{inh}$, C3, C4 and C5 were low or undetectable in the red cell concentrates.

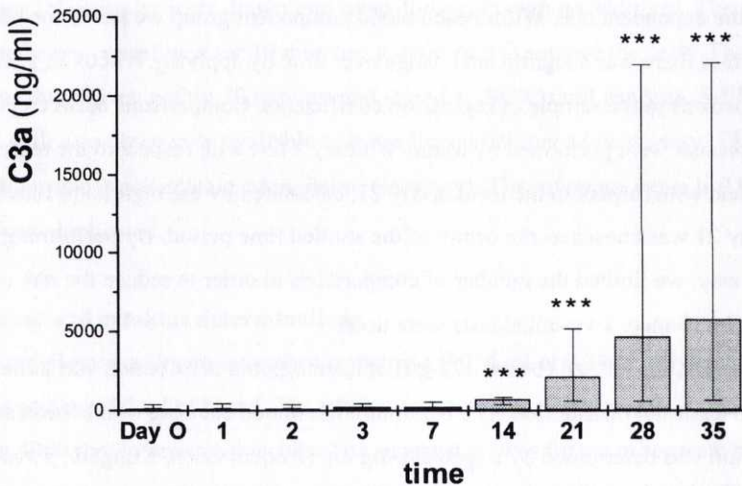
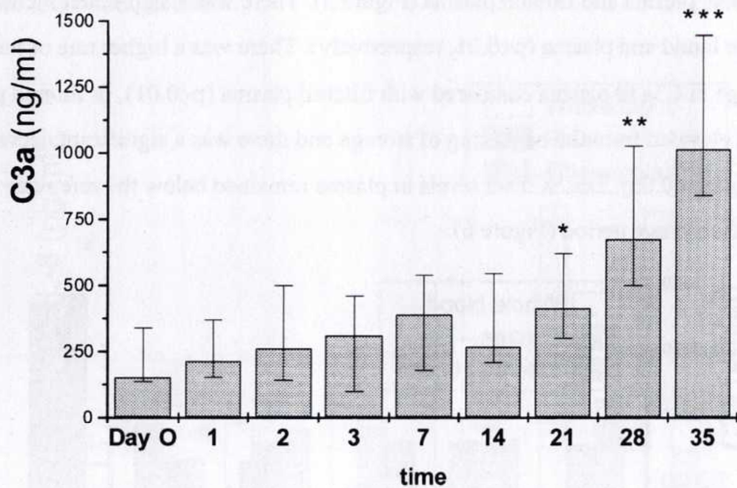


Figure 3. *C3a in plasma during storage. Median values and range.*

****= p<0.001.*



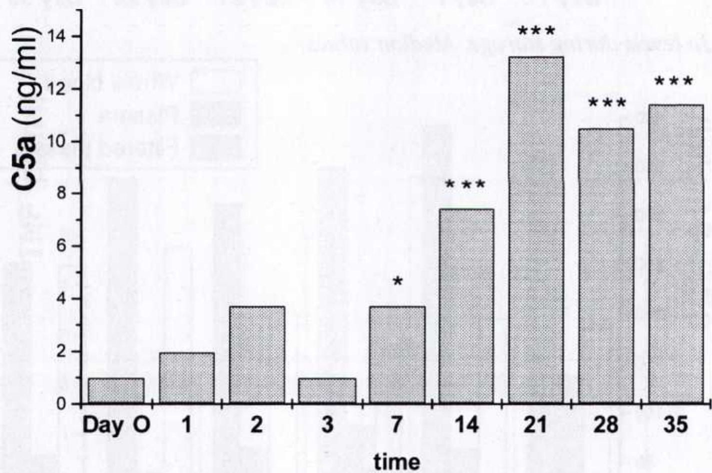


***Figure 4**. C3a and C5a in whole blood during storage. Median values and range.*
$*= p<0.05$, $**= p<0.01$ and $***= p<0.001$.

Complement activation and prestorage leucocyte filtration of plasma

Elevated levels of C3a and SC5b-9 were found in filtered plasma from the beginning of storage (**II**). The concentrations of anaphylatoxin C3a increased continuously during storage of whole blood, plasma and filtered plasma (Figure 5). There was a significant increase in C3a in whole blood and plasma ($p<0.01$, respectively). There was a higher rate of increase during storage in C3a in plasma compared with filtered plasma ($p<0.01$). In filtered plasma, SC5b-9 was elevated from the beginning of storage and there was a significant decrease during storage ($p<0.05$). The SC5b-9 levels in plasma remained below the reference value throughout the storage period (Figure 6).

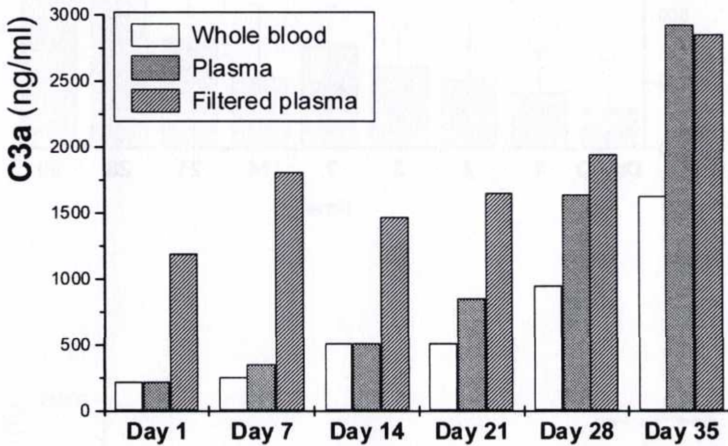


Figure 5. *C3a levels during storage. Median values.*

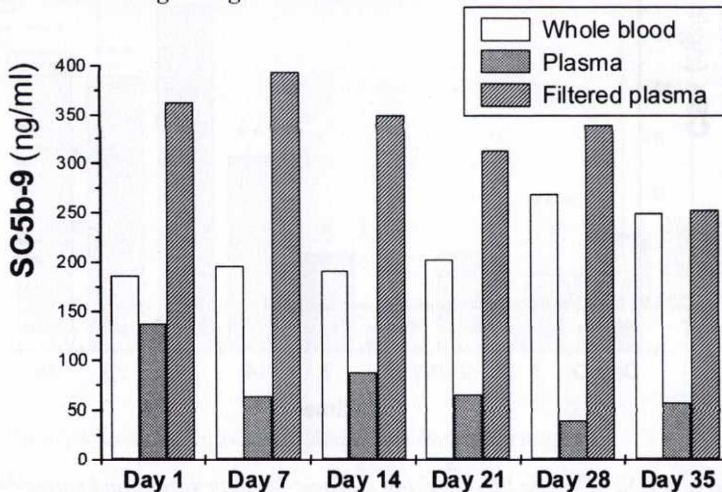


Figure 6. *SC5b-9 levels during storage. Median values.*

Effects of prestorage filtration and storage on cytokine release

IL-8 and TNF-α in whole blood increased significantly during storage, whereas IL-6 decreased significantly (**II**; Figure 7-8). The cytokine levels generated in plasma and filtered plasma were low or undetectable.

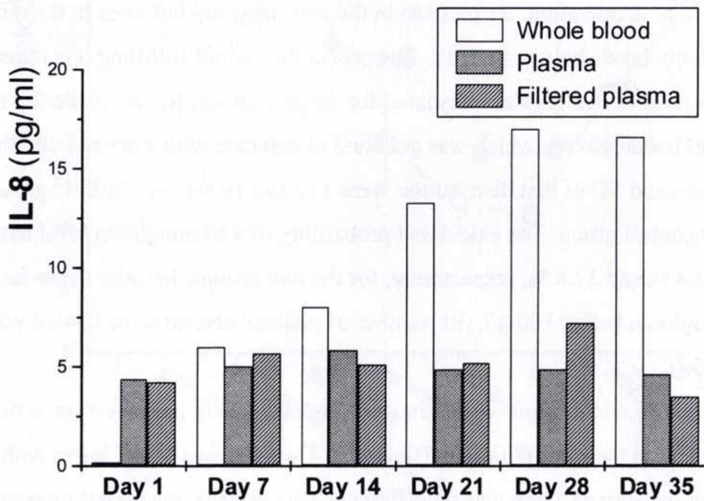


Figure 7. *IL-8 during storage. Median levels.*

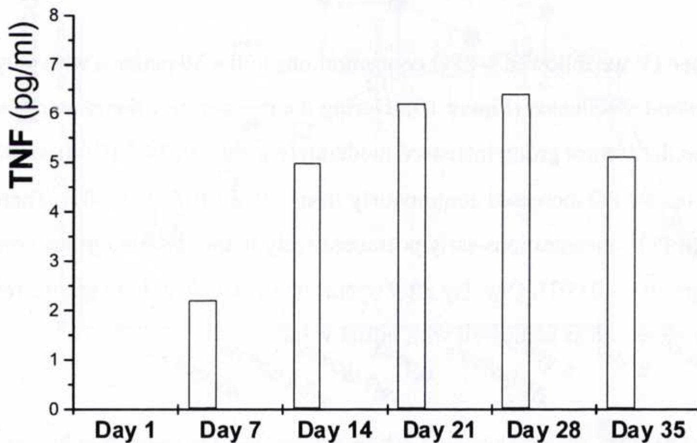


Figure 8. *TNF-α in whole blood during storage. Median levels.*

Erythropoietic response

Thirty-seven women predeposited three units of autologous blood each **(III)**. The last occasion of blood donation before operation is the most critical one with respect to a haemoglobin level of at least 100 g/l. Three patients in the control group (no rHuEPO therapy) and one patient in the rHuEPO group had venous haemoglobin levels below 100 g/l at this time. Prior to operation, six patients in the control group but none in the rHuEPO group had haemoglobin levels below the limit. The probability of not fulfilling this criterion of a haemoglobin level of 100 g/l was calculated for the two groups by use of the left tail of the distribution of haemoglobin, which was assumed to coincide with a normal distribution. The estimated mean and SD of that distribution were 118 and 10 for the rHuEPO group and 108 and 9 for the control group. The calculated probability of a haemoglobin level below 100 g/l was 3.4 % and 17.8 %, respectively, for the two groups. In order to obviate one patient with a haemoglobin below 100 g/l, the number of patients needed to be treated was 7 = 1/(0.178-0.034).

The haemoglobin median concentrations dropped during precollection in both groups, significantly less in the rHuEPO group (figure 9). The drop was 12 g/l lower with rHuEPO therapy. After the surgery there was no difference between the groups but on postoperative day 1 there was a significant difference with the highest haemoglobin value in the rHuEPO group. On postoperative day 5 and after 5 weeks there was no longer any significant difference between the two groups. The reticulocyte count increased in both groups during autologous donation, but earlier and to significantly higher values in the rHuEPO group (Figure 9).

In **paper IV** we followed s-EPO concentrations in the 30 patients who only received autologous blood transfusion (Figure 10). During the preoperative donation period, median s-EPO levels in the control group increased moderately from 7 to 14.4 IU/l ($p<0.05$). In the rHuEPO group, s-EPO increased continuously from 5 to 85 IU/l ($p<0.001$). There was an increase in s-EPO concentrations early postoperatively in the rHuEPO group compared with the control group ($p<0.001$). One day after operation the levels in both groups remained significantly increased as compared with initial values.

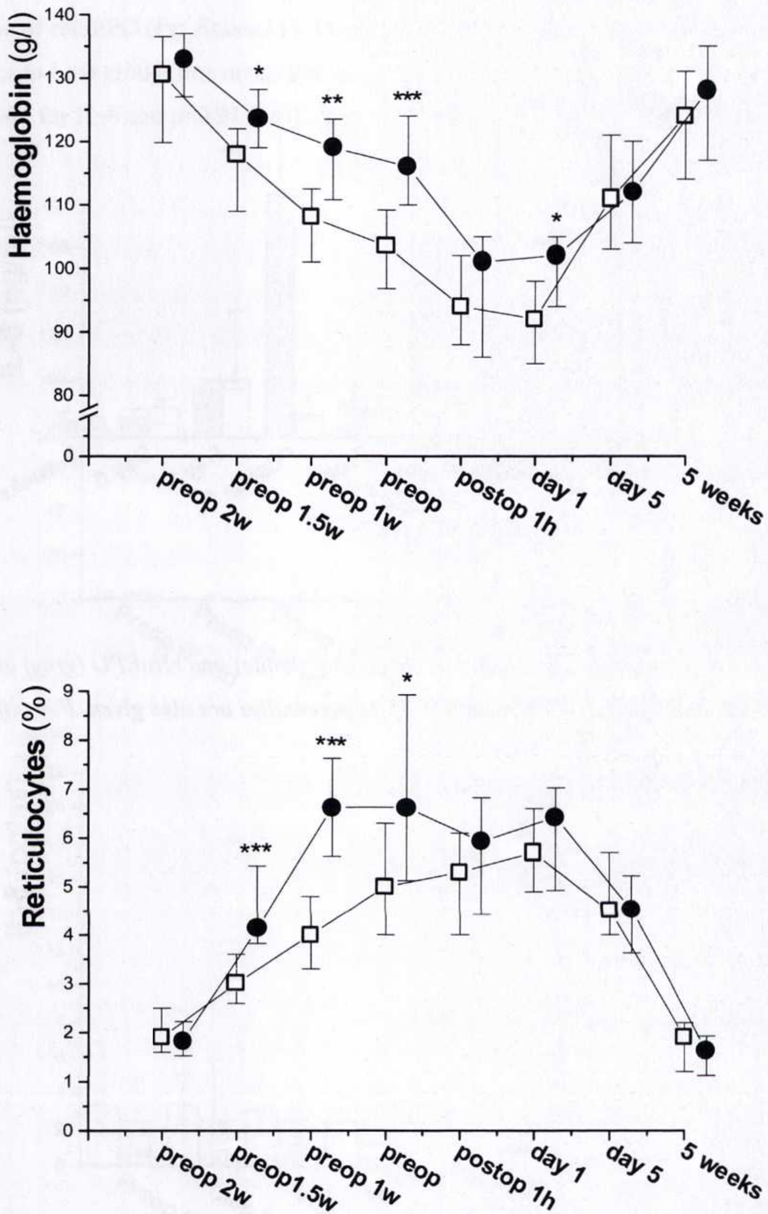


Figure 9. *Median blood values of haemoglobin and reticulocytes in the rHuEPO (●) and control (□) groups during the pre- and postoperative follow-up. 25-75 % percentiles are also given. For differences between the groups: *= p<0.05, ** =p<0.01, *** = p<0.001.*

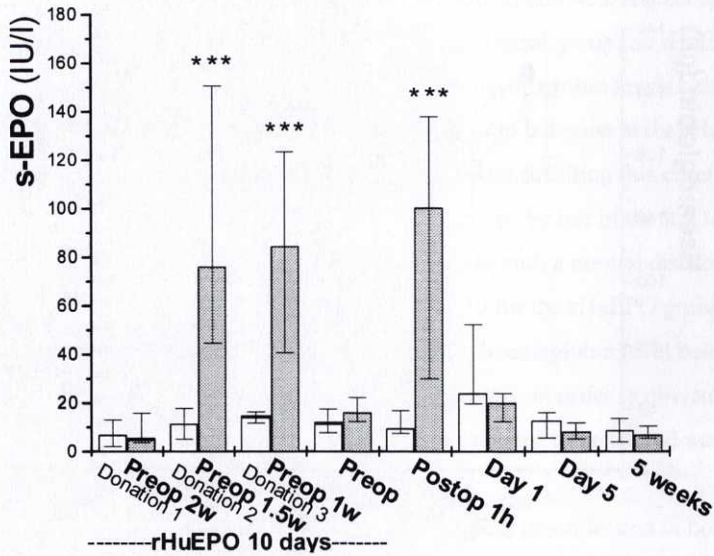


Figure 10. *The median s-EPO levels in the control (white) and rHuEPO (grey) groups during the pre- and postoperative follow-up. 25-75 % percentiles are also given. For differences between the groups: ***= p<0.001.*

Immune response

No changes in cytokine concentrations of IL-6 and IL-8 were found preoperatively and there was no significant difference in cytokine concentrations before and after intravenous administration of rHuEPO (**IV**; figure 11). There was a significant increase in IL-6 and IL-8 concentrations in both groups one hour after surgery and transfusion compared with initial values, $p<0.001$ for IL-6 and $p<0.01$ for IL-8.

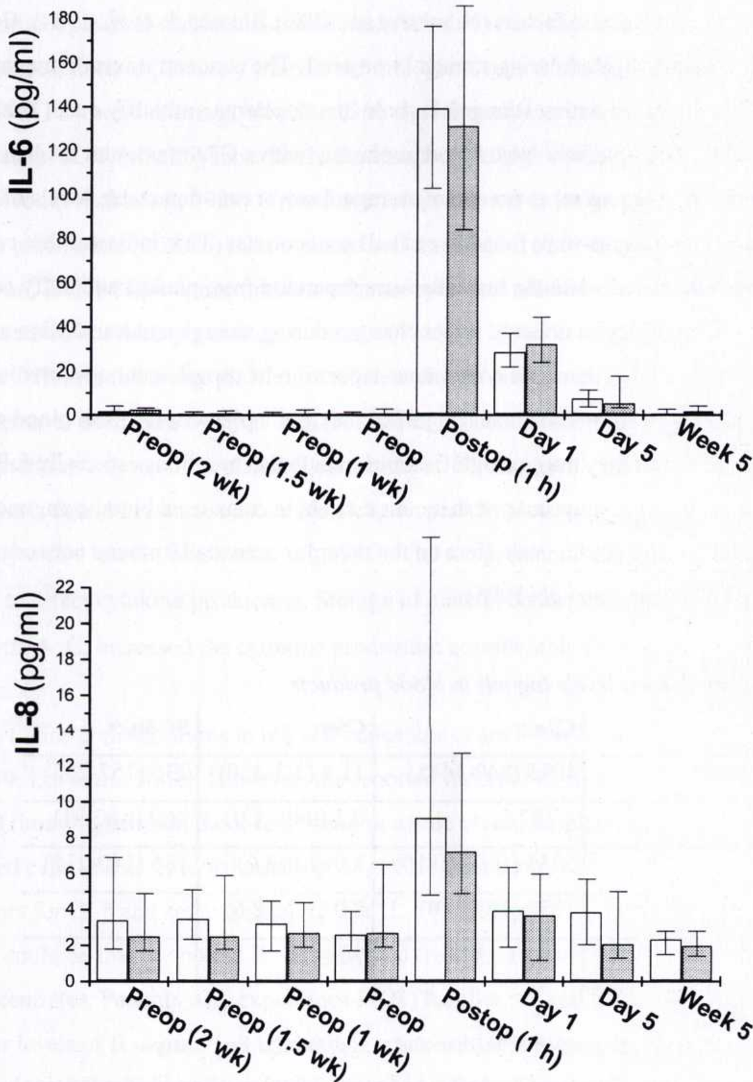


Figure 11. *The cytokine levels in the control (white) and rHuEPO (grey) groups during the pre- andpostoperative follow-up. Median values and 25-75 % percentiles.*

DISCUSSION

Activation of inflammatory mediators during storage of blood components

Complement

The components usually prepared when separating whole blood are red cells, plasma and platelets. Plasma is either frozen or stored as fluid plasma at 4 °C for several weeks. Fluid plasma is ready for immediate transfusion to patients (AABB, 1999). The changes during storage of such plasma have been studied earlier regarding fibrinolytic and kallikrein activation, and coagulation factors (Nilsson *et al.*, 1983; Blombäck *et al.*, 1984). Complement activation was investigated during storage in **paper I**. The concentrations of anaphylatoxins C3a and C5a increased during storage of whole blood, plasma and buffy coat (Table 1). The highest levels of complement were found in plasma, with a C3a maximum median concentration of 5735 ng/ml at the end of storage. Low or non-detectable levels of complement components were found in red cell concentrates. This indicates that complement proteins were removed when the red cells were separated from plasma and buffy coat. Furthermore, erythrocytes undergo major changes during storage and transfusion and it has been demonstrated that there is a continuous deposition of complement on stored erythrocytes (Szymanski *et al.*, 1984). The clinical significance of complement levels in blood components remains unclear, but they may be significant in transfusion reactions especially following massive transfusion. It is speculated that complement in transfused blood components may have an additive and detrimental effect on the complex activated immune network in severe conditions (Kristiansson *et al.*, 1996).

Table 1*. Complement levels (ng/ml) in blood products.*

	C3a	C5a	SC5b-9
Whole blood*	1015 (840-1445)	11.4 (3.3-250)	235 (157-295)
Red cells*	< 137.5	0.2 (0.08-1.0)	<60 (<60-91)
Plasma*	5735 (660-22110)	1.0 (1.0-3.0)	186 (140-215)
Fresh-frozen plasma†	1350 (10-2040)	9.0 (1.7-18.6)	
Platelets‡	9440 ± 1376	70 ± 10	

* Hyllner *et al.*, 1997. Expressed as median and range, storage for 35 days.

† Sonntag *et al.*, 1997. Expressed as median and range, samples collected after thawing.

‡ Schleuning *et al.*, 1994. Expressed as mean and standard deviation, storage for 7 days.

Cytokines

IL-8 in whole blood increased significantly during storage, whereas IL-6 decreased (**II**). TNF-α remained within low limits during the storage time, although there was a significant increase in the levels. Cytokines seem to be released from residual leucocytes in the blood products and are related to the leucocyte content and storage time (Muylle & Peetermans, 1994; Aye *et al.*, 1995). The viability of leucocytes in platelet concentrates is higher than 80 % after 5 days of storage (Hartwig *et al.*, 2002). The study suggests that cytokines are actively produced, and not passively leaked. Adverse transfusion reactions include immunologic responses and reactions to passively transferred biologic response modifiers e.g. cytokines. The transfusion of wound drainage blood leads to significantly increased plasma concentrations of IL-6, which is most likely the result of the high cytokine content of the transfused blood (Åvall *et al.*, 1999). Transfusion reactions are more frequently associated with platelet transfusion (30.8 %) than with red cell transfusion (6.8 %) (Heddle *et al.*, 1993; Patterson *et al.*, 2000). High concentrations of leucocyte- and platelet-derived cytokines have been demonstrated in stored platelet concentrates (Table 2; Bubel *et al.*, 1996, Wadhwa *et al.*, 2002). Most FNHTRs following platelet transfusion do not involve an immune-mediated event but appear to be caused by cytokines (Heddle *et al.*, 1994a; Muylle *et al.*, 1996). There are various methods of preparing platelets concentrates. The highest level of leucocyte contamination is found in random donor platelet-rich plasma products, which also accumulate the highest cytokine levels (Wadhwa *et al.*, 1996). The temperature during storage has also been shown to affect cytokine production. Storage of platelet concentrates at 22 °C, as compared with 4 °C, increased the cytokine production considerably (Table 2; Heddle *et al.*, 1994b).

The cytokine concentrations in red cell concentrates are lower than in platelets (Table 2; Heddle, 1999; Lin *et al.*, 2002). However, the reported levels of IL-8 in red cell concentrates are about 10 times higher than the levels found in whole blood, despite the lower leucocyte content of red cells (Table 2; Kristiansson *et al.*, 1996; Weisbach *et al.*, 1999). Erythrocytes have receptors for IL-8 and act as a reservoir for IL-8 (Darbonne *et al.*, 1991). A possible explanation could be that the bound IL-8 is released from these receptors during storage of red cell concentrates. Patients who experience FNHTRs after red cell infusion show increased intravascular levels of IL-6 and IL-8, and this is not related to the age or cytokine content of the transfused products (Sacher *et al.*, 1993; Lin *et al.*, 2002). The release of cytokines is suggested to be endogenous in response to the immune challenge of transfusion.

Table 2. *Cytokine concentrations (pg/ml) in blood components.*

	TNF-α	IL-1	IL-6	IL-8
Whole blood*	5.1 (2.8-10.1)		1.3 (0.2-77.3)	16.6 (13.3-27.5)
Whole blood†	2.4 + 1.4	5.2 ± 1.7	1.5	38.7 ± 14.3
Red cells‡	0.3 (0-2.4)	6.9 (3.1-12.6)	0.3(0-1.4)	261 (125-332)
Red cells§				512 ± 543
Plasma*	0		1.4 (0-4.6)	4.6 (2.5-7.2)
Platelets¶		48 (2-320)	2395 (112-8576)	32438 (250-70000)

* Hyllner *et al.*, in press. Expressed as median and range, storage for 35 days.

† Grunenberg *et al.*, 1995. Expressed as mean ± standard deviation, storage for 35 days.

‡ Shanwell *et al.*, 1997. Expressed as median and range, storage for 42 days.

§ Stack *et al.*, 1995. Expressed as mean ± standard deviation, storage for 42 days.

¶ Wadhwa *et al.*, 1996. Expressed as mean and range, storage for 5 days.

Effect of universal leucocyte reduction of plasma

The levels of anaphylatoxin C3a and terminal C5b-9 complement complex in prestorage leucocyte filtered plasma were elevated from the very beginning of storage, proving complement activation by the prestorage leucocyte filtration process (**II**). Leucocyte reduction filters may adsorb or activate anaphylatoxins depending on the electrostatic force between highly basic positively charged anaphylatoxins and filter membranes with different surface charge (Matsuda *et al.*, 1988; Cardigan *et al.*, 2001).

The levels of TNF-α, IL-6 and IL-8 generated in plasma and filtered plasma are low and not influenced by prestorage filtration. The cytokine levels are lower than reported for red cells (Stack *et al.*, 1995; Kristiansson *et al.*, 1996). This may be explained by low leucocyte counts in plasma as well as filtered plasma. It has previously been reported that a leucocyte count below 3×10^9 per l does not lead to cytokine synthesis (Muylle *et al.*, 1993). The cytokine levels generated in plasma and filtered plasma are unlikely to cause adverse transfusion reactions.

Prestorage leucocyte filtration of platelet concentrates and red cells diminishes the accumulation of leucocyte-derived cytokines during storage (Shanwell *et al.*, 1997; Wadhwa *et al.*, 2002). However, transfusion reactions are reduced but not eliminated (Uhlmann *et al.*,

2001; Heddle *et al.*, 2002). The activation and accumulation of complement anaphylatoxins may be one explanation of the remaining reactions.

How to store autologous blood

Autologous blood is predominantly predeposited and stored as whole blood in contrast to allogeneic blood, which is separated into components (Table 3). The current practice of separating allogeneic blood into components before storage and transfusion seems appropriate. Theoretically, this would also be an advantage for autologous blood. However, the volumes transfused and the storage time are limited for autologous blood. The clinical significance of processing autologous blood may therefore not be of vital importance.

An increasing number of blood banks have introduced universal leucoreduction by prestorage filtration of allogeneic blood components. Some countries have applied the standards of allogeneic transfusion to autologous blood and recommend leucocyte depletion of autologous whole blood or the preparation of red cells and plasma before storage. This is questioned though, because there is no evidence so far that leucocyte depletion of autologous blood will benefit the patient, and the production cost is considerably increased. There is a risk that guidelines recommending the processing of autologous blood will change transfusion practice without an improvement in quality.

The pro-inflammatory cytokines IL-8, TNF-α and the anaphylatoxins C3a and C5a are increased in whole blood (**I-II**). When autologous whole blood is retransfused to healthy volunteers a moderate immunomodulation occurs with a transient increase in plasma C3a and IL-6 concentrations (Frietsch *et al.*, 2001a). The impact of transfused cytokines, complement and leucocytes with the autologous blood may be negligible in comparison to the amounts generated by operation and anaesthesia. The humoral or cellular immune response in hip surgery was not altered by transfusion of autologous whole blood or autologous red cells and fresh-frozen plasma (Frietsch *et al.*, 2001b; Tolksdorf *et al.*, 2001). Furthermore, the study demonstrates no difference in infection rate or length of hospital stay.

Table 3. Allogeneic vs. autologous blood.

	Allogeneic red cells	Allogeneic plasma	Autologous whole blood
Complement activation	no	high	moderate
Cytokine release	low	low	moderate
Universal leucoreduction -Complement		increased	moderate
Universal leucoreduction -Cytokines	reduced	low	
Infectious disease transmission	yes	yes	no
Immunosuppression	yes	yes	no
Immunologic reactions	yes	yes	no

Preoperative autologous blood donation

In cancer surgery, the immunosuppression of allogeneic blood may be detrimental and it is attractive to offer PABD. The time period for collection of autologous blood is limited for patients with cancer as the time from diagnosis until operation should be short. The donation schedule has to be more aggressive than for elective operations. We have demonstrated that women scheduled for cancer operation can predeposit three units of blood in two weeks prior to operation **(III)**. All patients received autologous blood transfusion intraoperatively. Eighteen units of predeposited blood were not reinfused (15.5 %). This seems to be a reasonable wastage since a smaller amount deposited would result in a larger percentage of patients receiving allogeneic blood. Four patients in the rHuEPO group and three patients in the control group received additional allogeneic blood transfusions in connection with surgery. On the other hand, more than three units predeposited would most likely result in even more units not being reinfused.

Weekly PABD schedules have resulted in only a small increase in endogenous EPO levels in autologous donors, with a speculated suboptimal effect on erythropoiesis (Lorentz *et al.*, 1991). Despite a more intensive precollection program, we found only a moderate increase in s-EPO levels in the control group and the s-EPO levels stayed within the reference

values throughout the donation period (**IV**). The administration of rHuEPO is needed to increase EPO levels and erythropoiesis.

The role of recombinant erythropoietin in PABD

RHuEPO is expensive. The dose 100.000 IU used in **paper III** costs almost 10.000 SEK/patient. Although rHuEPO increases erythropoiesis its role in PABD is limited to a few situations, such as when the patient is already anaemic or needs to donate an unusually large amount of blood in a short time (Mercuriali *et al.*, 1993). Guidelines for better identification of those patients who would benefit most from PABD and rHuEPO therapy, would increase the efficiency and reduce the costs of PABD.

We found no evidence of IL-6 or IL-8 release during rHuEPO therapy as the cytokine levels stayed within reference values preoperatively and there was no release of cytokines after intravenous administration of rHuEPO (**IV**). About 15-20 % of dialysis patients show a poor response to rHuEPO therapy and one mechanism of unresponsiveness may be the induction of cytokines. RHuEPO stimulates the in vitro production of TNF-α and IL-1β, and this is reported as one explanation of persistent anaemia in dialysis patients receiving rHuEPO therapy (Takemasa *et al.*, 1996, 2000). Anaemia in critically ill patients and in chronic disease may be the result of an inadequate EPO response to the low haemoglobin levels, mediated by pro-inflammatory cytokines. Laboratory studies have shown that the cytokines IL-1α, IL-1β, IL-6 and TNF-α suppress EPO gene expression (Jelkmann, 1998).

The early postoperative endogenous EPO response was increased after rHuEPO therapy (**IV**). This is a novel result and the underlying mechanism is unclear. In an animal model it has been demonstrated that mice previously stimulated, conditioned, by rHuEPO injection produce more EPO in response to hypoxia than mice not previously stimulated (Bozzini *et al.*, 1994, 1997). Although there was no hypoxia in the patients we studied there was a decrease in haemoglobin concentration during operation. Conditioning from rHuEPO therapy preoperatively may explain why the women in the treated group required a higher EPO level for effect in response to operation-induced anaemia. Other reports have indicated that surgical procedures and rHuEPO therapy may suppress postoperative endogenous EPO production in response to anaemia (Levine *et al.*, 1991; Biesma *et al.*, 1993). This would be a clinically important disadvantage of rHuEPO therapy as regards postoperative haemoglobin recovery.

Endogenous EPO production and haemoglobin recovery after PAD and operation with and without rHuEPO therapy was investigated in 38 patients undergoing total hip joint

replacement operation (Åvall *et al.*, 2003). Patients donated whole blood at three, two and one week before operation. The EPO group received 10.000 IU/week at each donation visit and twice the last preoperative week, in all 50.000 IU. In patients with normal preoperative haemoglobin levels, rHuEPO therapy did not improve haemoglobin levels, nor reduce the need for allogeneic blood transfusion.

Previous studies compare doses in the 150-600 IU/kg range, often given daily, but there is evidence that lower doses than those used earlier are sufficient and it seems reasonable to begin rHuEPO therapy with doses of 100 to 150 IU/kg (Crosby, 2002). Therapy should be started at least two weeks before an operation and subcutaneous administration is as effective as intravenous administration (McMahon *et al.*, 1990). Weekly doses of epoetin alfa may be at least as effective and more convenient than daily administration (Goldberg *et al.*, 1996). Oral iron supplementation is usually effective (Olijhoek *et al.*, 2001). Patients undergoing more extensive operations with substantial blood loss may derive maximum benefit from a combination of rHuEPO therapy and aggressive PABD (Crosby, 2002).

Cost-effectiveness

Viral transmission and transfusion reactions are avoided with the use of autologous blood. However, it is often considered as expensive and logistically difficult, and the controversy about its cost-effectiveness is not yet settled. Decision models arrive at different costs depending on the effects considered. "Dollars per life-year saved" is a commonly used measure of a medical intervention and represents the cost in dollars to extend the life of a patient for one year. PABD can increase the cost considerably. Other models that consider the immunomodulatory effect of allogeneic transfusion on the rate of postoperative infection suggest the PABD is cost-effective (Blumberg *et al.*, 1996). Thus, it depends on what is considered. The additional cost of autologous blood depends primarily on the labour-intensive donation process and wastage of units donated but not transfused. Better identification of the patients most likely to benefit from autologous donation, would increase the efficiency and reduce the costs of PABD, as would more careful estimate of autologous blood needs. Nuttall et al. (2000) have derived guidelines for PABD and rHuEPO therapy based on retrospective analysis of 165 patients undergoing total hip arthroplasty. A patient with a predonation haemoglobin level higher than 147 g/l does not need PABD. Men with a predonation haemoglobin level less than 147 g/l and women with haemoglobin levels 132-147 g/l are recommended PABD. In women with a haemoglobin level less than 132 g/l, rHuEPO therapy should accompany PABD.

The discard rate is one of the variables that affect the cost-effectiveness and the rate varies considerably between Europe and the USA. The amount of blood discarded in orthopaedic operations in a recent European study was 13 % compared with 50 % reported for US medical centres (Rosencher *et al.*, 2003). There is a history of overcollection in the US since some states passed laws mandating the option of PABD.

"The safest blood is your own"

Autologous donations and transfusions increased dramatically with discovery of the human immunodeficiency virus. PABD is now the blood conservation strategy most often used. In 1997, about 4 % of transfused blood in Europe was autologous (Politis & Richardson, 2001). In the USA, 5 % autologous transfusions are reported the same year. PABD is most frequent in Italy with 8.9 %, but less than 1 % is reported from the Scandinavian countries. Overall, more than 670.000 autologous units were collected in Europe in 1997. In 2001, according to the Swedish National Board of Health and Welfare, 867 autologous units were collected compared with 451.447 allogeneic units (~0.2 %).

The public remains concerned about the safety of allogeneic transfusions and many patients would even be willing to pay a substantial amount of money for autologous services (Lee *et al.*, 1998). Despite information about the low risks of complications from allogeneic transfusions, an aversion to allogeneic transfusion persists as well as a willingness to pay for PABD.

There are a lot of pros and cons with PABD, depending on different beliefs and values. Patients who are healthy enough to undergo elective surgery will be able to donate blood preoperatively (**III**). Whose blood would *you* like to be transfused with if you could choose? What if you don't have a choice? Today, organisation for PABD at Swedish hospitals is very limited. Most patients receive no information about PABD as an alternative to allogeneic transfusion and can not be offered a choice. In some countries, patients have to sign a consent form where they agree to surgery and blood transfusion. This is one way to urge patients to decide on blood transfusion. A closer co-operation with the Blood Bank would make it possible to offer patients the advantage of autologous blood.

CONCLUSIONS

- The complement anaphylatoxins C3a and C5a are released during storage of whole blood, plasma and buffy coat. Low levels are detected in red cell concentrates.

- IL-8 in whole blood increased during storage. IL-6 decreased and TNF-α remained within low limits during the storage time. Cytokine levels in stored plasma and filtered plasma are low. It is clear that complement is activated and cytokines are released during the processing and storage of whole blood and blood components. Despite this, the current practice of separating blood into components before storage and transfusion seems accurate for allogeneic blood, but may not be appropriate for autologous blood.

- Leucocyte reduction by prestorage filtration of plasma with filter ASAHI RZ 2000 activates the complement cascade from the very beginning of storage with high levels of C3a and SC5b-9. Cytokine release is not influenced. It should be considered that leucocyte reduction filters might have different qualities, which will result in different activation of inflammatory mediators.

- The use of rHuEPO therapy to optimise PABD increases the postoperative endogenous EPO response. RHuEPO therapy does not influence IL-6 and IL-8 release. Thus, the present thesis could not demonstrate unsatisfactory effects of rHuEPO therapy.

- Short preoperative donation intervals resulted in only moderately increased s-EPO levels, and erythropoiesis was not increased compared with weekly PABD schedules.

- Women scheduled for cancer operation could predeposit three units of whole blood in only two weeks without the development of severe anaemia. A haemoglobin level below the 100 g/l donation limit can be prevented in one patient out of seven by treating women with rHuEPO. The present thesis suggests that PABD can be offered to female patients undergoing cancer surgery. The additional use of rHuEPO is limited to a few situations, e.g. when the patient is anaemic at the first visit for blood collection.

SAMMANFATTNING PÅ SVENSKA

Blodet innehåller komplexa kaskadsystem och substanser som kan aktiveras vid framställning av blodkomponenter och under lagring. Allogent blod (bankblod) separeras normalt i olika komponenter före lagring och transfusion, medan autologt blod (patientens eget blod) ofta används som helblod. Transfusion av bankblod är förenat med ett antal olika risker och därför har egenblodgivning inför en planerad operation blivit ett etablerat alternativ. För cancerpatienter kan den immunosupprimerande effekten av bankblod vara skadlig, men det är svårt att hinna med egenblodgivning eftersom man inte vill fördröja operationen. Egenblodgivningen börjar vanligen 4-6 veckor före en planerad operation och blod tappas en gång i veckan. Behandling med erythropoietin ökar volymen av eget tappat blod inför en planerad operation. Emellertid så indikerar andra studier att erythropoietinbehandling kan undertrycka den kroppsegna postoperativa produktionen av erythropoietin och stimulera frisättning av inflammatoriska mediatorer. Syftet med denna avhandling har varit att studera effekterna på blodnybildning och på frisättningen av inflammatoriska mediatorer under tappning och lagring av eget blod.

I denna studien tappades friska blodgivare på blod som lagrades som helblod eller delades upp i komponenter. Komplementaktivering och frisättning av pro-inflammatoriska cytokiner studerades under lagringstiden. Dessutom studerades effekten av leukocytfiltrering före lagring på frisättningen av inflammatoriska mediatorer. För kvinnor som skulle genomgå radikal hysterektomi planerades egenblodgivning av tre påsar blod under två veckor inför operation, med eller utan erythropoietinbehandling. Effekten på blodnybildning och immunsvar följdes före och efter operation.

Resultaten visar att komplementsystemet aktiveras under lagring av helblod och plasma, och att cytokinen IL-8 frisätts i helblod. Leukocytfiltrering av plasma aktiverar komplementsystemet men påverkar inte cytokinfrisättningen. Helt klart så kan kvinnor tappas på tre påsar blod under bara två veckor före en planerad operation. Genom att behandla kvinnor med erythropoietin förhindras en av sju patienter att hamna under blodvärdesgränsen på 100 g/l för tappning. Erythropoietinbehandling ökar det postoperativa kroppsegna erythropoietin svaret men påverkar inte cytokinfrisättningen. Avhandlingen föreslår att egenblodgivning kan erbjudas kvinnor som ska genomgå cancerkirurgi, och att autolog blod kan transfunderas som helblod.

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För en fullständig lista av ingående delarbeten, se avhandlingens början.

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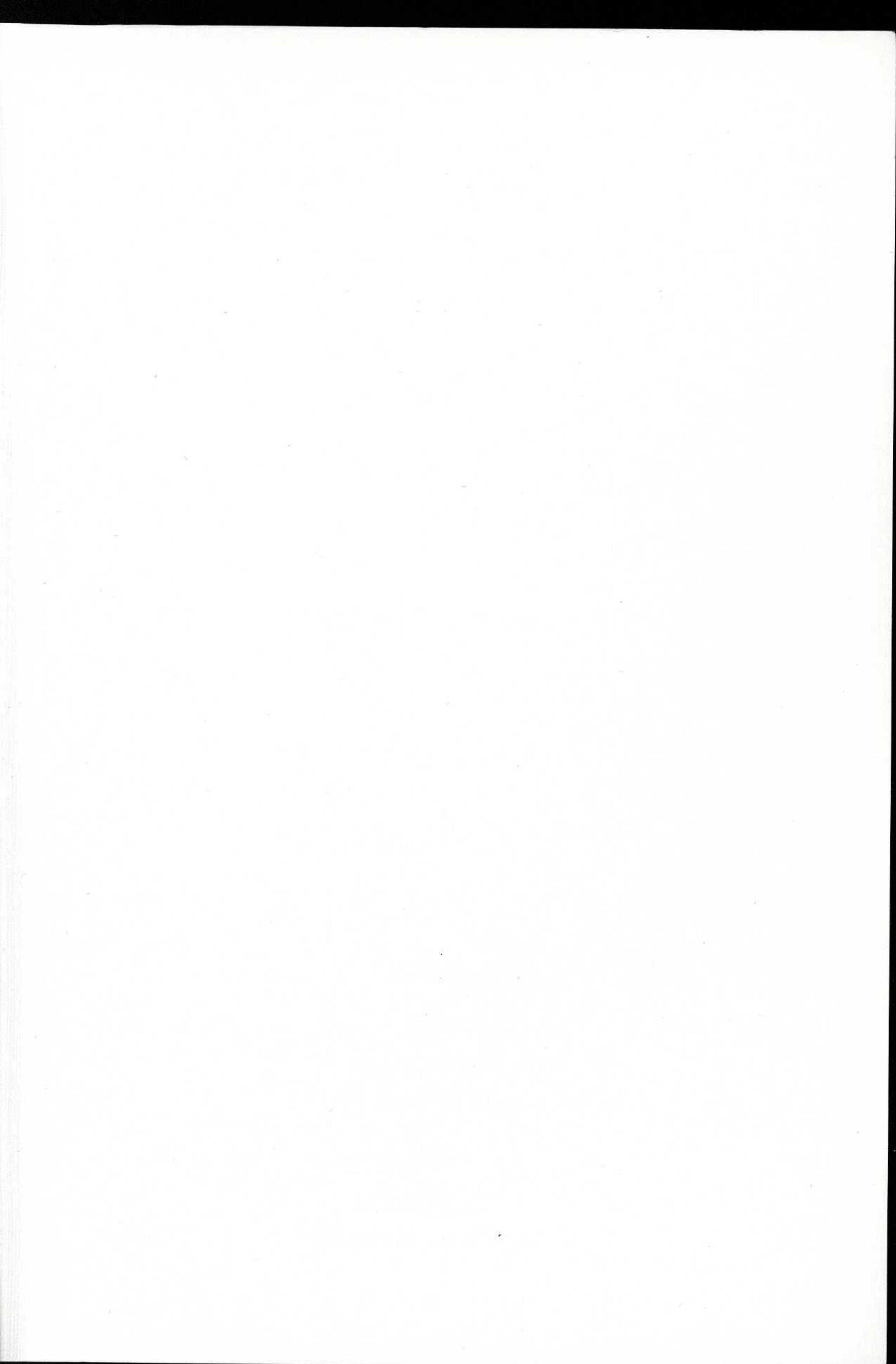

Table 3. Cytokine levels during storage

	IL-6			IL-8	
	Whole blood	**Plasma**	**Plasma: Filtered**	**Whole blood**	**Plasma**
Day 1	4.0 (2.1-98.4)	3.0 (0-11.9)	2.6 (1.0-16.2)	0.2 (0-3.0)	4.4 (2.2-7.2)
Day 7	2.3 (0-95.9)	2.0 (0.2-6.1)	2.2 (0.6-15.2)	6.0 (0-18.2)	5.0 (0.6-7.0)
Day 14	3.3 (0.8-43.7)	1.7 (0-5.5)	2.2 (0.8-15.7)	8.0 (0-18.7)	5.8 (4.6-10.2)
Day 21	1.8 (0-94.4)	1.0 (0.3-4.4)	1.2 (0.3-12.7)	13.3 (1.6-23.4)	4.9 (1.7-6.7)
Day 28	0.8 (0-91.0)	1.4 (0.5-4.9)	2.1 (0.9-11.6)	17.0 (10.1-28.7)	4.9 (2.7-13.1)
Day 35	1.3 (0.2-77.3)*	1.4 (0-4.6)	2.0 (0.8-10.9)	16.6 (13.3-27.5)†	4.6 (2.5-7.2)

Cytokine levels are expressed as pg mL^{-1}, values are given as median and range.

* $p<0.05$. Significance within each blood component group with respect to change during the study period.

† $p<0.01$

Table 1. Haematological variables during storage in whole blood and plasma

	K^+			Leucocytes		
Reference value	3.[illegible]			$4.0\text{-}10.0 \times 10^9 L^{-1}$		
	Whole blood	[illegible]	[illegible]	**Whole blood**	**Plasma**	**Plasma: Filtered**
Day 1	4.3 (3.7-4.8)	3.0 (2[illegible]	[illegible].1-3.6)	5.8(3.9-6.7)	<0.1	<0.1
Day 7	11.8 (9.1-15.1)	3.1 (2.7-3.7)	3.4 (3.1-3.6)	4.6 (3.5-5.3)	<0.1	<0.1
Day 14	16.4 (12.4-19.9)	3.1 (2.7-3.7)	3.4 (3.1-3.7)	4.5 (3.3-5.4)	<0.1	<0.1
Day 21	21.0 (16.8-25.0)	3.1 (2.7-3.7)	3.4 (3.1-3.6)	4.2 (3.1-6.6)	<0.1	<0.1
Day 28	25.0 (19.2-27.9)	3.1 (2.7-3.7)	3.[illegible] 1-3.6)	3.7 (2.7-6.7)	<0.1	<0.1
Day 35	26.6 (22.6-31.4)†	3.0 (2.7-3.7)	3.4 (3.1-3.6)	3.2 (2.4-3.9)†	<0.1	<0.1