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Haemostatic Changes in Plasma for Transfusion during Preparation and Storage

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Cover illustration

The stamps on the cover page were issued by various countries to promote blood donation. The individuals who are commemorated on some of these stamps are:

Karl Landsteiner, who discovered the ABO blood group system in 1900. For this, he received the Nobel Prize in Medicine in 1930.

Albert Hustin, who reported the use of sodium citrate and glucose as an anticoagulant solution in 1914.

Blood transfusions were originally performed by directly connecting a vessel of the donor to a vessel of the recipient. Techniques for such anastomoses were described in 1902 by Alexis Carrel, who received the Nobel Prize in Medicine in 1912.

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Natura nullibi magis perfecta, artificiosa magis quam in minimis.
Linné 1757

Till Moder Svea
och hennes 411 251 blod- och plasmagivare
2003

Abstract

The haemostatic quality of plasma for transfusion is liable to be affected by techniques for preparation and storage. We have studied changes in the contact system, coagulation and fibrinolysis under the following conditions: blood drawn into a half-strength citrate anticoagulant solution, virus inactivation of plasma with methylene blue and red light, and plasma storage in the fluid phase.

Blood is normally drawn into a citrate-phosphate-dextrose (CPD) anticoagulant solution. We evaluated the stability of factor VIII, other coagulation factors and their inhibitors in blood drawn into half-strength citrate CPD (0.5CPD) and kept at room temperature for eight hours before component preparation. We found no activation of the contact system or coagulation, but a significantly increased stability of coagulation factors VIII and IX.

During virus inactivation of single donor units of fresh plasma with methylene blue and red light, the concentration of clottable fibrinogen was unchanged but functional fibrinogen decreased in a light-dose dependent manner. Turbidity measurements of fibrin gel showed a lower fibrin fiber mass-to-length ratio after the treatment, indicating a tighter fibrin gel structure. Normal clot stability and fibrinolysis were found. L-histidine added to plasma before the treatment normalized the prolonged thrombin-induced coagulation time in a dose-dependent way.

We defined cold activation of plasma as an elevated kallikrein-like activity. During storage of plasma from male donors at +4 ^{o}C for 42 days, the cumulative frequency of cold activated plasma units increased in a time-dependent manner. When tested on two occasions, the majority of cold activators remained the same and so did the majority of non-cold activators. However, large intraindividual differences in the onset days of cold activation were observed in plasma of some of our male donors.

Cold activation was associated with a high degree of activation of the contact system and coagulation. Minor changes in fibrinolysis were observed. We have developed a method, to be used before storage, for the selection of transfusion plasma units stable at +4 ^{o}C for a certain period.

In plasma units with a short lag phase before cold activation, an imbalance was found between the functional levels of the contact proteins and their inhibitors on day 0, which suggests a mechanism leading to cold activation. An additional mechanism may be involved.

In conclusion, we have found an increased stability of factors VIII and IX in 0.5CPD. We have demonstrated changes in fibrin polymerization and gel structure after the methylene blue and red light treatment, and an activation of the contact system and coagulation during plasma storage at +4 ^{o}C. For a uniform quality of transfusion plasma units, it is important to maintain normal functional levels of all haemostatic proteins, including zymogen forms of proteinases, cofactors and proteinase inhibitors. An exclusion of cold-activated plasma units would increase the stability of several haemostatic proteins during storage at +4 ^{o}C.

Key words: transfusion plasma, cold activation, contact system, coagulation, fibrinolysis, citrates, methylene blue, fibrinogen, stability

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Contents

Sammanfattning

Hemostasändringar i transfusionsplasma under framställning och förvaring

Tekniker för framställning och lagring av transfusionsplasma kan medföra förändringar i plasmakvalitet. Vi har studerat hemostasförändringar under följande betingelser: halverad citratkoncentration i antikoagulationslösning, virusinaktivering med metylenblått och rött ljus och förvaring i flytande form.

Blod tappas normalt i antikoagulationslösning citrat-fosfat-dextros (CPD). Vi har undersökt inverkan av halverad citratkoncentration i CPD-lösning på aktivering av olika koagulationsfaktorer och deras hämmare i plasma under åtta timmars förvaring av helblod i rumstemperatur före komponentframställning. En halvering av citratkoncentrationen medförde inte aktivering av kontaktsystemet eller koagulation, men en signifikant bättre stabilitet av koagulationsfaktorer VIII och IX.

Under virusinaktivering av färsk plasma med metylenblått och rött ljus minskade koncentrationen av funktionellt fibrinogen med ökande ljusdos, medan den totala fibrinogenkoncentrationen var oförändrad. Turbiditetsmätningar av fibringelstrukturen visade att förhållandet fibermassa/fiberlängd var lägre efter behandlingen. Detta tyder på att gelstrukturen blev tätare. Stabilisering av fibrinnätverk eller fibrinolys påverkades inte. L-histidin tillsatt i plasma före behandling gav en dosberoende normalisering av förlängd trombininducerad koagulationstid.

Vi definierade köldaktivering av plasma som ökad kallikreinaktivitet. Vi fann en tidsberoende ökning av kumulerad frekvens av köldaktiverade plasmaenheter under lagring vid +4 ^{o}C i upp till 42 dagar. I upprepade plasmaprov från några givare fann vi stora intraindividuella skillnader mellan lagfaser före köldaktivering tydande på att denna inte är en kvalitativ utan snarare en kvantitativ egenskap hos den enskilda givaren.

I köldaktiverade plasmor blev kontakt- och koagulationssystemet kraftigt aktiverade, fibrinolyssystemet knappast alls. I icke-köldaktiverade plasmor skedde ingen eller obetydlig aktivering av de olika systemen under 42 dagar. Vi har utarbetat en metod för selektion av plasmaenheter som tål lagring vid +4 ^{o}C under en viss tid utan att bli köldaktiverade.

I jämförelse med plasmaenheter med lång lagfas före köldaktivering vid +4 ^{o}C, har vi i sådana med kort lagfas funnit en obalans mellan funktionella nivåer av kontaktfaktorer och deras hämmare. Detta verkar vara förklaringen till köldaktivering. Även andra mekanismer kan finnas.

Sammanfattningsvis har vi funnit en ökad stabilitet av faktorerna VIII och IX i CPD-lösning med halverad citratkoncentration. Vi har beskrivit förändringar i fibrinogenaktivitet under behandling av plasma med metylenblått och rött ljus, och i kontaktsystemet och koagulation under förvaring av plasma vid +4 ^{o}C. För en jämn kvalitet av plasmaenheter är det viktigt att bibehålla normala funktionella nivåer av alla hemostatiska proteiner, inklusive zymogenformer av proteinaser, kofaktorer och proteinashämmare. Uteslutning av köldaktiverade plasmor skulle kunna ge en bättre stabilitet av flera hemostasproteiner under lagring vid +4 ^{o}C.

List of papers included in the thesis

The thesis is based on the following original articles and manuscripts, referred to in the text by their Roman numerals:

I Suontaka AM, Åkerblom O, Blombäck M, Eriksson L, Högman CF, Payrat JM.
Stability of blood coagulation factors and inhibitors in blood drawn into half-strength citrate anticoagulant.
Vox Sanguinis 1996; 71: 97-102.

II Suontaka AM, Blombäck M, Chapman J.
Changes in functional activities of plasma fibrinogen after treatment with methylene blue and red light.
Transfusion 2003; 43: 568-575.

III Suontaka AM, Silveira A, Söderström T, Blombäck M.
Occurrence of cold activation of transfusion plasma during storage at +4 ^{o}C.
Vox Sanguinis 2005; 88: 172-180.

IV Suontaka AM.
A method for selecting transfusion plasma units that will be stable during storage at +4 ^{o}C.
Submitted for publication.

V Suontaka AM.
On cold activation mechanism of plasma for transfusion.
Submitted for publication.

Other papers on related topics

Suontaka AM, Bremme K, Åkerblom O, Blombäck M.
Blood component processing technique and plasma quality.
Infusionstherapie und Transfusionsmedizin 1992; 19: 110-114.

Åkerblom O, Bremme K, Dackland ÅL, Fatah K, Suontaka AM, Blombäck M.
Freezing technique and quality of fresh frozen plasma.
Infusionstherapie und Transfusionsmedizin 1992; 19: 283-287.

Wallvik J, Suontaka AM, Blombäck M.
Proteolytic activity during storage of platelets in plasma.
Transfusion medicine 1992; 2: 135-142.

Abbreviations and Acronyms

ACE	angiotensin-converting enzyme
APC	activated protein C
APTT	activated partial thromboplastin time
ATP	adenosine triphosphate
2,3 DPG	2,3 diphosphoglycerate
Ca^{2+}	calcium ion
CPD	citrate-phosphate-dextrose
0.5CPD	half-strength citrate CPD
D-dimer	fibrin D-dimer
ELISA	enzyme-linked immunosorbent assay
factor V – factor XIII	coagulation factors V – XIII
FVa – FXIIIa	activated coagulation factors V – XIII
FFP	fresh frozen plasma
g	acceleration due to gravity
HAV	hepatitis A virus
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
J	joule
MB	Margareta Blombäck
Mr	relative molecular mass
NADH	nicotinamide adenine dinucleotide
PAGE	polyacryl amide gel electrophoresis
PAI-1	plasminogen activator inhibitor-1
PAI-2	plasminogen activator inhibitor-2
PT(INR)	prothrombin time(international normalized ratio)
SDS	sodium dodecyl sulphate
serpin	serin proteinase inhibitor
TAFI	thrombin-activatable fibrinolysis inhibitor
TAT	thrombin-antithrombin complex
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TRALI	transfusion-related acute lung injury
t-PA	tissue-type plasminogen activator
u-PA	urokinase-type plasminogen activator
VWF	von Willebrand factor
Zn^{2+}	zinc ion

Introduction

Haemostasis

Blood is a tissue that must stay fluid in order to fulfil its many functions. The haemostatic mechanism serves to maintain blood in a fluid state in an intact vascular system, while in the setting of vascular injury it provides a response in an explosive fashion to seal vascular defects and stem blood loss. When this aim has been achieved, the haemostatic plug formed at the site of injury is degraded and the patency of the damaged blood vessel is restored.

The haemostatic mechanism in humans involves a complicated interaction between the vessel wall and the cellular and soluble components of the blood. Haemostasis includes endothelium, subendothelium, platelets, leucocytes, erythrocytes, adhesive proteins, plasma- and cell-based procoagulant, anticoagulant and fibrinolytic proteins, certain phospholipids and polysaccharides, biological response modifiers, e.g. cytokines, and inorganic matter, e.g. divalent cations such as Ca^{2+} ions. Furthermore, certain physical characteristics, such as vascular tone, blood flow, shear forces, pH, viscosity, and osmolality, also influence haemostasis.

The haemostatic system is one of several plasma proteolytic systems built up by inactive zymogens which are converted to enzymes. These conversions are accelerated by non-enzymatic protein cofactors that act either by altering the conformation of the zymogen or by binding converting enzymes and zymogens in close proximity on a surface. Both the conversion of the zymogens to the respective enzymes and the activities of the enzymes when they have formed, are controlled by inhibitors and feed-back mechanisms. The haemostatic system, including the contact system, coagulation and fibrinolysis, is closely interrelated to other plasma proteolytic systems, e.g. the kinin, complement and renin-angiotensin systems. An activation of one system is often associated with an activation of the others [for an overview of haemostasis, see Colman RW et al 2001, and references therein].

Under normal conditions, the interaction between the different haemostasis constituents is an ongoing process in balance, which includes a low level of consumption and synthesis of all biochemical reactants. A diminished haemostatic activity will give rise to an increased risk of bleeding, while an augmented activity can lead to an increased risk of venous thromboembolism or arterial occlusive disease.

Normal haemostasis *in vivo* depends on:

- Blood vessel and endothelial cell function – constriction of injured vessels
- Platelet function – formation of platelet plugs
- Coagulation – formation of fibrin clots
- Inhibition of coagulation – limitation of clot growth
- Fibrinolysis – dissolution of blood clots and wound healing

Blood vessel –- Endothelium

Rapid vascular constriction limits blood loss from an injured vessel. The vasoconstriction is induced by endothelins synthesized by endothelial cells lining the blood vessel [MacCumber MW et al 1989]. Other mechanisms, e.g. serotonin and thromboxane A_2 released from platelets, contribute to the vasoconstriction. The endothelial cells also modulate the vessel wall tone by releasing prostacyclin (PGI_2) and nitric oxide, which relax blood vessels.

Vascular endothelium plays an active role also in all other aspects of haemostasis. Von Willebrand factor released by endothelial cells mediates platelet adhesion to collagen exposed at the site of vascular injury. Von Willebrand factor is also the carrier of coagulation factor VIII in plasma. Tissue factor (TF) in contact with blood initiates coagulation.

Normal endothelium maintains blood fluidity by inhibiting platelet aggregation by adenosine diphosphatase, prostacyclin and nitric oxide, and blood coagulation by heparan sulphate and thrombomodulin. Heparan sulphate on endothelial cells binds plasma tissue factor pathway inhibitor (TFPI) and antithrombin, thus increasing their inhibitory efficiency. Thrombomodulin in complex with thrombin activates plasma protein C, which is another important downregulating mechanism of coagulation. Furthermore, the endothelial cells modulate fibrinolysis by releasing tissue plasminogen activator (t-PA) and plasminogen-activator inhibitor-1 (PAI-1).

Primary haemostasis – Platelets

Following vascular injury, platelets adhere to the damaged site, become activated, change shape, spread over the exposed subendothelial surface, and release aggregation promoting constituents from storage granules. Binding of fibrinogen to its receptor glycoprotein IIb/IIIa (integrin $\alpha_{IIb}\beta_3$) on the platelet surface results in formation of large platelet aggregates within one minute. The process leading to the platelet plug is called primary haemostasis.

After the initial limitation of bleeding by platelet plug formation, activated platelets contribute to the process of fibrin formation. Anionic phospholipids, especially phosphatidylserine, on activated platelets function as a catalytic environment for binding of activated coagulation factor/cofactor complexes producing the thrombin necessary for the formation of the fibrin clot. When the platelet-fibrin plug is formed, platelet mediated clot retraction occurs.

The haemostatic process is critically dependent on an adequate number of circulating platelets as well as on normal platelet function. Abnormal platelet function is seen e.g. in Glanzmann´s thrombasthenia (glycoprotein IIb/IIIa deficiency) and in the Bernard-Soulier syndrome (glycoprotein Ib/IX/V deficiency).

Secondary haemostasis – Plasma coagulation

The platelet plug may stop the bleeding temporarily but as the plug is fragile, it needs to be stabilized by a fibrin network to make it firm and durable. This fibrin network is produced by the coagulation system.

Prothrombin, coagulation factors VII, IX, X, XI and XII, prokallikrein and protein C are zymogens of serine proteinases (EC 3.4.21 serine endopeptidases). Factor V, factor VIII, kininogen(120 000) and protein S are non-enzymatic cofactors. Fibrinogen serves as one of the target substrates of thrombin. Activated factor XIII strengthens the stability of the fibrin network.

Activation of the zymogen occurs by cleavage at specific sites, most commonly by the action of another proteinase. Serine proteinases cleave their substrates by catalysing the hydrolysis of peptide bonds. They have several domains and the active site is located at the carboxy terminal region of the molecule. The active site, called the catalytic triad, contains the serine, histidine and aspartic acid residues responsible for the catalytic action. The haemostatic

serine proteinases cleave their substrates at the carboxy terminal side of the basic amino acids arginine or lysine at the preferred cleavage site(s). Only one or two bonds are cleaved during zymogen activation.

Earlier descriptions of the coagulation system as an enzyme cascade or waterfall depict an intrinsic or contact activation pathway and an extrinsic or tissue factor-dependent pathway independently activating factor X and hence prothrombin, resulting in fibrin polymerization [Macfarlane RG 1964, Davie EW and Ratnoff OD 1964]. This concept, derived from *in vitro* experiments, is retained as being useful for interpretation of coagulation screening tests such as activated partial thromboplastin time (APTT) and prothrombin time (PT, International Normalized Ratio, INR).

It is now apparent that numerous interconnections exist between these two pathways and that coagulation *in vivo* occurs mainly on surfaces rather than in solution. According to the present model, blood coagulation is not an enzyme cascade, but rather a cell-based system divided into three overlapping stages, i.e. initiation, amplification and propagation [reviewed by Hoffman M and Monroe DM 3rd 2001] (Figure 1).

Coagulation activation *in vivo* is mediated by the tissue factor pathway. Tissue factor is a cell membrane bound glycoprotein that is exposed to plasma proteins after tissue injury or upon monocyte activation. Endothelial and blood cells do not normally express tissue factor. Coagulation is *initiated* by the binding of factor VII (FVII) or activated factor VII (FVIIa) to tissue factor in the presence of Ca^{2+} ions. Factor VII bound to tissue factor can be activated to FVIIa by FXa, thrombin, FIXa, FXIIa, and FVIIa itself [Nemerson Y and Esnouf MP 1973, Nemerson Y and Repke D 1985, Seligsohn U et al 1979, Neuenschwander PF et al 1993]. Normally, about 1% of factor VII circulates in the active form (FVIIa) [Morrissey JH et al 1993, Wildgoose P et al 1992]. Binding of FVIIa to tissue factor enhances the former's activity against its substrates factor IX and factor X several thousandfold [Bach R et al 1981, Broze GJ Jr et al 1985]. The formed FXa can activate cofactor V [Monkovic DD and Tracy PB 1990a]. FXa in complex with FVa on the surface of TF-expressing cell produces a small amount of thrombin. Thrombin can then escape into the circulation.

Although platelets are already activated at the site of injury, the formed thrombin induces more secretion of platelet granule contents, including factor V in a partially activated form from α-granules [Monkovic DD and Tracy PB 1990b]. The platelet surface expressing phosphatidylserine is procoagulant in the presence of Ca^{2+} ions [Krishnaswamy S et al 1992]. Thrombin activates small quantities of cofactors V and VIII. Factor VIII is then released from

its carrier protein von Willebrand factor. Thrombin also activates factor XI on an anionic surface [Gailani D and Broze GJ Jr 1991]. The accumulation of FVa, FVIIIa and FXIa on the activated platelet surface has resulted in *amplification* of the initial procoagulant signal. At this stage, coagulation becomes independent of tissue factor expression. Tissue factor pathway inhibitor, forming a complex with free FXa, continuously blocks the complex FVIIa/TF. Thus, the relevance of the initial complex FVIIa/TF for the progression of coagulation decreases.

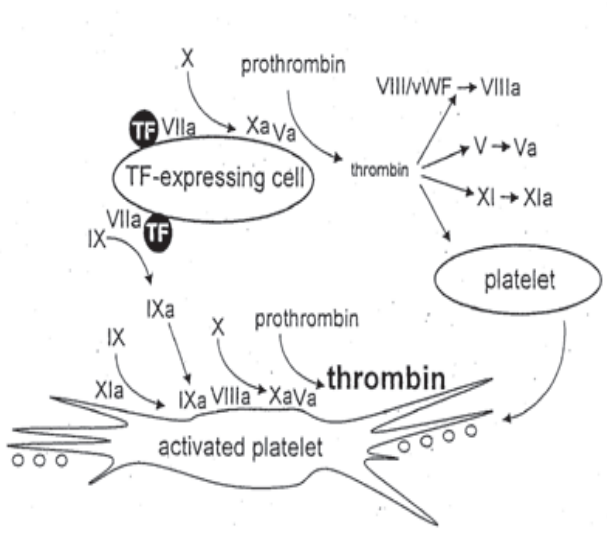

Figure 1. A cell-based model of coagulation [from Jurlander B et al 2001 with permission].
Ca^{2+} ions have been omitted for the sake of clarity.

The exposure of FVIIIa and FVa on the activated platelet surface recruits the initially formed FIXa and FXa, leading to formation of the complexes FIXa/FVIIIa and FXa/FVa. The complex FIXa/FVIIIa on the platelet surface is a much more potent source of FXa than the initial complex FVIIa/TF on the TF-expressing cell. The increased amounts of the complex FXa/FVa give rise to the formation of a large amount of thrombin, leading to an efficient conversion of fibrinogen into fibrin monomers and activation of factor XIII. Factor XIIIa is essential for the stabilization of fibrin.

Thrombin also plays a central role at this stage since it can *propagate* coagulation by activating larger quantities of cofactors V and VIII, and factor XI. The formed FXIa on the platelet surface can provide additional FIXa. Conversely, thrombin downregulates its own formation by activating the protein C pathway. Activated protein C (APC), with protein S as a

cofactor, inactivates FVIIIa and FVa by cleavage. Thrombin activity is also blocked by antithrombin. A balance between procoagulant and anticoagulant events is critical at this stage of coagulation.

Impaired or enhanced coagulation, due to either hereditary or acquired defects, may lead to hypocoagulable or hypercoagulable conditions. The most significant bleeding disorders are haemophilia A (factor VIII deficiency), haemophilia B (factor IX deficiency), and von Willebrand disease (von Willebrand factor deficiency). Deficiencies in other coagulation factors, e.g. fibrinogen, prothrombin, factor V, factor VII, factor X, factor XI and factor XIII, are very rare. Bleeding in patients with increased PT (INR) may be caused by anti-vitamin K therapy, vitamin K deficiency or liver injury. PT (INR) reflects the level of factor VII, factor X and prothrombin.

Individuals with an inherited deficiency of antithrombin, protein C or protein S have an increased risk of venous thromboembolism. The most common known inherited risk factor for venous thromboembolism in Western societies is a condition called APC resistance. A mutation in the factor V gene (1691G>A) causes FVa to be resistent to cleavage by activated protein C (FV Leiden mutation). A heightened tendency to venous thromboembolism can also be associated with high levels of prothrombin, which may be caused by a point mutation in the prothrombin gene (20210G>A). Elevated plasma concentrations of von Willebrand factor [Silveira A et al 1992], factor VII [Heinrich J et al 1994], fibrinogen [Wilhelmsen L et al 1984], and PAI-1 [Hamsten A et al 1985] have been shown to be independent risk factors for atherosclerotic cardiovascular disease.

Contact activation

Contact activation, formerly called the intrinsic pathway, is an alternative route of coagulation activation. Contact activation is initiated when factor XII, kininogen(120 000) and prokallikrein interact with negatively charged material. *In vitro,* such material includes non-physiological surfaces such as glass, kaolin, celite, silica, dextran sulphate and ellagic acid, as well as surfaces of biological activators, such as collagen, sulphatides, cholesterol sulphate and urate crystals. *In vivo*, an activating surface is not needed for contact activation that takes place on membranes of endothelial cells, platelets, neutrophils, and monocytes. The sequelae of contact activation include activation of coagulation factors XI and VII and production of bradykinin (Figure 2). Other proteolytic plasma systems connected with the contact activation

reactions, at least *in vitro*, are the complement system [Ghebrehiwet B et al 1983], the fibrinolytic system [Ichinose J et al 1986] and the renin-angiotensin system [Derkx FHM et al 1979] [for review see Colman RW and Schmaier AH 1997 and references therein].

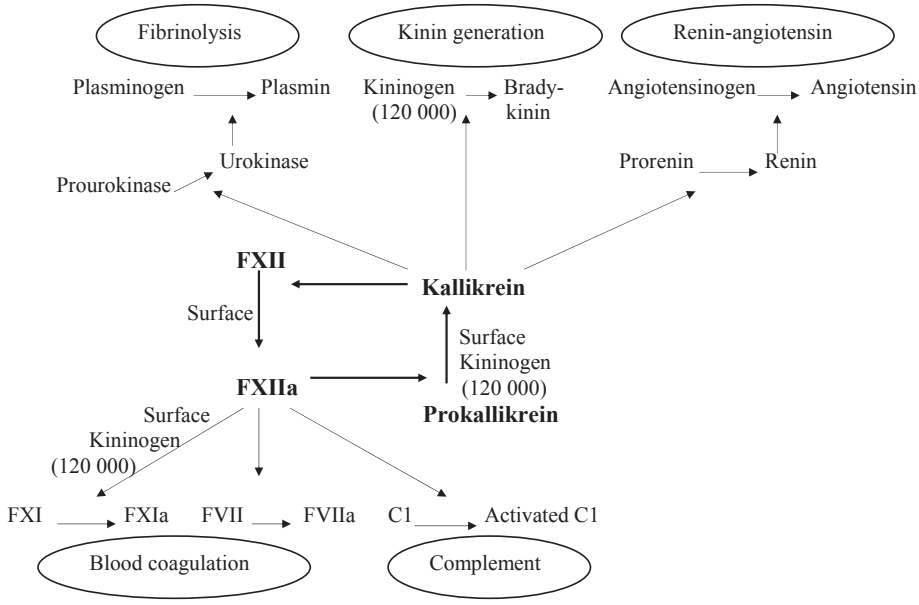

Figure 2. A schematic representation of the contact activation system and its interactions with other proteolytic systems. FXIIa denotes both surface-bound activated factor XII and factor XII fragment in the fluid phase.

Surface binding serves to bring the contact proteins into a close spatial orientation. Prokallikrein circulates in plasma in a non-covalent complex with kininogen(120 000). The latter has affinity to activating surfaces. Factor XII binds to activating surfaces at two binding sites located near the amino terminal end [Pixley RA et al 1987, Clarke BJ et al 1989]. Ca^{2+} ions are not needed for contact activation. Zn^{2+} ion binding to factor XII induces a conformational change that makes factor XII more susceptible to autoactivation, i.e., factor XII activation by FXIIa [Schousboe I 1993]. FXIIa converts available prokallikrein to kallikrein (Figure 2). Once formed, kallikrein can dissociate from the surface. As kallikrein is the essential activator of factor XII, it creates a positive feedback mechanism, making a rapidly

accelerating activation of both factor XII and prokallikrein possible. Since this eventually will lead to a rate of formation of FXIIa and kallikrein that exceeds the capacity of the inhibitors present, the reciprocal activation mechanism is crucial for further activation of the contact system and other interconnected systems. Surface-bound factor XII is 500 times more susceptible to activation by kallikrein than factor XII in solution [Griffin JH 1978]. At least two active forms of factor XII, namely FXIIa and FXII fragment, are produced by limited proteolysis by kallikrein. FXIIa with a relative molecular mass (Mr) of 80 000 is the active two-chain form of zymogen factor XII maintaining the surface-binding capacity of factor XII. FXII fragment with a Mr of 28 000 to 30 000 lacks the surface-binding capacity. (In this thesis, the term FXIIa sometimes represents both FXIIa and FXII fragment.)

Besides being an effective activator of factor XII, kallikrein is particularly potent in its action as a kininogenase. It liberates the biologically active nonapeptide bradykinin from kininogen(120 000). The kinin-free part of kininogen(120 000) has an increased surface-binding capacity, which enhances its procoagulant activity [Scott CF et al 1984]. Bradykinin elicits many effects that suggest its participation in acute inflammatory reactions: vasodilation, smooth muscle contraction, increased vascular permeability and production of pain [reviewed by Cyr M et al 2001]. Bradykinin has the ability to lower blood pressure, as seen e.g. after endotoxin-induced activation of the contact system [DeLa Cadena RA et al 1993]. FXII fragment and kallikrein have been identified as contaminants in some early plasma preparations with vasoactive effects when given intravenously [Alving BM et al 1978 and 1980]. Rapid infusion of plasma protein fractions contaminated with FXII fragment has been shown to cause hypotension combined with bradykinin generation in the circulation [Van Rosevelt RF et al 1982]. Some negatively charged surfaces used in filters for leucocyte reduction of plasma and platelet concentrates suspended in plasma medium have been reported to cause bradykinin generation [Shiba M et al 1997, Hild M et al 1998]. Such bedside filters have been associated with transfusion-induced hypotension, especially in patients treated with angiotensin-converting enzyme (ACE) inhibitors [Fried MR et al 1996, Hume HA et al 1996]. These patients are particularly susceptible to the vasodilator effects of bradykinin because ACE inhibitors decrease bradykinin degradation.

Procedures in which blood is exposed to artificial surfaces activating the contact system are e.g. cardiopulmonary bypass operation [Despotis GJ et al 1999] and haemodialysis with some membranes [Verresen L et al 1994]. Some radiographic contrast agents have also been shown to activate the contact system [Hoffmeister HM and Heller W 1996].

Activation of factor VII, with factor XII and prokallikrein involved, is seen after storage of blood and plasma in the fluid phase at low temperatures [Rapaport S et al 1955, Gjønnæss H 1972a, b, c].

A deficiency of factor XII, prokallikrein or kininogen(120 000) prolongs artificial surface-activated coagulation as measured e.g. by APTT, generally without being associated with bleeding. A deficiency of factor XI may, however, cause a bleeding diathesis. Factor XI used to be included in the contact system, because it circulates in a non-covalent complex with kininogen(120 000) and is activated by FXIIa. As already mentioned, factor XI is also activated by thrombin. The formed FXIa further activates factor IX in the presence of Ca^{2+} ions, which leads to formation of more thrombin.

Fibrin network

The formation of an insoluble fibrin network is the end product of the coagulation system. The rapid formation of this molecular net traps platelets, erythrocytes, leucocytes and serum into a clotted mass.

The fibrinogen molecule, the precursor of fibrin, is composed of two structurally identical halves with rotational symmetry. Each of the halves consists of three non-identical polypeptide chains, designated Aα-, Bβ- and γ-chains, held together by multiple disulphide bonds. The two halves are also joined by disulphide bonds through the Aα- and γ-chains near their N-termini, forming an N-terminal disulphide knot ($A\alpha 1\text{-}51, B\beta 1\text{-}118, \gamma 1\text{-}78)_2$. Electron microscopy has revealed that fibrinogen is a molecule with a central E-domain linked to two identical outer D-domains by coiled-coil segments (Figure 3). The core fragments obtained by plasmin digestion of fibrinogen are one N-terminal fragment E and two C-terminal fragments D. The dimeric fragment E has a considerable portion of its structure in common with the N-terminal disulphide knot [for review see Blombäck B 1996 with references therein].

The conversion of fibrinogen to fibrin network can be described as a three-stage reaction:

- Conversion of fibrinogen to fibrin – Release of fibrinopeptides A and B
- Fibrin assembly – Fibrin polymerization including lateral association and branching
- Stabilization of the fibrin structure – Covalent crosslinking of fibrin

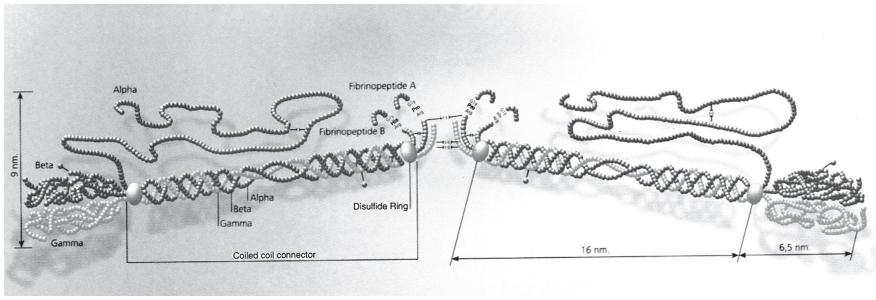

Figure 3. Primary structure model of human fibrinogen molecule (reproduced by permission from Dr. Xavier Soler).

Fibrinogen is converted to fibrin by thrombin, which quickly releases fibrinopeptides A from the N-terminal parts of the Aα-chains (Aα 1-16), followed by a slower release of fibrinopeptides B from the N-terminal parts of the Bβ-chains (Bβ 1-14). Fibrinopeptide A can also be released by thrombin-like enzymes, e.g. batroxobin purified from *Bothrops atrox* snake venom. The formed fibrin monomers initiate polymerization by constructing half-molecule overlapping dimers. These dimers extend linearly to form double-stranded fibrin protofibrils. An association occurs between D-domains in laterally aligned protofibrils, and thick fibrin fiber bundles of varying diameters are formed. The branching of fibrin fibers has been described to occur in a trimolecular branch point, in which one protofibril forms a junction with two separate protofibrils [Hermans J and McDonagh J 1982].

The three-dimensional fibrin gel is stabilised to the shear forces of the circulating blood and to proteolysis by covalent crosslinking. Coagulation factor XIII, being a protransglutaminase, is activated to transglutaminase (FXIIIa) by thrombin in the presence of Ca^{2+} ions. FXIIIa catalyses the formation of covalent bonds between glutamine and lysine residues in γ-chains of adjacent fibrin monomers. FXIIIa also catalyses crosslinking of fibrin α-chains. Crosslinking of γ-chains rapidly forms γ-dimers, while crosslinking of fibrin α-chains is both a slower and a more complicated process forming α-polymers. In addition, FXIIIa links plasmin inhibitor to fibrin [Aoki N and Sakata Y 1980].

Plasma fibrinogen is an acute phase protein. The levels are elevated in bacterial and viral infections, and in other inflammatory states. Afibrinogenemia is a very uncommon hereditary bleeding disorder. Hereditary hypofibrinogenemia rarely leads to spontaneous bleeding. Abnormalities in fibrinogen structure and function may lead to haemostatic

complications. Acquired low fibrinogen levels are found in many conditions with diminished synthesis or increased consumption of fibrinogen, such as in liver diseases and disseminated intravascular coagulation. Some dysfibrinogenemias are associated with an increased bleeding tendency, some with an increased thrombotic tendency, and some are asymptomatic.

Clot retraction

The completion of clot formation is followed by clot retraction. This is important for thrombus consolidation and wound heeling. Clot retraction is a thrombin-dependent, platelet-mediated contraction of the cellular clot mass resulting in the extrusion of serum. Platelet interactions with fibrinogen are involved, in part via the membrane glycoprotein complex GPIIb/IIIa [Peerschke EIB 2002]. Also essential are activation and assembly of the platelet cytoskeleton for contractile force via actin-myosin interactions.

Regulation of coagulation

Anticoagulant mechanisms limit growth of the blood clot to the vessel injury. Regulation of coagulation is exerted at each level of the pathway, either by enzyme inhibition or by modulation of the activity of the cofactors.

TFPI is a Kunitz-type inhibitor present in plasma and platelets. TFPI is bound to heparan sulphate when associated with endothelial cells**.** Initially, TFPI reacts with the active site of free FXa, inhibiting its activity. The FXa/TFPI complex effectively forms an inactive quarternary complex with FVIIa/TF on the surface of endothelial cells [Broze GJ Jr 1995].

Thrombin is self-regulatory by first activating and then inactivating factors V and VIII. The complex thrombin/thrombomodulin on endothelial cells initiates the formation of activated protein C. Activated protein C, with protein S as a cofactor, is required for the efficient neutralization of FVIIIa and FVa cofactor activity by proteolytic cleavage. Inactivation of FVIIIa and FVa stops the formation of the factor X activating complex FIXa/FVIIIa and the prothrombin activating complex FXa/FVa.

Activated protein C inhibitor is a serine proteinase inhibitor (serpin) without specificity. In addition to activated protein C, it inactivates some other haemostatic enzymes, among them kallikrein, FXIa, FXa, and tissue and urokinase plasminogen activators (t-PA and u-PA)

[Suzuki K et al 1989]. It has enhanced activity in the presence of exogenous heparin or heparan sulphate on the endothelial cell surface.

Other haemostatic serpins in the blood include antithrombin, heparin cofactor II, α_1-proteinase inhibitor (also called α_1-antitrypsin), C1 esterase inhibitor, plasmin inhibitor (antiplasmin), PAI-1 and plasminogen activator inhibitor 2 (PAI-2). The superfamily of serpins displays structural and functional similarities. Serpins act as substrates in which the amino acid sequence of the reactive center loop mimics that of an ideal natural substrate of target proteinases. Reaction with the target proteinase leads to formation of an initial non-covalent complex. After this, an acyl-enzyme intermediate forms that either develops into a covalent complex or dissociates to release the inactive cleaved serpin and the active enzyme.

Antithrombin inhibits most of the serine proteinases generated during activation of coagulation; mainly free thrombin and FXa, and to a lesser extent also FIXa, FXIa, FXIIa and kallikrein. Inhibition results into proteinase/inhibitor complexes, e.g. thrombin/antithrombin complex (TAT). Antithrombin is, in itself, an inefficient inhibitor. The complex formation is accelerated up to several thousandfold in the presence of pharmacological heparins or heparan sulphate on the endothelial cell surface.

Heparin cofactor II rapidly inhibits thrombin in the presence of heparin or heparan sulphate.

FXIa is primarily inhibited by α_1-proteinase inhibitor. The main function of this serpin is elimination of neutrophil elastase.

C1 esterase inhibitor contains a high amount of carbohydrate (about 35%) [Harpel PC et al 1975]. It accounts for more than 90% of the inhibition of both FXIIa and factor XII fragment [de Agostini A et al 1984, Pixley RA et al 1985]. C1 esterase inhibitor is also the predominant inhibitor of kallikrein [van der Graaf F et al 1983], and thus regulates the kinin system with bradykinin liberation. In the complement system, C1 esterase inhibitor is the only known efficient inhibitor of the C1r and C1s serine proteinases [Sim RB et al 1979]. A decrease in C1 esterase inhibitor levels occurs after activation of the contact system, e.g. in septic shock. Quantitative and qualitative defects of C1 esterase inhibitor are associated with angioedema.

α_2-macroglobulin is a secondary inhibitor of many serine proteinases, including kallikrein, thrombin and plasmin, which have Mr between 37 000 and 92 000. This multifunctional proteinase inhibitor, with a Mr of 725 000, traps enzymes within the molecule.

The trapped enzymes have no proteolytic activity against their large natural substrates, but retain an activity against small synthetic peptide substrates [Blombäck M et al 1974].

Fibrinolysis

When blood loss has been arrested at the site of injury and tissue repair is under way, it is the function of the fibrinolytic system to remove the fibrin deposits and recanalize the damaged blood vessel.

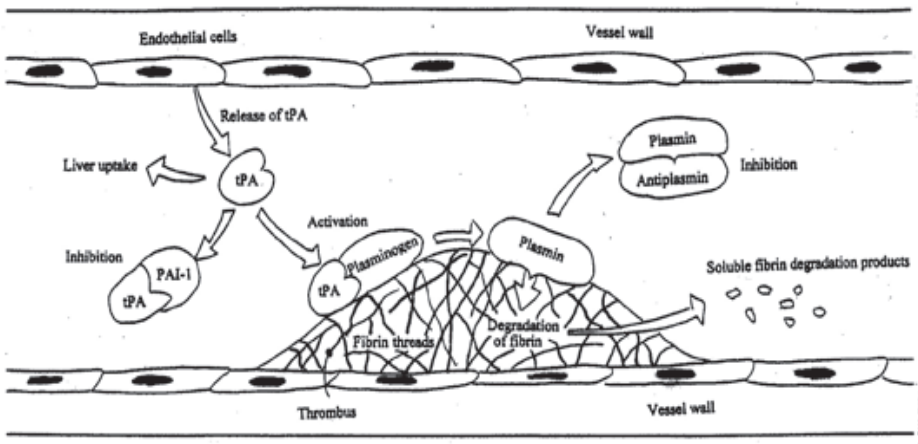

Figure 4. A schematic representation of the fibrinolytic system (modification of Wiman B, MRF informerar 2/87 with permission).

Activation of the fibrinolytic system is triggered by fibrin formation (Figure 4). Both the circulating zymogen plasminogen and the active serine proteinase t-PA released into the circulation by endothelial cells, have a high affinity for the fibrin clot. Plasminogen binds to lysine residues in fibrin, preferentially to carboxy terminal lysine residues [De Serrano VS et al 1989]. An efficient generation of the local fibrinolytic activity requires the formation of a ternary complex fibrin/t-PA/plasminogen [Wiman B and Collen D 1978]. After the conversion of plasminogen to the active enzyme plasmin by t-PA, plasmin degrades the fibrin network in a variety of ways to soluble fibrin degradation products with different molecular masses; the small ones are called fragments D and E. The degradation of covalent cross-

bound fibrin results in end products, in fibrin D-dimers, which are evidence of thrombin action, followed by FXIIIa and plasmin. In an early step in plasmin action on fibrin, fibrinopeptide Bβ(15-42) is cut off from each Bβ-chain, after Bβ(1-14), or fibrinopeptide B, has first been cut off by thrombin. The structure of the fibrin network is important for the fibrinolytic rate. A coarse network with thick fibres is degraded more rapidly than a tight network with thin fibres, even though the thin fibres are cleaved at a faster rate [Collet JP et al 2000]. When the fibrin network has become degraded, free plasmin is rapidly inactivated by circulating plasmin inhibitor (antiplasmin) forming the inactive plasmin/plasmin inhibitor complex [Wiman B and Collen D 1978].

The fibrinolytic system also involves another plasminogen activator, u-PA, as well as the two plasminogen activator inhibitors: PAI-1 and PAI-2. The latter is found in plasma of pregnant women. A fibrinolysis modifier, thrombin-activatable fibrinolysis inhibitor (TAFI), can be activated by the thrombin-thrombomodulin complex. Activated TAFI removes carboxy terminal lysine residues from partially degraded fibrin, and thus downregulates the clot lysis by decreasing the binding of plasminogen to fibrin before its activation [Nesheim ME et al 1997].

An increased fibrinolytic activity, e.g. due to PAI-1 or plasmin inhibitor deficiency, is associated with a bleeding tendency. An impaired fibrinolytic activity, e.g. due to poor t-PA release or an increased PAI-1 level, is often associated with thromboembolic diseases.

Plasma for transfusion

During the 1960s and 1970s it became evident that only one or a few of the constituents of blood are needed for effective treatment of a particular disease. For example, in most cases, treatment of anaemia with erythrocyte concentrates does not require platelet concentrates or plasma. Thus, a single unit of whole blood separated into blood components can serve several patients. Moreover, separation of blood components makes it possible to store each component under optimal conditions, which differ greatly from one component to another. For these reasons, whole blood is seldom used in an unseparated form. The introduction of plastic bags for blood donation led to convenient and safe separation of blood components by centrifugation of whole blood. Apheresis collection techniques were developed to extract one component, e.g. plasma, from whole blood and return the rest to the donor.

The quality of plasma constituents is best maintained in the frozen state. According to the international criterion, high-quality fresh frozen plasma (FFP) should be frozen within six hours of blood or plasma collection. Freezing should take place in a system that allows complete freezing within one hour to a temperature below -30 °C. This preparation contains a minimum of 0.70 kIU/L of coagulation factor VIII activity and at least similar quantities of the other coagulation factors and naturally occurring inhibitors. The permitted storage times and temperatures are: 36 months at below -25 °C and three months at -18 °C to -25 °C [general reference: Guide to the preparation, use and quality assurance of blood components. 12th edn. Strasbourg: Council of Europe Publishing; 2006].

Clinical indications for the use of plasma

When specific concentrates are not available, plasma transfusions are used to correct excessive bleeding or to prevent bleeding in those patients with abnormal coagulation tests that are undergoing an invasive procedure. The clinical conditions that may require the replacement of coagulation factors and inhibitors include:

- coagulation factor and inhibitor deficiencies for which specific concentrates are not available
- disseminated intravascular coagulation in the presence of bleeding
- surgical bleeding
- massive blood transfusion
- serious liver disease

• immediate reversal of warfarin effect in the presence of bleeding
• thrombotic thrombocytopenic purpura
• special pediatric conditions

[College of American Pathologists 1994, American Society of Anesthesiologists 1996].

The appropriate use of plasma in these clinical conditions is being investigated and evaluated [British Committee for Standards in Haematology 2004].

Plasma transfusion may be guided by screening tests, e.g. APTT and PT(INR). More specific information about haemostatic status is provided by analytes such as platelet concentration, levels of coagulation factor VIII activity, fibrinogen, antithrombin, D-dimer, soluble fibrin, and thrombin time and in special cases levels of other haemostatic factors and inhibitors.

Several instruments have been developed to monitor haemostasis at the bedside, using whole blood samples. The best known is the thrombelastograph, a device that measures the viscoelastic properties of the clot during its formation and subsequent lysis [Salooja N and Perry DJ 2001]. Another instrument, the Sonoclot instrument detects viscokinetic changes of whole blood or platelet-rich plasma as it undergoes coagulation, clot lysis and retraction [Hett et al 1995]. The use of various "near-patient" testing devices may be of some value as a qualitative screen for clot formation. Each of these methods has its own advantages and drawbacks.

Adverse effects of plasma transfusion

The transfusion of plasma is associated with a number of potential adverse effects. Immediate immunologic complications, although less common or very rare, include febrile nonhaemolytic reactions, allergic reactions, anaphylactoid reactions, and transfusion-related acute lung injury (TRALI). High titre human leucocyte antigen (HLA) or granulocyte-specific alloantibodies found in plasma mainly from women who have been pregnant are associated with TRALI when plasma is transfused to patients in a predisposing condition [Silliman et al 2003].

Besides these complications, there is always the possibility of plasma transmitting infectious agents due to viral, bacterial, or protozoal contamination. Such complications, however, are extremely rare today. A threat is posed by prions (variant Creutzfeldt-Jacob

disease). Transfusions also carry the risks of circulatory overload, hypothermia, and metabolic complications. Given these risks, transfusion of plasma should be appropriately minimized. Increasing the potency of plasma for transfusion could help to reduce exposure to plasma.

Haemovigilance is a national information system that is used in the surveillance of the blood transfusion chain, with particular reference to clinical use and adverse reactions.

Plasma preparation and storage

The main part of the collected plasma, prepared from whole blood by centrifugation or obtained by plasmapheresis, is used for fractionation into plasma-derived medicinal products. Such products include concentrates of coagulation factors VIII and IX, von Willebrand factor, antithrombin, C1 esterase inhibitor, protein C, albumin, and various immunoglobulins.

Plasma for transfusion in Sweden mainly originates from whole blood units; a smaller part originates from apheresis plasma. The current use of the amount of plasma for transfusion is about 32 000 kg, or almost 120 000 plasma units, a year [Berséus O et al 2006]. The mean volume of one unit is about 270 mL.

Plasma for transfusion should contain physiological levels of all haemostatic proteins, including zymogen forms of haemostatic factors, cofactors and inhibitors. Moreover, administration of plasma for transfusion should be as safe as possible. The quality of plasma depends on various conditions during blood collection and plasma preparation. Among them are: donor variables, venipuncture, choice of anticoagulant solution, the time and conditions under which the blood is stored between donation and plasma separation, centrifugation, separation of components in a closed system, reduction of cellular contaminants in plasma, and the temperature during storage of blood and plasma. Virus inactivation methods do also affect plasma quality. Some items are considered in the following sections.

Anticoagulant solution

The anticoagulant solutions used in blood collection have been developed to inhibit coagulation and to permit storage of erythrocytes for a certain period of time. While originally designed for whole blood storage, they have also been used in blood from which components are prepared. All solutions contain sodium citrate, citric acid and glucose (dextrose), some of them in addition phosphate and adenine. Citrate chelates Ca^{2+} ions, thereby inhibiting blood

coagulation. Glucose and adenine are cell nutrients used to sustain adenosine triphosphate (ATP) levels. Citric acid regulates pH. Phosphate added to a high concentration may function as a source of inorganic phosphate to maintain ATP levels and to yield high 2,3-diphosphoglycerate (2,3-DGP) levels in erythrocytes during storage [Högman et al 2006].

Most studies on plasma quality have focused on factor VIII activity, since factor VIII is the least stable haemostatic protein in plasma used for fractionation. The non-covalent complex factor VIIII/von Willebrand factor is stabilized by Ca^{2+} ions, optimally at the physiological Ca^{2+} ion concentration [Mikaelsson ME et al 1983]. The concentration of citrate in plasma, using the standard citrate-phosphate-dextrose (CPD) anticoagulant, is about 20 – 22 mmol/L, which is ten times higher than the calcium concentration. It has been shown that the stability of factor VIII in plasma can be improved by degreasing the concentration of citrate to 50% [Prowse C et al 1987]. Half-strength citrate CPD (0.5CPD) has also been shown to improve the maintenance of erythrocyte 2,3-DPG during storage of erythrocyte concentrates [Farrugia A et al 1992] and to give a satisfactory erythrocyte *in vivo* recovery [Griffin B et al 1988].

A lowered citrate concentration in transfusion plasma would reduce the total citrate load in the patient when plasma and erythrocytes are given simultaneously, e.g. in massive transfusion. Citrate lowers the level of plasma Ca^{2+} ions if several units are infused rapidly [Denlinger JK et al 1976]. Citrate binds magnesium as well as calcium. Hypomagnesemia has occasionally been documented in some massive transfusion situations leading to complications [McLellan BA et al 1984].

Cellular contaminants in plasma

Leucocyte contamination of blood components has been identified as a potent trigger of clinical side-effects of transfusion. Effective removal of leucocytes from the components has had several beneficial effects, reducing the risk of non-haemolytic febrile transfusion reactions and alloimmunization to human leucocyte (HLA) antigens [Bordin JO et al 1994]. There is good evidence that HLA alloimmunization can be prevented in most patients if transfused leucocytes are reduced to less than 1×10^6 per unit. Reduction of leucocytes also minimizes the risk of transmitting intracellular pathogens such as cytomegalovirus and Epstein-Barr virus [Meryman HT 1989].

Transfusion-associated graft-versus-host disease is a rare but potentially lethal condition caused by donor T lymphocytes. It cannot be prevented by leucocyte reduction alone; gamma irradiation of plasma units is needed.

A low platelet concentration reduces microaggregate formation. Moreover, the procoagulant surface provided by activated platelets and their fragments is diminished.

Previously, the main technique for making plasma leucocyte- and platelet-poor was centrifugation and removal of buffy coats. Filtration of whole blood or blood components is a technique that is easy to handle with currently available leucocyte and platelet filters. Filtration before storage is preferable to bedside filtration.

Virus inactivation of plasma

In spite of careful donor selection and sensitive methods for the detection of infectious agents, there is still a small risk of viruses being transmitted by transfusion. Screening tests such as human immunodeficiency virus (HIV), and hepatitis C virus (HCV) cannot detect potentially infectious donations, when the donor is in the antibody-negative (window) phase of infection. The most suitable methods for virus inactivation have been shown to be methylene blue and visible light and solvent-detergent treatment, since they provide an acceptable compromise between viral safety and impaired plasma quality. The methylene blue and light method is the only method that can be applied to plasma in its original plastic container [reviewed by Williamson LM et al 2003]. The dye can be reduced tenfold by adsorbent filtration before transfusion [AuBuchon JP et al 1998]. In the solvent-detergent technique, a plasma pool is incubated with a combination of a solvent tri-(n-butyl)-phosphate and a detergent Triton X-100. Both methods effectively kill viruses, mainly lipid-enveloped viruses, including HIV, hepatitis B virus (HBV) and HCV [Lambrecht B et al 1991, Mohr H et al 1995, Horowitz B et al 1992]. Non-enveloped viruses, such as hepatitis A virus (HAV) are not eliminated [Wagner SJ 2002, Mannucci PM et al 1994].

Methylene blue, a phenothiazine with a planar structure, has a high affinity for viral surface structures, nucleic acids and proteins. In proteins especially, an interaction with histidine residues has been shown [Inada Y et al 1978]. On exposure to red (visible) light at 620 – 670 nm, excitation of the dye causes chemical modification of adjacent molecules, a process which involves oxygen radicals [Lambrecht B et al 1991]. The process can result in a loss of individual clotting factors of at least 10%, with fibrinogen notably showing 20% to 39% loss [Lambrecht B et al 1991, Mohr H et al 1995, Zeiler T et al 1994, Aznar JA et al 1999].

Solvent-detergent treated plasma has been associated with low levels of plasmin inhibitor and protein S.

Since 1992, methylene blue and light treated plasma has been used in some European countries, while solvent-detergent treated plasma has been used in both Europe and the USA since 1991.

Other pathogen inactivation methods proven for plasma are being developed. The methods include the use of a synthetic psoralen (known as S-59 or Amotosalen HCl) and ultraviolet A light, riboflavin (vitamin B_2) and visible light, and ethylene imines (Inactine™). The inactivation targets of these methods are both enveloped and non-enveloped viruses, bacteria, parasites, and lymphocytes [Council of Europe expert committee in blood transfusion study group on pathogen inactivation of labile blood components 2001].

Plasma storage

Some of the many conditions that are known to influence the quality of fresh frozen plasma, in particular the activity of factor VIII, concern the time lag between blood collection and plasma freezing. The loss of factor VIII activity is about 1% per hour during the first 24 hours at room temperature, and slower after that [Pietersz RN et al 1989]. Six to eight hours is often applied as the maximum time by which the plasma must be frozen in order to ensure a high quality. Another important requirement is that freezing is rapid, e.g. less than one hour preferably to a temperature below the eutectic point of sodium chloride solutions (−23 °C) [Carlebjörk G et al 1986, Åkerblom O et al 1992, Swärd-Nilsson AM et al 2006]. To allow for temperature fluctuations during use, a freezer for storage of fresh frozen plasma should run at least at –30 °C or below (see page 23).

The highest plasma quality is obtained by storage in the frozen state. If the level of factor VIII in transfusion plasma is not important, storage at +4 °C is acceptable for a limited period of time. Since factor VIII is an acute phase reactant, its level is elevated in most patients. A decreased level of factor VIII is seen e.g. in severe disseminated intravascular coagulation, due to utilization and destruction, in haemophilia A and in von Willebrand´s disease.

Other haemostatic proteins, including factors V and XI, are more stable and do not decrease to levels associated with depressed haemostasis during 14-day storage of plasma at +4 °C [Nilsson L et al 1983, Blombäck M et al 1984, Suontaka AM et al 1992, Smak Gregoor PJ et al 1993, Boström F et al 2006]. The advantage of preserving plasma in the liquid state is its immediate availablity for transfusion without time-consuming thawing. However, haemostatic

activation has been observed in some plasma units during storage at +6 °C [Blombäck M et al 1984].

Aims of the study

The main purpose of the present study was to investigate and find out more about how some preparation and storage techniques affect the haemostatic quality of plasma for transfusion, with the further aim of developing methods for improving the plasma's stability.

The specific aims were:

1) To evaluate the stability of factor VIII, other coagulation factors and their inhibitors in whole blood drawn into half-strength citrate anticoagulant (0.5CPD) instead of normal full-strength citrate CPD *(Paper I)*.

2) To study the effects of virus inactivation of fresh plasma with methylene blue and red light on fibrinogen activities *(Paper II)*.

3) To investigate the frequency of the cold activation phenomenon during storage of plasma units at +4 ^{o}C and whether cold activation of plasma is an individually recurrent property of the donor *(Paper III)*.

4) To study the effects of storage of plasma at +4 ^{o}C on the contact system, coagulation and fibrinolysis *(Paper III)*.

5) To develop a method for the selection of plasma units that are stable during storage at +4 ^{o}C *(Paper IV)*.

6) To elucidate the cold activation mechanism in plasma during storage in bags at +4 ^{o}C *(Paper V)*.

Donors, Materials and Methods

Detailed descriptions of donors, materials and methods are given in the individual papers.

Donors and Donations

The healthy, volunteer donors fulfilled the national regulation criteria [Socialstyrelsens författningssamling 1989: 38] for blood or plasma donation. In the studies of cold activation of plasma (papers III − V) they were all men. Women were omitted because the possible use of oral contraceptives might affect haemostatic proteins and that could have complicated the interpretation of some results.

Plasma was obtained from whole blood separated into blood components (component plasma, papers I – V) and by apheresis technique (apheresis plasma, papers III and IV). Platelets used in viscokinetic measurements were harvested from CPD blood as platelet-rich plasma (paper II).

All studies were approved by the Human Research Ethics Committee of the Karolinska University Hospital Solna at Karolinska Institutet. All plasmapheresis donors gave their informed consent.

Plasma preparation, treatment and sampling

Whole blood (450 ml) was anticoagulated with 63 ml of CPD (papers I-V) or 0.5CPD solution (paper I). CPD-50 solution was used during plasmapheresis (papers III and IV). Calculated citrate concentrations in plasma at varying donor haemoglobin concentrations are presented in Table 3.

Table 3. Calculated citrate concentrations in plasma (mmol/L) (from paper I)

	Anticoagulant solution		
Donor haemoglobin	CPD	CPD-50	0.5CPD
120 g/L	18.5	11.6	9.7
140 g/L	20.0	12.8	10.5
160 g/L	21.9	14.2	11.5

In paper I, units of whole blood were collected into 0.5CPD solution (n=22) and into CPD solution (n=12) in a triple bag system under continuous mixing. The bags were made of the PL-2209 plastic (Optipac, Baxter, La Châtre, France). After holding for 8 ± 0.25 h at room

temperature, the blood units were centrifuged at 4100 *g* for 13 minutes. In all papers, plasma was separated by using automated separators. The plasma units (n = 12 of both types) were stored frozen for 15 months; 3 months at –70 °C and 12 months at –30 °C. The samples drawn from the bags after blood collection at 0 h were centrifuged immediately. The plasma units were sampled before (at 8 h after blood collection) and after the frozen storage. After centrifugation, supernatants were dispensed in small portions. In all papers, plasma samples were stored in plastic tubes at –70 °C to –80 °C until analyses. Some samples were analysed immediately.

In paper II, units of whole blood (n=13) were collected into CPD solution in the triple bag system (Optipac, Baxter). The blood bags were centrifuged at 2700 *g* for 13 minutes at room temperature. During the methylene blue and red light treatment of 228 to 275 mL of fresh plasma, the methylene blue concentration was 0.9 – 1.1 µmol/L of plasma. The diodes in a light box (Baxter) emitted red light with a bandwidth of 640 – 670 nm. The plasma units with methylene blue were irradiated in the light box for 53 minutes without agitation to obtain a light dose of 48 J/cm^2. Some plasma units with methylene blue were irradiated for up to 120 minutes (light dose 109 J/cm^2). The plasma samples were collected before and after the irradiation, and in some experiments also during the irradiation. The samples were then centrifuged at 2200 *g* for 20 minutes at room temperature before freezing of supernatants.

In papers III, IV and V, units of whole blood (n=100) were collected into CPD solution by using a quadruple bag system (Imuflex WB-RP, Terumo Corporation, Tokyo, Japan) with an integrated whole blood filter for reduction of the content of leucocytes and platelets. The filter material was neutrally charged polyurethane. The bags were made of Teruflex® (polyvinyl chloride plastic). Whole blood was filtered 1 – 6 h after donation. The blood units were centrifuged at 4275 g for 11 minutes at +22 °C. After recentrifugation of the plasma at 5660 g for 5 minutes, the plasma units were stored at a median temperature of +4 °C (range +2° to +5 °C) for 28 days. Samples were removed on storage days 0, 7, 14, 21 and 28. Some samples on day 0 were frozen and some were stored in sterile 0.5-mL polypropylene tubes (a cryotube with a screw stopper containing an O-ring, Sarstedt, Nümbrecht, Germany) at the median temperature of +4 °C (range +2° to +5 °C) for 7, 14, 21, 28 or 42 days before freezing (paper III).

In papers III, IV and V, plasmapheresis donors underwent a standard plasmapheresis performed by using a PCS 2® (Haemonetics Corporation, Braintree, MA, USA). Samples from apheresis plasma units were collected from 100 male donors. Samples from repeat apheresis

plasma units were collected from 32 of the 100 donors after a median of 14 months (range 12-20 months). Some samples on day 0 were frozen and some were stored in sterile 0.5-mL cryotubes at the median temperature of +4 °C (range +2° to +5 °C) for 7, 14, 21, 28 or 42 days before freezing (papers III and V). In paper IV, samples from both component plasma units and apheresis plasma units were stored at 0 °C, +4 °C, +22 °C and +37 °C for varying times.

Bacterial control

Each of the apheresis plasma units was found to be negative when the rest plasma stored in the bag at +2° to +5 °C for 42 days was cultured for bacterial growth, using conventional methods (papers III and IV). Sterility was tested in some samples from component plasma units during storage by using methylene blue staining.

Cell concentrations

Residual platelets and erythrocytes in plasma were counted on day 0 by using a standard technique in a Bürker chamber. Leucocytes were counted in a Nageotte chamber after sample dilution (1 + 4) in Türk's solution [Rebulla P et al 1994, Moroff G et al 1994].

Analytical methods

The methods used for quantitation of the haemostatic proteins and other analytes listed in Table 4 were activity methods, using either natural or synthetic substrates, and immunochemical methods. Other methods include turbidity measurements, electrophoretic procedures and viscokinetic measurements.

Coagulation methods

Coagulation factor XII, prokallikrein, kininogen(120 000) and *factor IX* were quantified using a coagulation method based on the measurement of APTT. The appropriate deficient plasma served as the substrate [Veltkamp JJ et al 1968, Hardisty RM and Macpherson JC 1962].

Activated factor VII (FVIIa) concentration was measured by a coagulation method using soluble recombinant truncated tissue factor (a kind gift from Professor James Morrissey, College of Medicine, University of Illinois at Urbana-Champaign, IL) [Morrissey JH al 1993].

Table 4. Plasma analytes in the different papers

	Paper I	Paper II	Paper III	Paper IV	Paper V
Residual cell concentrations			√		
Coagulation factor XII					√
Kininogen(120 000)					√
Prokallikrein					√
Kallikrein-like activity on S-2302	√		√	√	√
Proteolytic activity on S-2288	√				
Coagulation factor IX	√				
Coagulation factor VIII	√	√			
Coagulation factor VIIa			√		
C1 esterase inhibitor	√		√		√
Protein C	√				
Activated protein C inhibitor					√
Antithrombin	√				
Thrombin-antithrombin complex	√				
Fibrinogen	√	√			
Fibrinopeptide A	√	√	√		
Soluble fibrin	√	√			
Batroxobin-induced coagulation time		√			
Thrombin-induced coagulation time		√			
Turbidity measurements of fibrin gel		√			
Coagulation factor XIII		√			
Clot stability		√			
Coagulum retraction		√			
Plasminogen			√		
Plasmin inhibitor			√		
Plasmin-plasmin inhibitor complex			√		
Fibrin D-dimer		√			
Total fibrinogen and fibrin degradation products		√			
Plasmin digestion products		√			

Amidolytic methods

Spontaneous proteolytic activity (SPA), using the chromogenic substrate S-2288, measures the general amidolytic activity of plasma; using the substrate S-2302, it mainly measures kallikrein-like activity [Gallimore MJ and Friberger P 1982, Blombäck M et al 1984]. When *kallikrein-like activity* was used for detection of cold activation (papers III -V), the method was modified according to the manufacturer of the chromogenic substrates (Chromogenix, Mölndal, Sweden).

The following analytes were measured using commercial reagent kits:

- *Factor VIII* using a kit from Chromogenix [Rosén S et al 1985]
- *C1 esterase inhibitor* using a kit from Immuno (Vienna, Austria) [Wiman B and Nilsson T 1983, Kleindel M et al 1983]
- *Protein C* using a kit from Chromogenix [Ødegård OR et al 1987]
- *Antithrombin* using a kit from Chromogenix [Abildgaard U et al 1977]
- *Soluble fibrin* using a kit from Chromogenix [Wiman B and Rånby M 1986]
- *Plasminogen* using a kit from Chromogenix [Gram J and Jespersen J 1985]
- *Plasmin inhibitor* using a kit from Chromogenix [Clason SB et al 1999]

Coagulation factor XIII activity was measured photometrically by a NADH-coupled enzymatic reaction using a kit from Behringwerke (Marburg, Germany) [Fickenscher K et al 1991].

Immunochemical methods

Enzyme linked immuno sorbent assay (ELISA) methods were used for measurements of the following analytes:

- *Thrombin-antithrombin complex* using a kit from Beringwerke [Pelzer H et al 1988]
- *Plasmin-plasmin inhibitor complex* using a kit from Dade Behring (Marburg, Germany)
- *Fibrin D-dimer* using a kit from Biopool [Umeå, Sweden] [Elms MJ et al 1983, Rylatt DB et al 1983]
- *Total concentration of fibrinogen and fibrin degradation products* using a kit from Organon Teknika (Boxtel, The Netherlands) [Legnani C et al 1990]
- *Functional activated protein C inhibitor* was measured by a modified ELISA method using a kit from Technoclone (Vienna, Austria)

Fibrinopeptide A concentration was measured with a radioimmuno assay [Nossel HL et al 1974] as modified by Kockum C and Frebelius S [1980] (papers I and II) or with a competitive enzyme immunoassay kit (Diagnostica Stago, Asnieres-Sur-Seine, France) [Amiral J et al 1984] (paper III).

The concentration of total thrombin clottable fibrinogen was measured using the syneresis clot method [Blombäck B 1958]. *The concentration of functional fibrinogen* was measured using one of two methods: the coagulation rate method as described by Vermylen C et al [1963] was used in paper I and a modification of the Clauss coagulation rate method [1957] as described by Marbet GA and Duckert F [1992] was used in paper II.

Batroxobin-induced coagulation time was measured using batroxobin purified from *Bothrops atrox* snake venom and *thrombin-induced coagulation time* using bovine thrombin [Latallo ZS and Teisseyre E 1971].

Turbidity measurements of fibrin gel

The turbidity profile of a fibrin gel formed in the presence of calcium chloride (20 mmol/L) and bovine thrombin (0.5 NIH unit/mL) at room temperature was monitored at 600 nm in a spectrophotometer. A tangent was drawn to the steepest part of the first wave of the biphasic sigmoidal turbidity curve. Its intersection with the time axis was defined as the clotting time [Blombäck B et al 1994]. After 20 to 24 hours, the maximal turbidity was recorded at 600 nm and the gel was scanned between 670 nm and 850 nm. Fibrin fiber mass-to-length ratio and fiber diameter were calculated from the wavelength dependence of turbidity [Carr ME and Hermans J 1978, Carr ME and Gabriel DA 1980].

Electrophoretic procedures

Clot stability (solubility) was screened in 2% acetic acid [Schwartz ML et al 1971].
For the electrophoretic procedure for detecting fibrin crosslinking of γ-chains and α-chains, fibrin gels treated under reducing conditions were tested with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using Phast Gel 7.5 (Pharmacia, Uppsala, Sweden) [Laemmli UK 1970].

Plasmin digestion products were also detected by using SDS-PAGE.

Viscokinetic measurements

Coagulum retraction was evaluated in a viscoelastometer (Sonoclot Coagulation Analyzer, Sienco, Morrison, Colo, USA). Changes in clot impedance were measured using a vibrating probe in recalcified plasma after platelets had been added [Saleem A et al 1983].

The error of the coagulum retraction method was estimated from double measurements. Standard deviation was calculated according to the formula $SD = \sqrt{\sum d^2/(2n)}$, where d is the difference between the double values and n is the number of the pairs of values. Standard deviation was 1.0 chart division/min at the interval of 3.1 – 20.8 chart divisions/min (n = 8).

Statistical methods

Results were presented as arithmetic means and standard deviations (papers I and II). When most of the data were not normally distributed, results were given as medians and ranges (papers II and III) or presented as box plots (paper V).

In paper I, statistical analyses were performed using Student´s *t* test for paired observations.

In paper II, the Wilcoxon signed rank test was used for the analysis of differences between pairs of values. The measure of correlation was the Spearman rank-order correlation coefficient.

In paper III, the Kruskal-Wallis one-way analysis of variance by ranks, followed by the Bonferroni method for adjusted P-values, was used for comparisons of donor age and duration of apheresis between more than two donor groups. The Kolmogorov-Smirnov two-sample test was used for comparisons of cumulative frequencies of cold-activated plasma units. The Fisher exact test was used to assess whether the frequency of cold-activated plasma units was associated with age group or blood group. The Wilcoxon signed rank test was used for within group-analysis and the Mann-Whitney U-test for comparisons between two independent groups. The Friedman two-way analysis of variance by ranks, followed by the Bonferroni method for adjusted P-values, was used to analyse data from more than two repeated measurements over time.

In paper V, the Mann-Whitney U-test was used for comparisons between two independent groups.

P-values <0.05 were considered significant. The tests were performed by using StatView 4.5 (Abacus Concepts, Inc., Berkeley, CA, USA) or manually.

Still confused
but on a significantly higher level,
P <0.01

Results and Comments

Stability of contact factors, coagulation factors and inhibitors in blood drawn into half-strength citrate anticoagulant (Paper I)

When blood is collected into the standard citrate-phosphate-dextrose (CPD) solution, the stability of coagulation factor VIII is impaired. We have evaluated the effects of using the half-strength citrate anticoagulant (0.5CPD) on the contact system and coagulation. Whole blood was drawn into 0.5CPD solution and, for comparison, into the standard CPD solution under continuous mixing. The blood units were stored at room temperature for eight hours before component preparation. Samples were collected at 0 hour and at 8 hours after blood donation.

In the plasma obtained from 0.5CPD blood, no statistically significant changes were found between 0-hour and 8-hour samples with one exeption: protein C decreased from 0.98 U/mL to 0.95 U/mL ($p<0.001$). Factor VIII activity decreased during eight hours by 1%, from 1.08 ± 0.25 IU/mL (mean ± SD) to 1.07 ± 0.25 IU/mL. Factor IX activity decreased by 1%, from 1.03 ± 0.16 IU/mL to 1.02 ± 0.15 IU/mL.

In the plasma obtained from CPD blood, factor VIII decreased during eight hours by 8%, from 1.09 ± 0.22 IU/mL to 1.00 ± 0.18 IU/mL ($p<0.001$). Factor IX decreased by 7%, from 1.05 ± 0.20 U/mL to 0.98 ± 0.18 U/ml ($p<0.01$). Small but significant changes ($p<0.05$) were found in the levels of antithrombin, protein C and C1 esterase inhibitor but were considered to be of no clinical importance. The levels of other analytes (thrombin-antithrombin complex, fibrinogen, fibrinopeptide A, soluble fibrin, spontaneous proteolytic activity on S-2288 and on S-2302) did not change.

After 15 months in the frozen state (three months at –70 ˚C and 12 months at –30 ˚C), the beneficial effects of 0.5CPD for factor VIII or factor IX were no longer found. The 0.5CPD plasma lost 16% of the factor VIII present at 8 hours ($p<0.05$), while in the CPD plasma no significant further loss was noted. In both the 0.5CPD plasma and the CPD plasma the mean levels of protein C decreased significantly ($p<0.001$) to values just above the lower reference limit and the mean levels of soluble fibrin increased significantly ($p<0.001$) to values just above the upper reference limit. Although there were no significant changes in the levels of thrombin-antithrombin complex and fibrinopeptide A, these results may indicate that some activation had occurred.

Comments

The loss of factor VIII activity is dependent on the concentration of citrate and the time between blood collection and separation into its components [Mikaelsson ME et al 1983]. In whole blood, factor VIII seems to be best preserved at +20˚ to +24 ˚C [Pieterz RNI et al 1989].

After holding blood for 8 hours at room temperature, we found a significant decrease of factor VIII in the CPD plasma (8%) but not in the 0.5CPD plasma (1%). Similar results have been described by other authors. When blood units were cooled to +20 ˚C after collection and plasma was separated after three hours, Solheim BG et al [1998] observed no significant difference between the levels of factor VIII in the CPD plasma and 0.5CPD plasma. The collections were paired, so that the CPD blood and the 0.5CPD blood were collected from the same donors in a single session. After storage of blood for 24 hours at +20 ˚C, they found significantly better preservation of factor VIII in the 0.5CPD plasma. The decrease compared with the 3-hour levels was 23% in the CPD plasma and 10% in the 0.5CPD plasma. After storage of blood for 18 hours at room temperature, Prowse CV et al [1987] found a markedly greater decrease of factor VIII in the CPD plasma than in the 0.5CPD plasma (35% vs. 12%).

The significant decrease of factor IX which we found in the CPD plasma (7%) was not expected, since factor IX is generally regarded as a stable coagulation factor. The better preservation of factor IX at the lowered citrate concentration has been confirmed by Beek H et al [1999]. They also found a better preservation of factor V at the lowered citrate concentration, which is in accordance with earlier findings by Blombäck B and Blombäck M [1963].

The improved stability of factor VIII and factor IX should be a welcome benefit to the plasma fractionator. However, fractionators have tended to be reluctant to use plasma from blood collected in 0.5CPD due to a lack of clear evidence that the lowered citrate concentration has no negative consequences for the fractionated products. The lowest concentration of citrate that is safe for all the products proceesed from plasma is still a matter for debate.

The use of 0.5CPD in blood collection also provides possibilities for improving the preservation of erythrocytes. A new additive solution has been developed to guarantee optimal storage of erythrocytes prepared from such blood units. Superior maintenance of both ATP and 2,3-DPG has been found in this solution [Högman CF et al 1993 and 2002].

The lowered citrate concentration in plasma for transfusion would be an advantage particularly in massive transfusion, when cells and plasma are given simultaneously. It exerts less influence on the recipient´s level of ionized calcium and there is less citrate to be metabolized by the liver.

In conclusion, anticoagulation with 0.5CPD was sufficient during the 8-hour holding time of blood at room temperature. No negative effects on the contact system or coagulation, including their inhibitors, were found. Instead, a significantly better stability of coagulation factors VIII and IX was shown.

Changes in functional activities of plasma fibrinogen after treatment with methylene blue and red light (Paper II)

A virus inactivation system for single donor plasma units, consisting of a blood pack with the photoactive phenothiazine dye methylene blue and a light box, was developed by Baxter. The diodes in the light box emitted red light with a bandwidth of 640 to 670 nm. Fresh plasma units obtained from CPD blood were treated with 1 μmol/L methylene blue in 260 mL of plasma and a light dose of 48 J/cm^2. The effects of the treatment with methylene blue and red light on the functional activities of plasma fibrinogen were investigated.

The effect of the photodynamic treatment depends on the combined action of the photosenzitizer (i.e., methylene blue) and red light. Treatment with methylene blue without red light or red light without methylene blue was found to have no effect on the thrombin-induced coagulation time.

After the treatment with methylene blue and red light, both batroxobin-induced coagulation time and thrombin-induced coagulation time were significantly prolonged. L-histidine in final concentrations between 0 and 10 mmol/L added to plasma before the treament normalized the thrombin-induced coagulation time in a dose-dependent way.

No effect of the treatment was detected on the concentration of total thrombin clottable fibrinogen measured by the syneresis method, apart from the dilution effect of the added methylene blue solution (4%).

A light-dose dependent decrease was found in the functional fibrinogen concentration measured by the Clauss method. In the original method and the actual modification, no calcium was added. The effects of added calcium chloride on final concentrations between 0 and 30 mmol/L on fibrinogen concentration were evaluated. The maximal fibrinogen concentration was achieved in the presence of 7 mmol/L calcium chloride. The proportional effect of added calcium chloride was greater on treated than on untreated plasma.

The initial release rate of fibrinopeptide A, measured during the first minute after addition of thrombin, was slightly increased in treated plasma. The concentration of fibrinopeptide A, measured before and after the treatment, was unchanged.

The concentration of soluble fibrin was slightly but significantly ($p < 0.05$) increased in treated plasma.

Turbidity measurements of fibrin gels formed after addition of 0.05 mol/L thrombin and 20 mmol/L calcium chloride (final concentrations) to treated plasma showed significantly ($p < 0.05$) prolonged clotting time, lower fibrin fiber mass-to-length ratio and smaller fiber diameter compared with untreated plasma. At a given clotting time, a gel with a lower fiber mass-to-length ratio was produced in treated plasma.

Kinetics of turbidity development during clotting of treated plasma showed a turbidity curve with less steep initial and second phases compared with untreated plasma. The addition of about 1 U/mL of factor VIII concentrate to treated plasma before turbidity measurements resulted in a steeper second phase of the turbidity curve and a slightly increased mass-to-length ratio. The effects of added factor VIII on untreated plasma were marginal.

Factor XIII activity was slightly decreased after the treatment. The clot solubility test showed normal insolubility. The same electrophoretic patterns of factor XIIIa-induced γ-dimer and α-polymer chain formation were found in the gels formed in untreated and treated plasma.

No significant increase in the fibrin D-dimer concentration was found after the treatment. The total concentration of fibrinogen and fibrin degradation products was unchanged. Plasmin digested fibrin clots formed in untreated and treated plasma showed identical electrophoretic patterns.

No significant change in the coagulum retraction rate in the presence of platelets and calcium chloride was found after the treatment.

Comments

After the treatment with methylene blue and red light, a discrepancy was found between fibrinogen concentrations measured by the coagulation rate-independent syneresis method and the rate-dependent Clauss method. While the former remained unchanged, the latter showed a light-dose dependent decrease during the treatment. The partial loss of fibrinogen clottability is comparable to findings by others in studies on methylene blue and light-treated plasma [Lambrecht B et al 1991, Zeiler T et al 1994, Mohr H et al 1995, Aznar JA et al 1999, Depasse et al 2005]. There are several modifications of the Clauss method. An effect of the amount of

added calcium chloride on the fibrinogen concentration was demonstrated, particularly in treated plasma.

The prolonged coagulation times induced by thrombin (as also detected by the Clauss method and turbidity measurements) or batroxobin could not be explained by the well-known inhibitory effect of fibrinogen and fibrin degradation products on fibrin polymerisation, since these were not increased after the treatment. The prolonged coagulation times were not caused by a delayed initial release rate of fibrinopeptide A because this was, in contrast, slightly increased in treated plasma. Previously, the initial release rates of fibrinopeptides A and B were reported to be normal in methylene blue and light treated plasma [Inada Y et al 1978].

The prolonged coagulation time (clotting time, gel point) found in the turbidity measurements indicates that a fibrin network (gelation) formed later in methylene blue and red light-treated plasma. At a constant fibrinogen concentration, the clotting potential is presumably represented by either the initial rate of fibrinogen activation or by the extent of activation required to induce gelation [Blombäck B et al 1994]. Thus, with a slightly increased initial rate of fibrinogen activation, the prolonged clotting time was presumably due to the need for a higher concentration of activated fibrin monomers the gel point.

An alteration in the fibrin gel structure was found after the treatment. A property of a fibrin gel, expressed as the fiber mass-to-length ratio obtained from turbidity measurements, provides structural information that is directly related to the porosity of a gel obtained from permeability measurements [Blombäck B et al 1994]. The fiber mass-to-length ratio was significantly decreased after the treatment, which indicates a less porous (tighter) fibrin gel. The addition of factor VIII only slightly increased the fiber mass-to-length ratio. No effect of added factor VIII on the ratio of untreated plasma was found. This is in accordance with the earlier statement that after the addition of thrombin within certain limits, the initially formed network structure is not qualitatively influenced by the excess of thrombin generated after the gel point [Blombäck B et al 1994].

The linear relationship between corresponding clotting times and fiber mass-to-length ratios in fibrin gels confirmed that the gel structure was determined by the clotting potential of the system [Blombäck B et al 1994]. The results showed that at a given clotting time, a tighter gel structure was produced in treated plasma.

Furthermore, differences in fiber hydration can alter the porosity of gels [Blombäck B et al 1989]. At a constant fiber density, a decrease in the fiber mass-to-length ratio is normally accompanied by a decrease in the average fiber diameter. A lower fibrin fiber mass-to-length

ratio means fewer fibrin molecules per fiber length unit; thus, thinner fibers are formed. After the treatment, however, the fiber diameter was only slightly decreased. This suggests swelling of fibers; a presumably more hydrated gel was formed [Blombäck B et al 1989].

The treatment with methylene blue and light has been reported to photooxidize histidine residues [Inada Y et al 1978, Henschen-Edman A 1997]. A modified histidine residue Bβ16 in the fibrinogen molecule has been identified as a cause of impaired fibrin polymerisation [Shimizu A et al 1983 and 1986]. The histidine Aα24 is possibly also relevant for fibrin polymerization [Henschen-Edman A 1997].

In our experiment, L-histidine added to plasma before the treatment normalized the thrombin-induced coagulation time in a dose-dependent way. Added L-histidine is supposed to be a target molecule of singlet molecular oxygen generated during methylene blue and red light treatment, thus quenching the effect of the treatment. The singlet oxygen quenching activity of histidine has been found to correlate with the inhibitory effect of histidine on virus inactivation [Müller-Breitkreutz K et al 1995].

Conclusions

During the treatment of single donor units of fresh plasma with methylene blue and red light, the concentration of clottable fibrinogen was unchanged, but a light dose-dependent decrease was found in the concentration of functional fibrinogen. Turbidity measurements of fibrin gel showed prolonged clotting time, lower fibrin fiber mass-to-length ratio, and slightly smaller fiber diameter. At a given clotting time, a gel with lower fibrin fiber mass-to-length ratio was produced. Clot stability and fibrinolysis remained normal. L-histidine added to plasma before methylene blue and red light treatment normalized the prolonged thrombin-induced coagulation time in a dose-dependent way.

Storage of plasma for transfusion at +4 °C (Paper III)

In this paper the frequency of cold activated transfusion plasma units during storage at +4 °C was investigated, as well as whether cold activation of plasma is an individually recurrent property of the donor. The effects of storing plasma at +4 °C on the contact system, coagulation and fibrinolysis were studied by using various analytes as markers of the activation of these systems.

Residual cell concentrations in plasma

A reduced concentration of platelets in plasma diminishes the procoagulant surface provided by activated platelets and their fragments. Low leucocyte and erythrocyte concentrations reduce the risk of adverse effects of plasma transfusion.

Filtration of whole blood effectively reduced the platelet and leucocyte concentrations compared to non-filtered plasma (Table 5). It also reduced the platelet concentration compared to apheresis plasma.

Table 5. Residual cell concentrations expressed as median (range) on day 0 in component plasma (whole-blood filtered double-centrifuged plasma, paper III), non-filtered double-centrifuged plasma (later calculations of median and range were not shown in the paper by Suontaka AM et al 1992) and apheresis plasma (paper III).

Type of plasma	n	**Platelets** 10^6/L	n	**Leucocytes** 10^6/L	n	**Erythrocytes** 10^6/L
Whole-blood filtrered component plasma	20	16 (8-41)	20	<0.05 (<0.05-0.15)	20	34 (5-306)
Non-filtered component plasma	10	3 500 (2 000-9 000)	10	12 (2-22)	10	64 (20-80)
Apheresis plasma	100	19 900 (6 600-39 600)	20	0.05 (<0.05-0.20)	20	56 (19-161)

Frequency of cold activation of transfusion plasma units during storage at +4 °C

Component plasma units prepared from whole-blood filtered CPD blood from 100 male donors were stored in bags for 28 days and in cryotubes for up to 42 days. Furthermore, samples from apheresis plasma units from 100 male donors were stored in cryotubes for up to 42 days. The median storage temperature was +4 °C (range +2° to +5 °C). Cold activation was measured weekly as kallikrein-like activity of plasma. The highest value of kallikrein-like

activity found on day 0 was chosen as the cut-off value for cold activated plasma (absorbance 0.252).

The cumulative frequency of cold activated plasma units gradually increased in a time-dependent manner during storage. The comparisons between the two plasma preparation methods and between the storage containers (bag and cryotube) are shown in Figure 5. The frequency of cold activation during storage of component plasma units in bags was 5% on day 7 and increased to 18% on day 28. A comparison of component plasma and apheresis plasma, both stored in cryotubes, showed a higher frequency of cold activation during storage of the latter, though the difference was not significant (Figure 5). The apheresis plasma samples contained more platelets than the component plasma samples (Table 5). However, no correlation was found between the platelet concentration and increased kallikrein-like activity during storage of apheresis plasma samples at +4 ºC for 42 days (Spearman rank-order correlation, $p = 0.76$, $n = 100$) (Suontaka AM, unpublished results).

The frequency of cold activation was not associated with donor age, blood groups 0, A or AB, or Rh groups. A positive association, although not significant ($p = 0.08$), was found for blood group B. Four cold activated component plasma units were found among the 10 of blood group B tested. Two of these cold activated units were those with the highest rates of cold activation among the 18 cold activated component plasma units found on day 28. A larger number of plasma units from donors with blood group B needs to be investigated in order to determine whether this blood group is overrepresented in cold activation.

Using the Thrombotest time test, Gjønnæss H [1972a, b, c, 1973a, b] detected cold promoted activation in plasma samples stored in plastic tubes at 0 ^{o}C for 20 hours, indicating activation of factor VII. He observed an intermediate or strong cold activation of plasma from about 12% of healthy men, from about 20% of women without any medication, and from about 65% of women on oral contraceptives containing oestrogen [1973b]. Czendlik C et al [1985] found cold activation of plasma from about 40% of women on oral contraceptives containing oestrogen.

The inclusion of female donors in our study, particularly women on oral contraceptives, would presumably have resulted a higher frequency of cold activated plasma units. Ethinyl oestradiol is the usual oestrogen in oral contraceptives. Before 1980, the dose was generally 50 to 150 µg, whereas it now is usually 35 to 50 µg. Combined oral contraceptives contain a progesteron in addition to ethinyl oestradiol. How high the frequency of cold activators is among oral contraceptive users today remains to be proven.

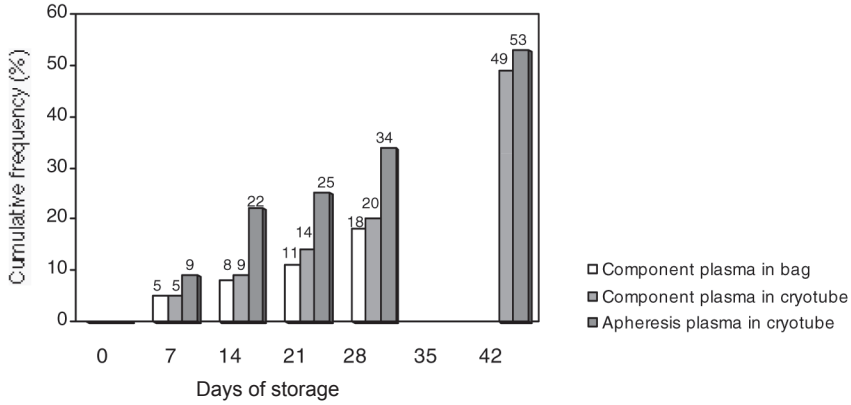

Figure 5. Cumulative frequencies of cold activated component plasma units obtained from 100 male whole-blood donors and samples from apheresis plasma units from 100 male donors. Component plasma units were stored at +2° to +5°C, in bags for 28 days and in cryotubes for up to 42 days, and samples from apheresis plasma units were stored in cryotubes for up to 42 days. The data were analysed by using the Kolmogorov-Smirnov two-sample test. The cumulative frequencies of cold-activated units did not differ significantly either between component plasma units stored in bags compared with cryotubes or between component plasma units compared with apheresis plasma units, both stored in cryotubes (from Paper III).

Is cold activation of plasma an individually recurrent property of the donor?

Samples from repeat apheresis plasma units from 16 strong cold activators on days 7 and 14, and from 16 non-cold activators on day 42 were collected after a median of 14 months (range 12 – 20 months). The intra-individual rate of cold-activation reaction was not constant, although the majority (9/16) of the cold activators on days 7 and 14 remained the same and so did the majority (13/16) of non-cold activators on day 42. The intra-individual time differences in the onset-day of cold activation ranged from 7 days shorter to 35 days longer.

Rare cases of spontaneous intra-individual changes from positive to negative cold activation, or vice versa, have been reported (Gjønnæss H 1973b, Czendlik C et al 1985). Thus, the rate of the cold activation reaction of plasma seems to be a quantitative rather than a qualitative property of the individual donor, as also indicated by the cumulative frequency distribution.

The reason for changes in the rate of the cold activation reaction in repeat plasma samples from men is unknown. In women, such changes have been shown to depend on changes in the oestrogen level, e.g. with the use of oral contraceptives and in pregnancy

(Gjønnæss H 1973b, Czendlik C et al 1985). Cold activation has been shown in plasma from 93% of women in the third trimester of pregnancy (Gjønnæss H 1973b).

Effects of cold activation on the contact phase, coagulation and fibrinolysis

In samples from apheresis plasma units cold activated on day 7 or day 14, an increase in kallikrein-like activity was accompanied by a decrease in C1 esterase inhibitor activity and increased concentrations of factor VIIa and fibrinopeptide A. Kallikrein-like activity peaked between days 14 and 28. The decrease in C1 esterase activity was continuous, except in five of 16 samples where this activity had already decreased to <30 kU/L on day 7 or 14. Unmeasurable or very low C1 esterase activity (0.00 – 0.10 kU/L) was found in four of 16 samples between days 14 and 42. The factor VIIa concentration peaked between days 7 and 21 and then decreased in all samples. The highest peak value (194.3 µg/L) was found on day 7. This peak value was about 40% of the normal factor VII concentration. On day 14 this value was 24.4 µg/L and on day 42 it was 3.8 µg/L.

The functional plasminogen level did not change during storage for 42 days. A minor decrease in plasmin inhibitor activity and a corresponding minor increase in the plasminogen-plasmin inhibitor concentration were found.

In non-cold activated apheresis plasma samples, no activation of the contact system, coagulation or fibrinolysis was found on day 42, as measured with the above methods, apart from a varying increase in the fibrinopeptide A concentrations at the end of storage. However, an ongoing increase in kallikrein-like activity, albeit at a very low rate, was observed in most of the non-cold activated plasma units. This was valid both in component plasma and in apheresis plasma during storage, both in bags and in cryotubes.

Clinical follow-up

Of the 100 component plasma units included in the study, 81 were transfused on storage days 29 – 42; 15 units were cold activated and 66 units were non-cold activated on day 28. No transfusion reactions were reported according to the patients' files at the blood centre.

Furthermore, all plasma units prepared from whole blood from the 100 component plasma donors within a six-year period were checked for transfusion reactions. In addition to the 81 units mentioned above, 230 units stored at +4 ºC were found to have been transfused on days 2 – 42; 28 units were from cold activators on day 28 and 202 were from non-cold

activators on day 28. No transfusion reactions were reported. Plasma recipients were random patients. Fifty-three percent of the plasma units were transfused in intensive care units.

Conclusions

The cumulative frequency of cold-activated plasma units increased in a time-dependent manner during storage at +4 ºC for 42 days. The intra-individual onset-day of cold activation of plasma was constant in the majority of male donors, but varied widely between plasma samples of some donors. Cold activation was associated with a high degree of activation of the contact phase and coagulation. Minor changes in the fibrinolytic system were found.

A method for selecting transfusion plasma units that will be stable during storage at +4 ºC (Paper IV)

This work comprises the development of a method for selecting transfusion plasma units that are stable during a specified storage time at +4 ^{o}C. The method is based on incubation of samples, drawn from plasma units on day 0, at optimal conditions for cold activation. The optimal conditions were determined by measurement of kallikrein-like activity on the chromogenic substrate S-2302. The shortest lag period was found when 0.5-mL samples were incubated in 15-mL plastic tubes at 0 ^{o}C (ice water). The method can be modified to suit an automatic procedure, e.g. the end-point measurement used in this paper can be replaced by measurement of the change in absorbance over time.

Cold activated samples found after incubation in tubes at 0 ^{o}C for 24 hours corresponded to cold activated component plasma units found after storage in bags at +4 ^{o}C for 28 days (paper III). Tests of samples from 100 component plasma units and 100 apheresis plasma units resulted in cold activation of all samples with time. The longest incubation time of plasma (lag period) found in tubes at 0 ^{o}C was 70 days.

In conclusion, the simple method presented for use before storage is suitable for identifying plasma units with a short lag phase before cold activation. The stability of plasma for transfusion can be improved by excluding such units from storage at +4 ^{o}C.

On the cold activation mechanism of transfusion plasma units during storage (Paper V)

During the course of cold activation of plasma at +4 ^{o}C, we found a simultaneous increase in kallikrein-like activity and a decrease in C1 esterase inhibitor activity (paper III). These were signs of an activation of the contact system.

The involvement of the contact system in cold activation was confirmed by the use of the inhibitors of factor XII and prokallikrein (paper V). The presence of either corn trypsin inhibitor (inhibitor of FXIIa) or soybean trypsin inhibitor (kallikrein inhibitor) prevented cold activation measured as kallikrein-like activity for at least 42 days. These results agreed with the findings of Gjønnæss H [1972c, 1973a], who showed both factor XII and an agent with properties resembling kallikrein to be indispensable for cold activation of plasma. The agent was later identified as being prokallikrein [Stormorken H and Abildgaard CF 1974, Saito H et al 1975]. Gjønnæss H [1972c] also showed that cold activation did not require the presence of either factor V, factor VIII, factor IX, factor X, factor XI or calcium. Furthermore, he showed that the kallikrein system was activated even in factor VII-deficient plasma [Gjønnæss H 1972c].

Cold activation includes a lag period with large inter-individual differences, as demonstrated in papers III and IV. The hypothesis behind paper V was that the disposition for cold activation of transfusion plasma is a consequence of an imbalance between the contact factors and their inhibitors. To test this hypothesis, functional levels of factor XII, prokallikrein, kininogen(120 000), C1 esterase inhibitor, and activated protein C inhibitor were measured on day 0 in the component plasma units with a short lag phase. Such units were cold activated within 14 days during storage in bags at +4 ^{o}C (paper III). The component plasma units with a long lag phase used for comparison were not cold activated on day 28 in bags or on day 42 in cryotubes (paper III).

The plasma units with a short lag phase had higher levels of functional coagulation factor XII ($p = 0.01$) on day 0, as compared with plasma units with a long lag phase. They also had slightly higher (not significant) levels of functional prokallikrein and kininogen (120 000), but lower functional levels of activated protein C inhibitor ($p = 0.02$) and slightly lower (not significant) functional levels of C1 esterase inhibitor. These differences may be a potential mechanism for the propensity to cold activation in plasma units with a short lag phase.

Some investigators have described changes leading to a similar imbalance in conditions connected with a high incidence of cold activation. Increased levels of factor XII and prokallikrein, and decreased levels of C1 esterase inhibitor have been found in plasma from women on oral contraceptives containing oestrogen and in pregnant women [Bühler R et al 1995, Halbmeyer WM et al 1991, Gordon EM et al 1980 and 1982, Czendlik C et al 1985]. The functional level of activated protein C inhibitor has been shown to decrease during pregnancy [He S et al 2000].

The results presented in this study suggest that factor XII and activated protein C inhibitor are the key proteins for the cold activation reaction. High levels of factor XII have been associated with spontaneous cold activation [Gjønnæss H 1972c, Gordon EM et al 1982]. On an anionic surface, factor XII becomes susceptible to autoactivation and activation by plasma kallikrein, non-covalently complexed with kininogen(120 000). The formed FXIIa and factor XII fragment are the links between the contact phase and coagulation, since both are able to activate factor VII to FVIIa [Seligsohn U et al 1979, Radcliffe R et al 1977].

The finding that activated protein C inhibitor is the most important kallikrein inhibitor at +4 ^{o}C has been reported earlier by España F et al [1991]. α_2-macroglobulin is a secondary inhibitor of kallikrein. C1 esterase inhibitor is the major inhibitor of FXIIa, FXII fragment and kallikrein at +37 ^{o}C [Colman RW 2001]. The efficacy of C1 esterase inhibitor is reduced at temperatures below +37 ^{o}C [Weiss R et al 1986, Harpel et al 1985]. In the present study, the C1 esterase inhibitor level was slightly (not significantly) lower in plasma units with a short lag phase on day 0. Addition of C1 esterase inhibitor to plasma before storage at +4 ^{o}C prevented cold activation. Addition of increasing amounts of C1 esterase inhibitor has been shown to progressively reduce kallikrein-like activity during storage of plasma samples at +6 ^{o}C [Czendlik C et al 1986].

During cold activation of plasma units stored at +4 ^{o}C for 28 days, the level of activated protein C inhibitor fell to 19% or below. This decrease was sharper than that in the level of C1 esterase inhibitor.

An additional cold activation mechanism may exist, since one plasma unit with factor XII activity as low as 0.04 kU/L had a short lag phase. Activated protein C inhibitor in this unit was 28%. However, it has been shown that in the presence of an activating anionic surface, kaolin, only 3% of the normal plasma concentration of factor XII was sufficient for cold activation at 0 ^{o}C [Gjønnæss H 1972c]. Furthermore, the concentration of anionic sulphatide

vesicles has been found to control the activation of the contact system [Mitropoulos KA 1999]. There are numerous physiologically relevant contact activating surfaces, but direct demonstration of their role in surface activation of factor XII has proved difficult [Colman RW 2001].

In conclusion, the results suggest that an imbalance between the contact factors and their inhibitors is associated with a short lag phase before cold activation. An additional mechanism may exist.

Conclusions

The present study was performed to add to knowledge of effects of some preparation and storage techniques on the haemostatic quality of plasma for transfusion. The following conclusions were reached with reference to the specific aims presented on page 30:

1) The use of half-strength citrate CPD (0.5CPD) instead of full-strength citrate CPD anticoagulant significantly increased the stability of coagulation factors VIII and IX in blood kept at room temperature for eight hours before component preparation. No negative effect was found on contact or coagulation factors, or their inhibitors *(Paper I)*.

2) Virus inactivation of single donor units of fresh plasma with methylene blue and red light caused a light dose-dependent decrease in the concentration of functional fibrinogen and the formation of a tighter fibrin gel structure, whereas normal clot stability and fibrinolysis were maintained *(Paper II)*.

3) The cumulative frequency of cold activated plasma units increased in a time-dependent manner during storage at +4 ^{o}C for 42 days.

   Large intra-individual differences in the onset day of cold activation were observed in plasma of some donors when tested on two occasions *(Paper III)*.

4) Cold activation of plasma was associated with a high degree of activation of the contact and coagulation systems. Only a minor activation of the fibrinolytic system was observed *(Paper III)*.

5) Using the new method for the selection of plasma units that are stable during storage at +4 ^{o}C, units with higher functional levels of several haemostatic proteins could be obtained.

   Testing of samples from 200 plasma units demonstrated cold activation of all samples at 0 ^{o}C with time *(Paper IV)*.

6) The short lag phase before cold activation seems to depend on an imbalance between the functional levels of the contact proteins and their inhibitors, especially coagulation factor XII and activated protein C inhibitor. An additional mechanism may be involved. *(Paper V)*.

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SKÅNSKA RESA PÅ HÖGA ÖVERHETENS BEFALLNING FÖRRÄTTAD ÅR 1749

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