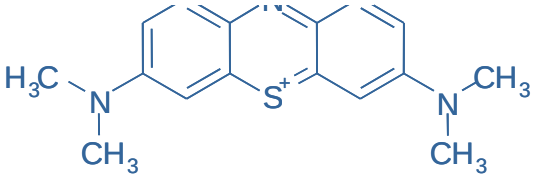
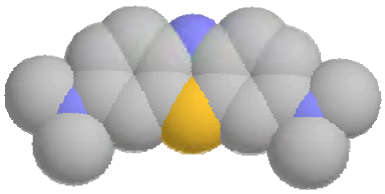
THERAFLEX-MB Plasma

Processing principle



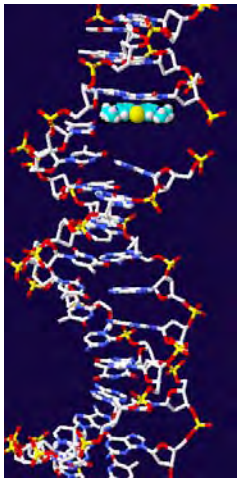
Methylene Blue molecule



MacoPharma Methylene Blue Pill (85µg / unit of plasma)

Illumination of plasma + Methylene Blue (590 nm, 180J/cm^2)

- Intercalation of MB into nucleic acids
- Excitation of MB by visible light
- Oxidation of Guanosine
- Degradation of nucleic acids



The combined action of Methylene Blue and light is a photodynamic process which blocks transcription and replication of viral RNA and DNA.

Photo-inactivation procedure of THERAFLEX-MB PLASMA

200-315ml of plasma (Aphaeresis or Whole Blood)	Filtration of plasma and dissolution of the MB Pill	Illumination of plasma+MB with the Macotronic V4	MB removal by filtration with Blueflex	Plasma freezing
				

THERAFLEX-MB Plasma

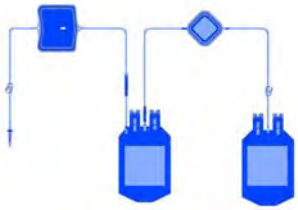
Examples of systems and treatments

Whole blood plasma
Min 250mL - max 330 mL*

Whole Blood Plasma with MB treatment

BSV system : plasma filtration with Plasmaflex > MB Pill dissolution > illumination

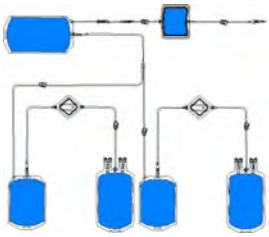
Whole Blood Plasma with MB treatment and MB removal



SDV system : plasma filtration with Plasmaflex > MB Pill dissolution > illumination > MB removal with Blueflex

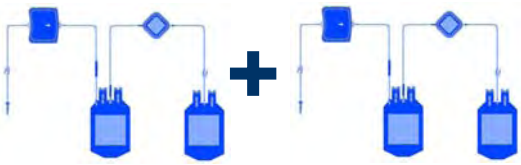
Aphaeresis plasma
Min 485 mL – max 650mL*

Aphaeresis Plasma : distribution in 2 units



SPV system : filtration of the aphaeresis unit with Plasmaflex MB Pill dissolution and distribution in 2 units > illumination > MB removal with Blueflex

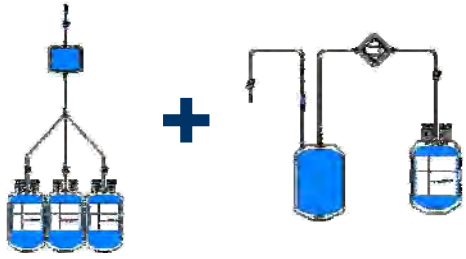
Aphaeresis plasma
Min 500 mL – max 665mL*



SDV system : plasma filtration with Plasmaflex > MB Pill dissolution > illumination > MB removal with Blueflex

Aphaeresis plasma
> 690 mL*

Aphaeresis Plasma : distribution in 3 units



BSV + ZDV system : filtration of the aphaeresis unit with Plasmaflex and distribution in 3 units with the BSV system. Connection of each unit to a ZDV system : MB Pill dissolution > illumination > MB removal with Blueflex

* *Volume ranges for plasmas to be treated with THERAFLEX-MB Plasma, based on process requirements.*

Methylene blue-treated fresh-frozen plasma: what is its contribution to blood safety?

Lorna M. Williamson, Rebecca Cardigan, and Chris V. Prowse

With current donor-selection criteria and virus genome testing, fresh-frozen plasma (FFP) in the developed world is probably safer than it ever has been. In the UK, where FFP is not manufactured from first-time or lapsed donors, it has been estimated that the residual virus risks from a single unit of FFP are 1 in 10 million for HIV, 1 in 50 million for HCV, and 1 in 1.2 million for HBV (Eglin R, written communication, January 2003). Against these levels of risk, it has been questioned whether pathogen reduction of FFP is a necessary strategy and/or the best use of healthcare resources.[1] However, the appearance of West Nile virus in blood components in the US in 2002, with fatal transmissions in immunocompromised recipients,[2] reminds us that sometimes viruses move ahead of our ability to test for them. Also, background viral incidence in a population can change, as is currently observed in Scotland, with HIV levels showing an increase to three per million population (Soldan K, written communication, February 2003). It is now over 10 years since a photodynamic system using methylene blue (MB) and visible light was developed in Springe, Germany, for virucidal treatment of FFP. The method has been used at various times since then in Germany, Denmark, Portugal, Spain, and the UK, so it is timely to review its potential contribution to overall FFP safety.

MB is a phenothiazine compound (Fig. 1), which was first used clinically by Paul Ehrlich in the 1890s and has been used to kill viruses since work at the Walter Reed Hospital in the 1950s.[3] When activated by visible light, MB generates reactive oxygen species, mainly singlet oxygen, through a Type II photodynamic reaction, and it is these that are responsible for its pathogen inactivating properties.[3-5] The original system developed in Springe, Germany, used an initial freeze-thaw step to disrupt intact WBCs, then added an amount of MB solution calibrated to the weight of the plasma pack, to achieve precisely the same MB concentration in every pack. Later systems (Baxter and Macopharma) developed for small-scale use in blood centers involve sterile connection of the plasma pack (before or after freezing) to a pack with a WBC-reduction filter upstream of a liquid pouch or a dry pellet containing 85 to 95 μg of MB (Fig. 2). To achieve the desired final MB concentration of 1 μ*M*, the input plasma volume has to be within a 200-to-300-mL range, so 600-mL apheresis units require splitting. In both the Springe and commercial systems, the MBFFP packs are then exposed to visible wavelengths of light to activate the MB. Because it is not possible to use the equivalent of radiation-sensitive labels to confirm illumination, the light-exposure system must be designed to ensure good manufacturing practice (GMP)-compliant control of both light intensity and duration. Radio-frequency chips for this purpose are in development. During illumination, MB is converted to its bleached leuko- form and to demethylated components (azure A, B, and C, and thionine; Fig. 1). A recent feature has been the development of commercial filters for post-treatment MB removal, which reduce the residual MB concentration to 0.1 to 0.3 μ*M*. The plasma is then ready for freezing or refreezing.

One of the attractions of the technique is that it is applied to single units of FFP, without the need for pooling. Commercial systems are available that can be set up in standard blood center GMP conditions, without the need to install specialized plant, and it is this model that is in operation in the UK. Plasma is frozen locally, sent to one of three central MB-treatment points, then returned for distribution to hospitals.

PATHOGEN-REDUCTION SPECTRUM

The ability of MB to inactivate viruses is dependent on its binding to nucleic acid, being greater for double stranded

ABBREVIATIONS: APTT = activated partial thromboplastin time; FFP = fresh-frozen plasma; MB = methylene blue; MBFFP = methylene blue-treated fresh-frozen plasma; PT = prothrombin time; TTP = thrombotic thrombocytopenic purpura.

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than single stranded, although viruses containing genomes of either type may be efficiently inactivated (see below). Activation results in a mixture of strand cross-linking, guanosine oxidation, and depurination. MB may also modify proteins and lipids, the relative rates depending on the MB and local oxygen concentrations. For virus-infected cells, this may be influenced by the reducing and detoxifying mechanisms present inside the cell. MB is not considered useful for inactivation of intracellular viruses or to attain bacterial or protozoal reduction, although it does enter cells.[5-7] Its only application in transfusion has been to achieve virus inactivation of plasma, with prior cell removal by filtration or freeze-thaw lysis[8-10] (Flament J, Mohr H, and Walker W, written communication, 2000).

Photodynamic treatment with MB results in efficient virus inactivation for all lipid-enveloped viruses tested to date, including all those for which the UK and US currently routinely screen blood donations, as well as West Nile virus.[3-5,10] The extent of removal for such viruses is usually at least 5 logs, this being true for both double- and single- stranded RNA and DNA viruses (Table 1). Nonlipid-enveloped viruses show a more diverse spectrum of susceptibility, some being totally unaffected (EMC, polio, HAV, porcine parvovirus), whereas others (SV40, HEV models, human parvovirus B19) show reduction factors of 4 logs or more (Table 1). More recently, testing using PCR methods has shown direct removal of HIV, HBV, HCV, and parvovirus B19 reactivity from infected donations,[11-14] the last of these demonstrating 4-log reduction by a newly developed B19 bioassay on the KU 812 EP 6 cell line (Flament J, Mohr H, and Walker W, written communication, 2000).

Are such reduction factors sufficient to assure that a single plasma donation, taken during the peak of viremia, is rendered noninfectious? The answer will depend on whether the donation is also subjected to NAT or serologic testing and on the level of viremia. For most viruses, we know that the answer is almost certainly yes, but in a few cases such as parvovirus B19, in which the peak of viremia is around 10^7 genome equivalents per mL, this conclusion is more dubious. However, for viruses of major concern, peak viremia levels are either within the clearance range of the system, or screening with assays of high sensitivity will have ensured that only donations with lower levels of viremia enter the processing laboratory (handling errors excepted). In the pregenome testing era, there was a possible HCV exposure from a unit of MBFFP taken from a donor in the sero-negative window period (Flament J, written communication, March 1998). The patient seroconverted for HCV but remained genome negative. The precise events remain unproven, but it is possible that the patient generated an antibody response against inactivated virus.

Although MB and other phenothiazine dyes have been suggested as having inhibitory action against transmissible spongiform encephalopathies,[15] there is no evidence of in vitro inactivation of infectivity at the concentrations used in the transfusion setting.

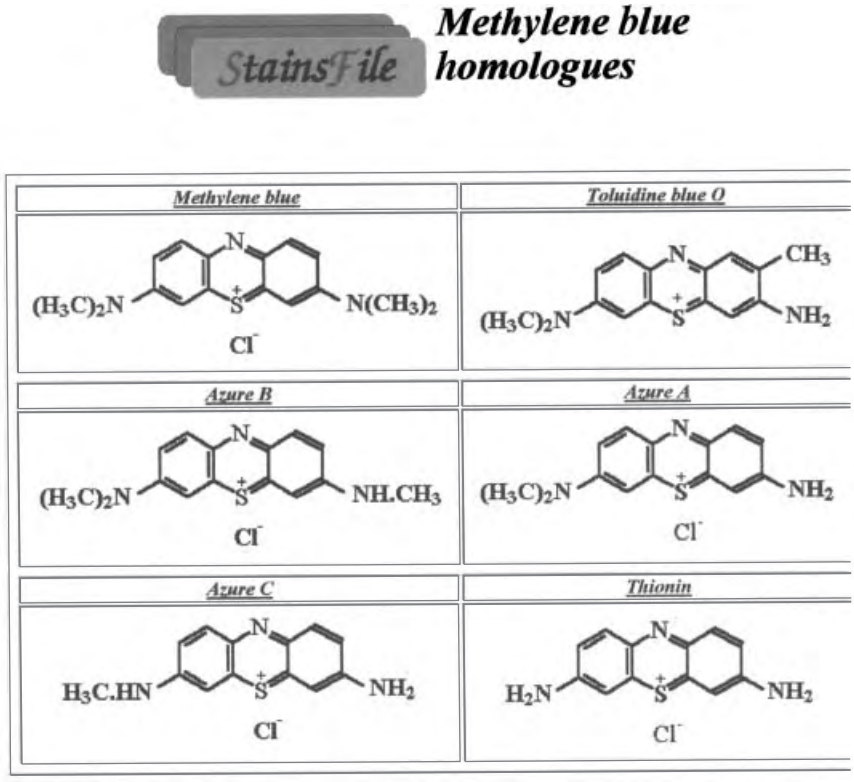

Fig. 1. MB and its photodegradation products.

TABLE 1. Virus reduction

Lipid enveloped		Non-lipid enveloped	
Virus	log reduction factor	Virus	log reduction factor
HIV	>5.5	HAV	0.0
Bovine viral diarrhea	>6.2	Encephalomyocarditis	0.0
Duck HBV	3.9	Porcine parvovirus	0.0
Influenza	5.1	Polio	0.0
Pseudorabies	5.4	SV40	4.3
Herpes simplex	>6.5	Adenovirus	4.0
Vesicular stomatitis	>4.9	Human parvovirus B19	≥4.0
West Nile virus	>6.5	Calicivirus (HEV)	>3.9

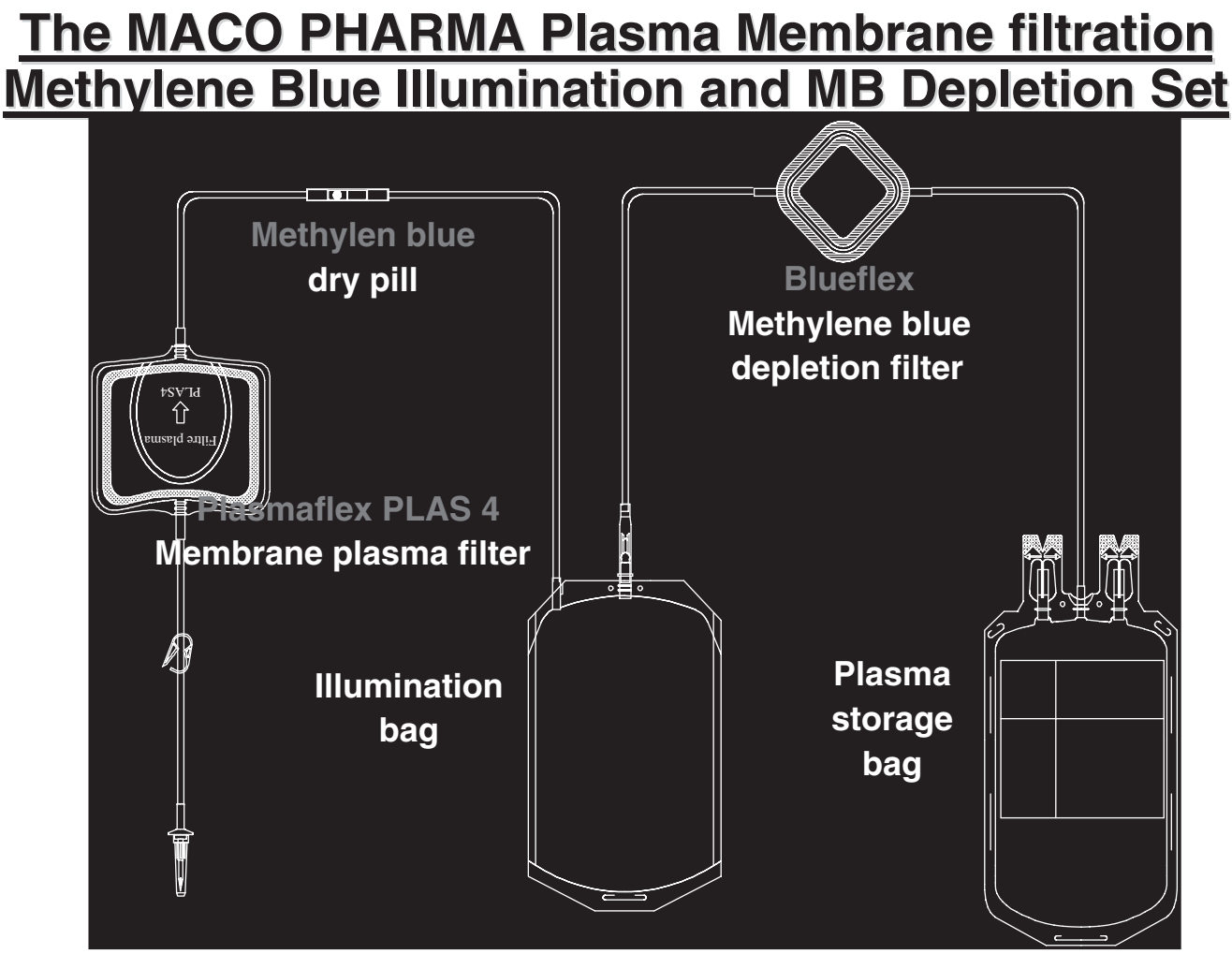

Fig. 2. Schematic representation of the closed bag system for MB treatment of fresh-frozen plasma.

EFFECT OF MB TREATMENT ON COAGULATION PROTEINS

It is well established that MB treatment of plasma affects the functional activity of various coagulation proteins and inhibitors (Table 2). The proteins most severely affected by MB treatment of plasma are FVIII and fibrinogen, where activity is reduced by 20 to 35 percent. The decrease in fibrinogen is seen when assayed by the method of Clauss, but not in antigenic assays,[16] suggesting that MB treatment effects the biologic activity but not concentration of fibrinogen. It has been suggested that this is due to the photo-oxidation of fibrinogen inhibiting polymerization of fibrin monomers.[17] The effects on fibrinogen are probably due to an interaction of MB with histidine residues and may result in a modified in vivo clearance.[16,18-20] However, fibrinogen isolated from MB-treated plasma retains normal ability to bind to glycoprotein IIb/IIIa receptors on platelets,[21] an important mechanism in platelet activation and aggregation. The inhibitory effects are ameliorated by the presence of ascorbate[22] but do not appear to result in the formation of any neoantigens[16,18,19] or positivity in tests for the formation of IgE antibodies (Flament J, Mohr H, and Walker W, personal communication, 2000).

Unsurprisingly, the changes in coagulation proteins observed in MB-treated plasma are associated with a prolongation of the prothrombin time (PT) and activated partial thromboplastin time (APTT).[16,23]

Original studies on MB inactivation were reported on plasma freeze-thawed before treatment, but later work on the Baxter Pathinact and Maco Pharma Theraflex systems was performed on fresh plasma (Table 2). However, we have recently shown that the major cause of coagulation factor loss is the MB treatment itself and not the freeze-thawing.[16,24] Fortunately, changes in coagulation proteins induced by WBC-reduction and MB-removal filters appear to be negligible compared to the effect of the MB process itself. Filtration of plasma using a filter (Hemasure)

TABLE 2. Changes in coagulation factor proteins and inhibitors in MBFFP

Parameter*	Percent change due to MB treatment†‡§	Mean residual levels‡§
Fibrinogen (Clauss) g/L	↓ 24,[10] 24,[23] 39[29]	1.65,[10] 1.80,[16] 2.01,[23] 1.97,[28] 2.05[29]
Fibrinogen (antigen) g/L		2.74[16]
Prothrombin (FII) (U/mL)	↓ 8,[10] 8,[16] 18,[23]	1.15,[10] 1.05,[16] 1.00[23]
FV (U/mL)	↓ 4.5,[10] 21,[16] 32,[23] 10,[28]	0.84,[10] 0.73,[16] 0.79,[23] 0.76[28]
FVII (U/mL)	↓ 8,[10] 9,[16] 7,[23]	1.10,[10] 0.90,[16] 0.90[23]
FVIII (U/mL)	↓ 13,[10] 33,[16] 28,[23] 26,[28] 29[29]	0.78,[10] 0.58,[16] 0.58,[23] 0.83[28]
FIX (U/mL)	↓ 17,[10] 23,[23] 11[28]	1.00,[10] 0.72,[23] 0.88[28]
FX (U/mL)	↓ 13,[10] 7[23]	1.05,[10] 0.90[23]
FXI (U/mL)	↓ 17,[10] 27,[23] 13[28]	1.00,[10] 0.73,[23] 0.84[28]
FXII (U/mL)	↓ 17[10]	1.20[10]
FXIII (U/mL)	↓ 7,[23] 16[29]	1.02,[23] 1.12[29]
vWF antigen (U/mL)	↓ 7,[23] 5[29] →[28]	0.94,[23] 0.83,[29] 1.00[28]
vWF:ristocetin cofactor(U/mL)	↓ 8,[23] 18[29]	0.92,[23] 0.79[29]
C1-inhibitor (U/mL)	↓ 23,[10] →[16]	0.88,[10] 1.03[16]
Antithrombin (U/mL)	↓ 8,[10] 3[23] →[16,23]	0.78,[10] 0.95,[16] 1.00,[23] 0.96[28]
Protein C (U/mL)	→[16,28]	1.03,[16] 0.89[28]
Protein S (U/mL)	→[16]	1.11[16]
α_1-antitrypsin (U/mL)	→[16]	155 mg/dL
Plasminogen (U/mL)	→[10,16]	0.90,[10] 0.98[16]
α_2-antiplasmin (U/mL)	→[16]	0.96[16]

* Results given as U/mL because not all studies were calibrated against international standards. Assays are functional unless otherwise stated.
† Arrows indicate direction of change, with horizontal arrow indicating no change.
‡ [10,16,23]Studies used frozen-thawed plasma.
§ [28,29]Studies used fresh plasma (<8 hr from collection).

designed to remove both WBCs and MB simultaneously results in a prolongation of the APTT but has no effect on the PT or fibrinogen when measured by manual techniques.[25] Filters to remove residual MB in plasma developed more recently by Pall and Maco Pharma are reported to result in a small increase in the APTT but minimal loss of coagulation factor activity.[26,27] It has been suggested that the increase in the APTT in the latter studies may be a result of some activation of the contact system of coagulation after contact of plasma with the artificial surface of the filter.[26]

Levels of thrombin-antithrombin complexes are not elevated in MB-treated plasma,[16] indicating that MB treatment is also not associated with excessive thrombin generation. Functional measurements of the naturally occurring anticoagulants protein C & S and antithrombin also appear to be relatively unaltered in MB-treated plasma.[10,16,23,28] MB treatment is reported to have little effect on levels of plasminogen, alpha-2-antiplasmin (the main inhibitor of plasmin), fibrin monomer, and D-dimers,[16] suggesting that the use of MBFFP is unlikely to result in enhanced fibrinolysis. vWF activity in plasma, as measured by ristocetin-induced agglutination of platelets, is reduced by 10 to 20 percent,[23,29] but vWF multimeric distribution and cleaving protease activity are reported to be unaffected.[23,28-30]

After transfusion of MB-treated plasma to healthy adults, there was no significant difference from baseline values in APTT, PT, TT, FVIII, FXI, Clauss fibrinogen, fibrin degradation components, or platelet aggregation induced by collagen or ADP, suggesting no major influence on coagulation or fibrinolytic systems.[31]

There have been relatively few studies examining cryoprecipitate and cryosupernatant produced from MB plasma. Levels of FVIII and fibrinogen activity in cryoprecipitate are 20 to 40 percent lower than untreated units[23,32] but remain within Council of Europe Guidelines. The effect on levels of vWF antigen and activity seem more variable: one study reports no significant difference,[23] whereas in a two-center study, one center also reported no change, while the other saw 15 to 20 percent lower values in MB units.[32] These differences might be explained by variation in the methodology used to prepare the cryoprecipitate. However, both studies show that the multimeric distribution of vWF is unaltered. Cryoprecipitate produced from MBFFP has not yet been introduced in any country that provides MBFFP, but work is ongoing in the UK to optimize fibrinogen concentration.[33]

Cryosupernatant produced from standard or MB-treated plasma lacks the largest molecular weight forms of vWF.[23] The main clinical indication for cryosupernatant is for the treatment of thrombotic thrombocytopenic purpura (TTP). Patients with TTP tend to have unusually large molecular weight vWF multimers,[34] which are known to promote platelet aggregation, and some believe that treatment with a plasma component that lacks the high molecular weight forms of vWF may be beneficial. However, no clinical data are available to answer this question. Levels of vWF cleaving protease have not been measured in cryosupernatant produced from MB-treated plasma, but given

that levels appear to be relatively unaltered in the source plasma,[30] one would not expect them to differ significantly. It would thus appear that MB-treated cryosupernatant would be suitable for the treatment of TTP, but it has yet not been manufactured for clinical use.

If MB plasma is used to suspend single-donor platelets, there is no significant effect on platelet numbers, morphology scores, osmotic recovery, or levels of LDH, CD62P expression, lacate, pH, and glucose compared to standard plasma.[35] Similarly, if MB-treated plasma is added to RBCs, there appears to be no appreciable effect on leakage of potassium, hemolysis, or osmotic fragility during 28 days of storage.[35] This is in contrast to direct treatment of RBCs with MB and light, which results in membrane leakage and enhanced surface binding of IgG.[5-7]

PHARMACOLOGY AND TOXICOLOGY

The major clinical application of MB in the past has been as a redox reagent in the reversal of methemoglobinemia and cyanide poisoning using intravenous doses of 1 to 5 mg per kg. It has also been used at higher oral doses for the treatment of manic depression (300 mg/day) and renal calculus disease (195 mg/day). Intravenous doses of 2 to 5 mg per kg have also been used for heparin neutralization and for perioperative staining of the parathyroid gland.[3,4,9,10,20] For comparison, the plasma pathogen-reduction systems described here result in a MB concentration of 1 μ*M* in the FFP, equivalent to an intravenous dose per 250 mL FFP unit of 0.0012 mg per kg. If MB-removal filters are used during processing,[25,36] this level is reduced approximately ×10, to a final concentration of 0.1 to 0.3 μ*M*. For a 70-kg adult receiving the recommended 15 mL per kg of FFP, this equates to a total MB dose of approximately 33 μg, or less than 1 μg in a 2-kg premature infant. Infused MB is rapidly cleared from the circulation and marrow (half-lives in rats are 7 and 18 min, respectively) to an extent that its presence in blood (half-life in man approx. 60 min) is difficult to detect after infusion of MBFFP. There is some tissue uptake, but the majority of MB is excreted via the gastrointestinal tract and in urine within 2 or 3 days[3] (Flament J, Mohr H, and Walker W, written communication, 2000).

A US toxicologic report summarizes its use to assess membrane rupture during amniocentesis, noting mild and transient side effects at most.[37] In mammals, the half lethal dose for MB is of the order of 100 mg per kg, with photo-illumination products having similar, or lesser, toxicity profiles to the parent compound.[3,5] Chronic dosing of animals with MB at doses up to 0.2 g per kg day for 13 weeks are nontoxic. Chronic exposure of rats to a diet containing 4 percent MB had no carcinogenic or cirrhotic effects, while testing in both rodents and Drosophila revealed no genotoxic effects at near lethal doses. Testing for induction of birth defects at doses up to 5 mg per kg per day has also given negative results,[3,5] although recently higher doses have been reported as inducing fetal growth retardation.[38] In contrast to this, in vitro tests, such as the Ames test for mutagenic effect in selected bacteria, have yielded some mutagenic and genotoxic data, particularly in the presence of a liver microsomal (S9) fraction. Testing on human lymphocytes and the mammalian V79 cell line has been reported by some to show no mutagenicity, although in the presence of the microsomal S9 fraction, some chromosomal aberrations were seen in lymphocytes at 1 to 2 μg per mL (Flament J, Mohr H, and Walker W, written communication, 2000). Wagner et al.[39] has reported genotoxic effects in mouse lymphoma cells at 30 μg per mL of MB, which was enhanced by S9 addition, but failed to detect any activity in vivo in a mouse micronucleus assay.

Between 1992 and 1998, more than a million units of MBFFP were used in Germany, Switzerland, Austria, and Denmark. Use has continued in the UK, Portugal, and Spain using the Grifols, Baxter, and Macopharma versions of the technology. The latter two systems have a European Medical Devices licence (CE mark), granting of which includes a toxicologic assessment. Both passive and active surveillance[40] have yielded adverse event rates that do not differ from those for standard FFP. In neonates, where the concern is greater due to the immature detoxification system, there are few reports on surveillance, but data from both Germany and Spain indicate no acute adverse events, even when MBFFP is used for exchange transfusion (Castrillo A, Pohl U, written communication, 1999). Concern over the potential in vitro mutagenic effects of MB and its derivatives, particularly in the presence of the S9 fraction, was the reason for the failure to re-license the product (without MB removal) in Germany in 1998. An opinion has not been reached on whether the system including the MB-removal step will be granted a German license. However, a large amount of clinical usage and in vivo toxicology testing suggest that despite the effects seen in vitro, in vivo side effects are minimal, presumably mainly due to the dilution on infusion and the rapid clearance of the compound. One toxicology expert in the field has suggested the risk is on a par with smoking a pack of cigarettes over a lifetime (Flament J, Mohr H, and Walker W, written communication, 2000).

CLINICAL STUDIES

Most studies in patients have been small and/or have used laboratory rather than clinical endpoints. Despite usage of more than 1 million units in Europe, there have been no full reports of large, randomized trials of MBFFP using relevant endpoints such as blood loss or exposure to other blood components. Early studies described successful use of MBFFP in either single or small groups of patients with

deficiencies of FV or FXI, TTP, and exchange transfusion in neonates.[41,42] One study of 71 patients compared MBFFP with S/D-treated FFP in cardiac surgery and showed better replacement of protein S and alpha$_2$-antiplasmin with MBFFP but no difference in blood loss.[20] However, one hospital in Spain has reported that after a total switch to MBFFP, FFP demand rose by 56 percent, with a two- to three-fold increase in demand for cryoprecipitate, which was not MB treated.[43] The authors suggest that the increase in demand, particularly for cryoprecipitate, may have been required to offset the reduced fibrinogen level in the component. Indeed, after orthopedic surgery, transfusion of MBFFP has been associated with increased reptilase clotting times and ratio of immunologic to functional measured fibrinogen,[44] suggesting that MB may interfere with fibrin polymerization in vivo. However, the data from the Spanish study need to be interpreted with care. In the period studied, which spanned introduction of MBFFP, 2967 patients received no fewer than 27,434 units of plasma, but only 24,607 units of RBCs, with 26 percent of admissions receiving FFP only. The very high FFP to RBC ratio (1.11) contrasts sharply with the recent corresponding figure for the UK Transfusion Services (0.14).[45] This suggests very different prescribing practices for FFP between Spain and the UK, including routine use of FFP in all cardiac surgery procedures in Spain.[43] Nevertheless, their study emphasizes the importance of monitoring clinical demand after any change to MBFFP, to see whether the in vitro effects truly result in a requirement for larger doses.

No specific data are available from studies in neonates, but no specific problems have been found. The only report of MB toxicity in a neonate was a case of severe bullus formation and desquamation was reported in a baby who received phototherapy for hyperbilirubinemia after administration of 10 mL of 1 percent MB to the mother to investigate possible rupture of amniotic membranes.[46] Although neonatal blood levels of MB were not reported, the skin of the baby was visibly stained blue, suggesting blood and tissue levels many times higher than would be achieved after infusion of MBFFP. No problems with MBFFP-treated infants requiring phototherapy have been reported in Europe, and glucose 6 phosphate dehydrogenase deficiency is not a contra-indication to its use (Walker W, written communication, 2002). Similarly, digital capillary measurement of oxygen saturation by colorimetric means is not affected by infusion of MBFFP.

Limited data are available on the use of MBFFP for plasma-exchange procedures for TTP.[47] Although levels of vWF cleaving enzyme in MBFFP are normal,[30] one study of two small cohorts of patients (13 treated with FFP and 7 with MBFFP) reported an increase in the number of plasma-exchange procedures and days in hospital in the MBFFP group.[48] This is of concern, although the small patient numbers make it difficult to draw conclusions; clearly, larger studies are required to establish the role of MBFFP in TTP.

FFP SAFETY: WHERE ARE WE GOING?

Five years ago, an editorial in this journal accompanied the availability in the USA of pooled S/D FFP.[1] Despite the impact of the previous HIV and HCV transmissions on transfusion services in many countries, S/D FFP did not subsequently become a standard of care in the USA, although it has become so in Norway, Belgium and Portugal. Other European countries have chosen quarantining of FFP with donor re-test as their method of minimizing virus risk from FFP. This avoids potential toxicity or loss of activity, but provides no protection against new agents such as West Nile virus. In virus reduction terms, the MB system appears to have acceptable efficacy, and has the advantage of being a single unit system, so that potentially increased risks from new agents unaffected by the system, such as prions, are minimized. The major disadvantage is loss of coagulation factors, such as fibrinogen. The as yet unlicenced single unit psoralen S59 pathogen reduction system for FFP appears to result in much better preservation of fibrinogen, with only 3-13 percent reduction.[49] However, toxicity will be a concern for any pathogen reduction system which interacts with nucleic acids, especially if administered to very young recipients.

In the UK, provision of MBFFP is linked to the most recent Department of Health precautionary decision to minimize the unknown risk of variant CJD from UK blood components. In August 2002, UK Transfusion Services were instructed to seek supplies of US plasma for FFP production for children born after January 1, 1996, a date from which the UK food supply has been considered safe from bovine spongiform encephalopathy. This imported FFP will be subjected to MB treatment, and, in preparation, UK Transfusion Services have already introduced MBFFP for this age group. No immediate problems with side effects or loss of efficacy have been reported, although the number of children treated is still small. Hospitals also have access to S/D FFP from commercial sources.

But to take an overview of FFP safety, 5 years' hemovigilance data in the UK reveal that virus transmission is a much smaller risk than that of TRALI. From 1996 to 2001, there were 15 TRALI cases in which FFP was clearly implicated, and another 4 where FFP was among a range of components transfused. In the same time period, there was not a single proven virus transmission from FFP.[45] Single-unit pathogen-reduction systems by themselves contribute nothing to TRALI prevention, which may be helped by selection of male donors for FFP[50] and/or screening of parous females for WBC antibodies. Interestingly, the pooling of several hundred donations required in the S/D FFP process may provide benefit against TRALI

by diluting out those with high-titer WBC antibodies. The National Blood Service in England has begun a formal option appraisal of TRALI-prevention strategies, beginning with plasma-rich components. The relative cost effectiveness and long-term role of pathogen reduction of FFP in an overall blood safety strategy remain to be elucidated.

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The effect of methylene blue photoinactivation and methylene blue removal on the quality of fresh-frozen plasma

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BACKGROUND: The effects of using fresh or frozen-thawed plasma, WBC reduction of plasma before freezing, and the use of two different methylene blue (MB) removal filters on the quality of MB-treated plasma were compared.

STUDY DESIGN AND METHODS: In a paired study (n = 11/arm) plasma was frozen within 8 hours of collection, thawed, MB photoinactivated, and then filtered using one of two MB removal filters. Fresh plasma (n = 16) and plasma WBC reduced before freezing (n = 19) were MB inactivated.

RESULTS: Freeze-thawing resulted in loss of activity of FXII and VWF of 0.06 and 0.04 units per mL, respectively, but no significant loss of activity of factors II through XI or fibrinogen. Further loss of activity occurred after MB treatment: FII (0.07 IU/mL), FV (0.11 U/mL), FVII (0.08 IU/mL), FVIII (0.28 IU/mL), F IX (0.12 IU/mL), FX (0.16 IU/mL), FXI (0.28 U/mL), FXII (0.15 U/mL), VWF antigen (0.05 IU/mL), VWF activity (0.06 U/mL), and fibrinogen (0.79 g/L). Losses due to this step were significantly (5-10%) lower in fresh plasma compared to frozen-thawed plasma. Neither MB removal filter resulted in significant loss of activity of any factor studied.

CONCLUSION: MB removal, by either of the available filters, has little impact on the coagulation factor content of plasma, but freezing of plasma before MB treatment results in a small additional loss.

Due to stringent donor selection and testing procedures, fresh-frozen plasma (FFP) in the developed world offers a high degree of viral safety. For example, the risk of an infectious FFP donation entering the blood supply in England is estimated to be 1 in 10 million for HIV, 1 in 50 million for HCV, and 1 in 1.2 million for HBV (Eglin R, written communication, 2002). Nevertheless, viral transmission from blood components continues to occur, with 16 cases reported in the UK in the last 6 years.[1] There is, therefore, considerable research activity in pathogen inactivation of single-unit components because methods suitable for single components offer reassurance that no increased infectious risks are added due to pooling. For FFP only, one licensed single-unit system is currently available (methylene blue photoinactivation). It is desirable that there is as much flexibility as possible in the handling conditions for plasma before inactivation, to enable production of FFP from collection centers distant from the processing site. This is particularly relevant because the UK Departments of Health have recently recommended that FFP is imported from North America for neonates and children born after 1995 (after the introduction of relevant food bans to limit BSE transmission) as a precautionary measure against vCJD transmission. Previous studies have

ABBREVIATIONS: APC = allophycocyanin-conjugated; APTT = activated partial thromboplastin time; FFP = fresh-frozen plasma; MB = methylene blue; PMN, = neutrophil; PRP = platelet-rich plasma; PT = prothrombin time; VWF:CB = VWF collagen-binding activity.

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demonstrated vCJD infectivity in plasma of rodents infected with prion diseases,[2,3] and a recent report describes interim results from a study that demonstrate transmissions of bovine spongiform encephalopathy (BSE) and scrapie between sheep by whole-blood transfusion.[4] Because background levels of virus marker positivity in the North American population are significantly higher than in the UK, it has been deemed sensible to subject imported plasma to a pathogen-inactivation step.

The methylene blue (MB) photoinactivation process for viral inactivation of human plasma has been well described[5] as has its effect on the loss of coagulation factor activity of plasma.[5-9] The original Springe MB process, also used by Grifols in Spain, described by Lambrecht,[5] used freeze-thawing of plasma before MB inactivation to expose intracellular viruses to the action of MB. However, recently, blood collection packs that integrate WBC reduction and MB addition before inactivation of plasma (Baxter Pathinact, Baxter Healthcare, Compton Newbury, Berkshire, UK, and Maco Pharma Theraflex, Middlesex, UK) remove the need to freeze-thaw plasma.[10-12] There are also differences between the systems in how MB is added to plasma. With two of the systems (Springe and Baxter), a variable dose of MB solution is added to achieve a standard final concentration of 1 μ*M* MB. The other system (Maco Pharma Theraflex) incorporates an 85-μg pellet of MB hydrochloride per plasma unit, therefore the concentration can vary slightly (0.84-1.13 μ*M*) depending upon the plasma volume (recommended range, 235-315 mL).

For MB photoinactivation of plasma to be centralized, but plasma from remote sites used as a start material, it is essential to be able to freeze and thaw plasma before treatment. Although we have previously evaluated the use of the two systems (Baxter and Maco Pharma) using fresh plasma,[10-12] we have not evaluated freeze-thawing of plasma before MB treatment using such systems. Although it is known that freeze-thawing itself has minimal effect on the coagulation factor activity of plasma,[7] there are no comparative data available on whether the loss of coagulation factor activity due to the MB inactivation step is affected by prior freeze-thawing. Furthermore, in the UK, there was concern that freeze-thawing non-WBC-reduced plasma could potentially increase exposure to vCJD due to fragmentation of platelets and WBCs, which are known to contain normal cellular prion protein (Prp^{c})[13] and might therefore host the infective abnormal prion protein Prp^{sc}. We therefore assessed the effect of removing these cells by an additional WBC reduction step before freezing on the quality of MB plasma.

Following concerns over possible side effects of residual MB in plasma, a further recent development is the ability to remove MB by filtration before final component storage. Evaluations of two removal filters (Pall MB1, Pall Biomedical, Portsmouth, UK, and HemaSure LeukoVir, Marlborough, MA) MB have been previously reported,[14,15] but there are no data available on the use of a new MB removal filter (Maco Pharma Blueflex). The aim of this study was therefore to evaluate the combined effect of WBC reduction before freezing, freeze-thawing, MB photoinactivation, and MB removal using two different filters, on coagulation factor activity and activation markers in FFP. We also examined the effect of freeze-thawing and subsequent filtration of non-WBC-reduced plasma on its cellular constituents, to provide assurance that the process is not likely to increase the risk of vCJD transmission after transfusion to patients.

MATERIALS AND METHODS

Blood collection and processing

The experimental design is shown in Fig. 1. Twenty-four units of whole blood (group A, n = 12; group O, n = 12) were collected into "Top and Bottom" configuration blood packs (Pall Medsep 789-94 U, Pall Biomedical). Blood was then centrifuged (Heraeus Cryofuge 6000, Kleinostheim, Germany) at 3300 rpm for 12 minutes at 22°C and processed to RBCs and plasma (Compomat G4 system, Fresenius-Hemocare NPBI, Abingdon, UK). In Experiment A, plasmas were pooled in groups of two units of identical ABO group into 600-mL transfer packs (Baxter FGR2089, Baxter Healthcare). The pools were mixed thoroughly and divided equally between two 300-mL transfer packs (Fresenius Hemocare P4164, Fresenius Hemocare). All units of plasma were frozen within 8 hours of collection in a freezer (Thermogenesis MP1101, Cheshire, UK) to –45°C within 45 minutes and stored frozen at –40°C for 4 days to 4 weeks. The units were then thawed at 37°C and immediately WBC reduced (PLAS 4, Maco Pharma, Middlesex, UK) and MB photoinactivated (Maco Pharma Maco-Tronic system) as previously described.[11] For each pair of plasmas, MB was removed (either Pall MB1 or Maco Pharma Blueflex) according to the manufacturers' instructions. Plasmas were refrozen in a freezer (Thermogenesis MP1101) and stored at –40°C. In addition, 19 units of plasma were WBC reduced before freezing using one of two filters (RZ2000, Baxter Healthcare, or LPS1, Pall Biomedical), MB inactivated, and then MB removed (MB1 filter, Experiment B). These filters were selected because they are known to have minimal effect on coagulation factors in plasma.[16] A further 16 units of plasma (group O, n = 8; group A, n = 8) were MB inactivated without the freeze-thaw step and MB removed (Blueflex filter, Experiment C).

We took 15-mL samples by sterile connection of a sample pouch at four time points: 1) before freezing, 2) after thawing and before WBC reduction and MB addition, 3) after MB treatment before MB removal, and 4) after MB removal. Samples were frozen at –80°C for coagulation assays, and two aliquots were frozen in EDTA for C3a des arg assays.

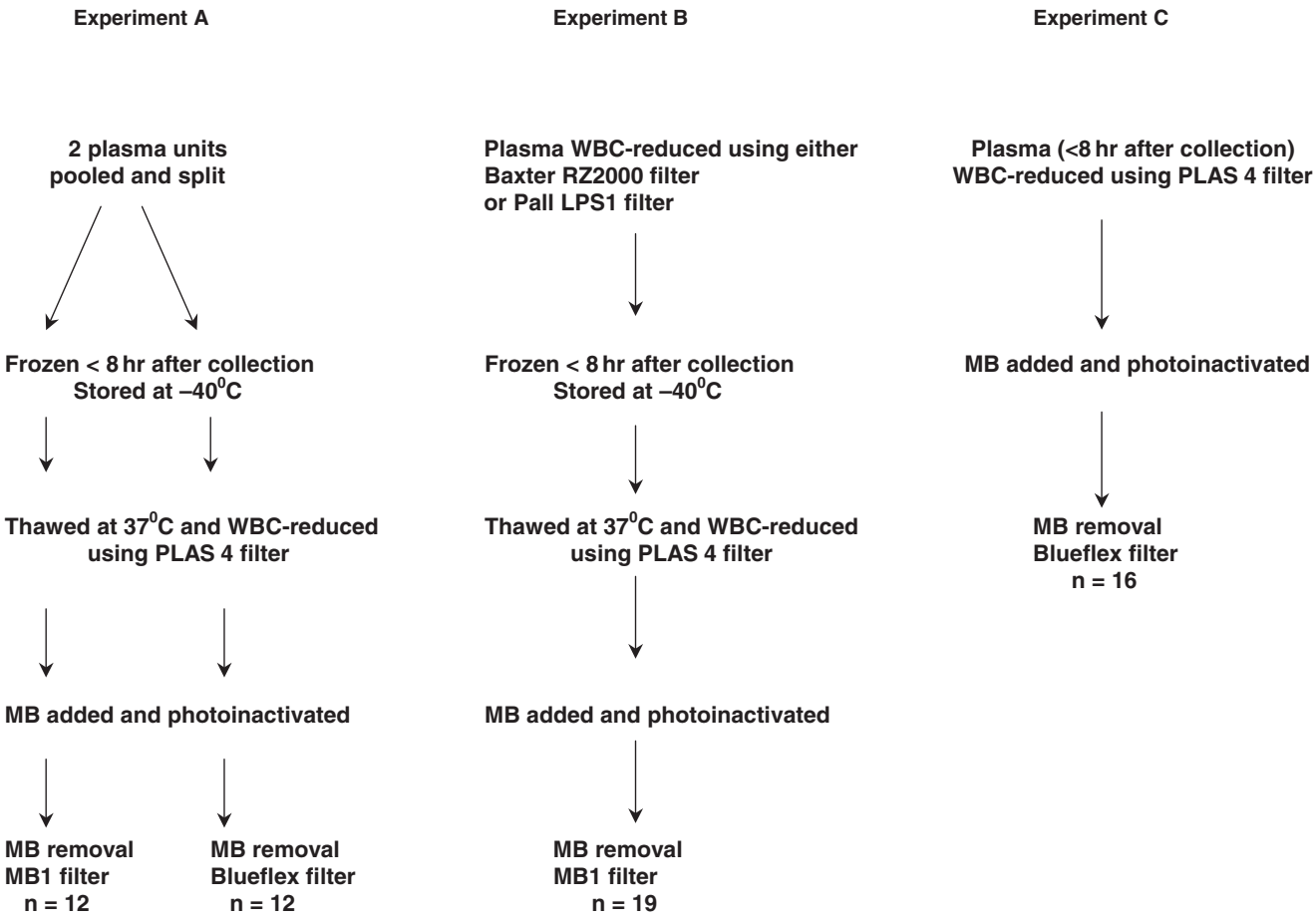

Fig. 1. Study design.

Plasma factors

All coagulation assays were performed using commercially available analyzers (Sysmex CA 1500 analyzer, Sysmex, Milton Keynes, UK; Coagamate X2 analyzer, Organon-Teknika, Cambridge, UK; or Amelung KC 4 A micro analyzer, Sigma Diagnostics, Poole, Dorset, UK). FII, FV, FVII, and FX were assayed by one-stage prothrombin time (PT)-based assays and F IX and FXII using a one-stage activated partial thromboplastin time (APTT)-based assay, using deficient plasma (Dade Behring, Marburg, Germany). The PT and APTT were expressed as a ratio to the geometric mean result of 20 normal citrated plasmas. These types of samples were chosen as "normal" plasma to provide a standard reference point between studies. VWF antigen was measured by latex agglutination (STA Liatest Kit, Diagnostica Stago, Asnieres, France). FVIII and FXI were assayed using one-stage clotting assays with deficient plasma (Diagnostics Scotland, Edinburgh, Scotland; and Sigma-Aldrich Company, Poole, Dorset, UK, respectively). Fibrinogen was measured using a Clauss assay with Fibriquick reagents (Organon-Technika, Cambridge, UK). FVIII assays were standardized using the British plasma standard (NIBSC, South Mimms, UK). All other assays were standardized using Coagulation Reference plasma 100 percent (Technoclone, Dorking, UK). A control plasma of known potency was assayed on each occasion for all coagulation assays.

Commercially available ELISA kits were used to determine levels of prothrombin fragment 1 + 2 (Dade-Behring), FXIIa (Axis-Shield, Dundee, Scotland), and VWF collagen-binding activity (VWF:CB, Immuno, Vienna, Austria). C3a des arg was assayed by radioimmunoassay (Amersham Pharmacia Biotech, Buckshire, UK). VWF cleaving protease activity was measured as previously described[17] and results expressed as a ratio to that of a pooled normal citrated plasma.

Effect of freeze-thawing plasma and filtration steps on cellular content of plasma

Double WBC-reduced plasma (LPS1 filter, Pall Biomedical) was spiked with WBCs (<1-200 $\times 10^6$/L) with or without platelets (<1-100 $\times$ 10^9/L), both of which were prepared from fresh whole blood by density gradient centrifugation,

to represent levels of cellular contamination that may be expected to occur in non-WBC-reduced plasma. Plasma was then blast-frozen, thawed, WBC-reduced by sterile connection with the (PLAS 4) filter, MB added, and MB removed using a filter (the Pall MB1 filter). Samples were taken at four stages: before freezing, after thawing, after PLAS4 LD filter, and after MB removal. Samples were analyzed for platelet count by a hematology analyzer (Sysmex SE9000, Sysmex) and WBC count by flow cytometry (FACSCalibur, Becton Dickinson, Oxford, UK) using LeucoCount reagents (Becton Dickinson). Release of the neutrophil primary granule marker elastase was measured by ELISA of α_1-proteinase inhibitor: neutrophil (PMN) elastase complexes (Pathway Diagnostics, UK) and release of LDH by enzymatic assay in supernatant plasma (Vitros DT60II, Axis-Shield, Dundee, Scotland). RBC microparticles were measured by flow cytometry (FACSCalibur) as previously described using antibodies to glycophorin A.[18]

Analysis of platelet microparticles (PMP) was determined as follows: Plasma (5 µL) was incubated for 20 minutes at room temperature with 5 µL allophycocyanin-conjugated anti-CD61 (APC-CD61, Caltag-Medsystems, Towcester, UK), 5 µL rhodophycoerythrin-conjugated anti-CD42b (Caltag-Medsystems), 10 µL FITC annexin V (FITC-AV, Caltag-Medsystems), 5 µL 10 × HBSS (Sigma, Poole, UK) and made up to 50 µL with HEPES-calcium buffer (2.8 m*M* $CaCl_2$, 20 m*M* HEPES). Samples were resuspended in 0.45 mL of 1 × HBSS and transferred to a tube containing a known amount of beads (Trucount, Becton Dickinson), and analyzed using a flow cytometer (FacsCalibur, Becton-Dickinson). Platelet microparticles were defined using forward scatter as events falling in a region, which includes less than 2 percent of platelets in plasma (PRP) from 20 normal donors, and of less than 1 µm as determined by APC fluorescent beads (Spherotec, Libertyville, IL). Platelet-derived events were defined by fluorescence due to APC-CD61 binding above that of an isotype-matched control. Annexin-V-positive events were defined as events binding FITC-AV above a control containing 5 m*M* Na_3 EDTA. In normal subjects (n = 20), less than 1 percent of unstimulated platelets bind FITC-AV. To control assay variability, a negative control of unstimulated PRP and a positive control (PRP incubated with 10 µ*M* A23187 [Calibochem-Novabiochem, San Diego, CA] for 15 min) were included for platelet microparticle assays.

Effect of MB on assays

To assess the effect of the presence of MB itself on coagulation factor assays, a MB pellet (from the Maco Pharma pack) was dissolved in each of six units of plasma. Samples were collected before and after addition of MB, and once MB had been added, the plasma was not photoilluminated and was protected from light at all times. All parameters were performed as for the main study with the exception that all coagulation assays were performed using a particular analyzer (Sysmex CA 1500 analyzer) and C3a des arg levels were performed by ELISA (Quidel, San Diego, CA).

Statistical analysis

Since the distribution of some data were non-Gaussian with positive skew, nonparametric tests were applied. The Wilcoxon rank sum test was used for paired data and the Mann-Whitney U-test for unpaired data. A p value less than 0.05 was considered significant. All results are given as median with range.

RESULTS

Plasma processing

From the 24 paired units (Experiment A), one unit of plasma fractured on thawing, therefore data on 11 paired units of plasma are presented, all of which were within the required volume range before MB inactivation. Due to sampling, 2 out of 16 fresh plasma units (Experiment C) were slightly below the lower range limit (233 and 234 mL). Filtration time for one WBC-reduction filter (PLAS 4) was 8 to 15 minutes, with a loss of 20 mL of plasma. Filtration times for two other removal filters (Pall MB1 and Maco Blueflex) were 2 to 5 minutes and 5 to 11 minutes, respectively, with a loss of 10 mL of plasma for each.

Effect of freeze-thawing and MB on loss of coagulation factors

The change in coagulation activity due to freeze-thawing and the MB process is shown in Table 1. There was a significant loss of FXII (2%) and VWF:CB (9%), and a small increase in levels of FVII (1%) and FXI (4%) due to freeze-thawing. This was associated with an increase in both PT ratio (1.06 [0.97-1.12] vs. 1.05 [0.95-1.11], $p < 0.0001$) and APTT ratio (1.04 [0.91-1.20] vs. 1.02 [0.89-1.18], $p < 0.0001$). The degree of loss of coagulation activity due to the MB process varied between factors, the highest losses occurring with FVIII (29%), fibrinogen (28%), and FXI (25%), therefore the evaluation of plasma treated fresh was mainly restricted to these factors. For FV, FVIII, FXI, and fibrinogen, the loss of activity due to the MB process was approximately 8-percent higher in the frozen-thawed plasma units compared with fresh plasma (Table 1). In addition, the increase due to this step in both PT ratio (0.09 [0.04-0.16] fresh vs. 0.14 [0.08-0.28] frozen-thawed, $p < 0.0001$) and APTT ratio (0.11 [0.05-0.15] fresh vs. 0.16 [0.11-0.26] frozen-thawed, $p < 0.0001$) was also higher. However, there was no significant difference in changes in levels of FVII, FXIIa, and C3a due to MB treatment between units which were treated fresh or after freeze-

TABLE 1. Percentage change in coagulation factor activity due to freeze-thawing and MB treatment of plasma

Factor	Due to freeze-thawing median (range)	Due to MB treatment + WBC reduction* (freeze-thawed plasma) median (range)	Due to MB treatment + WBC reduction (fresh plasma) median (range)
Number	22	22	16
Fibrinogen (g/L)	–10 (–27 to 14)	–28 (–51 to –20)	–21 (–38 to –7)†
FII (IU/mL)	0 (–4 to 4)	–8 (–11 to –2)	NA
FV (U/mL)	0 (–5 to 4)	–13 (–20 to 4)	–5 (–11 to 4)†
FVII (IU/mL)	1 (–1 to 4)‖	–7 (–10 to –1)	–4 (–9 to –1)
FVIII (IU/mL)	–3 (–20 to 9)	–29 (–42 to –9)	–24 (–37 to –11)†
F IX (IU/mL)	1 (–3 to 4)§	–13 (–20 to –11)	NA
FX (IU/mL)	0 (–2 to 3)	–15 (–22 to –10)	NA
FXI (U/mL)	4 (–19 to 11)‖	–25 (–35 to –7)	–15 (–23 to –6)†
FXII (U/mL)	–2 (–6 to 1)‖§	–18 (–31 to –14)	NA
VWF:Ag (IU/mL)	–1 (–4 to 3)§	–6 (–11 to –3)	NA
VWF:CB (U/mL)	–9 (–17 to 5)‖§	–8 (–16 to 3)	NA
FXIIa (ng/mL)	0 (–25 to 43)	–20 (–43 to 33)	–14 (–43 to 0)
Prothrombin *F*1 + 2 (n*M*)	–19 (–55 to 32)¶	91 (36 to 160)	27 (–12 to 180)‡
C3a des arg (ng/mL)	0 (–41 to 65)	–10 (–45 to 155)	16 (–27 to 295)

* WBC reduction was performed with an integral PLAS 4 WBC-reduction filter in the Maco Pharma MB pack configuration.
† $p < 0.01$ refers to significance from the Mann-Whitney U-test between fresh and frozen plasma.
‡ $p < 0.05$.
§ $n = 11$.
¶ $p < 0.01$ refers to significance from the Wilcoxon rank sum test between plasma before freezing and after thawing.
‖ $p < 0.05$.

thaw. The increase in prothrombin *F*1 + 2 levels due to MB treatment was higher in frozen-thawed units compared to fresh.

When the influence of MB on the assays was studied (in the absence of photoinactivation), there was no significant difference between before or after the addition of MB for any parameters, apart from FXIIa, which was significantly lower after MB addition (1.59 [0.76-2.13] ng/mL before, 0.72 [0.56-1.05] ng/mL after, $p < 0.05$ before vs. after).

Effect of MB removal filters on coagulation activity

To assess the difference between the two MB removal filters, pairs of units were pooled and half of each pool MB-treated and processed through each of the removal filters in parallel. Due to logistical problems, it was not possible to process and assay these two sets simultaneously. A small difference was apparent in levels of some coagulation factors between the two arms of the study, probably due to small differences in processing and storage. However, the percentage change in activity due to freeze-thawing and MB treatment was the same for each arm of the study (data not shown), and therefore these data were combined. To evaluate the effect of MB removal filters, a comparison of pre- and postcoagulation activity for each arm of the study was examined (Table 2). There was no apparent decrease in any parameter studied with either removal filter, apart from a reduction in levels of C3a using one of the filters (Pall MB1). There was a slight increase in levels of fibrinogen, FII, FV, FVII, and FX, using the Pall filter. There was an apparent increase in levels of FXIIa after filtration with both removal filters, which was probably due to the influence of MB on the assay and was comparable between filters. There was an extremely small variation in the PT and APTT ratios with both filters (Table 2).

We compared the final levels of coagulation activity in frozen-thawed MB-treated plasma to a reference range based on 66 samples of WBC-reduced plasma that had not been MB treated. Because there was no loss of activity with either MB removal filter, both sets of data were pooled. The reference data was not collected as part of this study but from previous studies carried out by the National Blood Service over the past 4 years. The methodology used in these studies[16] for either plasma processing or assay did not differ significantly from the current study. Over 90 percent of MB units were within our reference range for all coagulation factors, apart from prothrombin *F*1 + 2 and PT ratio, the MB-treated plasma having 23 percent and 50 percent of values above the range, respectively (Table 3). The range of PT ratios observed in reference and MB-treated plasma is shown in Fig. 2.

We did not evaluate MB removal by the filters used in this study, but previous studies have shown that the MB1 filter removes 81 to 95 percent of MB[14] and the Blueflex filter removes more than 95 percent MB.[19]

Effect of WBC reduction before freezing

WBC reduction of plasma before freezing appeared to have little influence on final levels of FVIII (0.67 [0.44-1.23] WBC reduced vs. 0.62 [0.48-0.86 IU/mL] non-WBC reduced) or fibrinogen (1.88 [1.45-3.24] WBC reduced vs.

TABLE 2. Effect of MB-removal filters on plasma coagulation activity using frozen-thawed MB photoinactivated plasma

	MB1 filter median (range)		Blueflex filter median (range)	
Factor	Before MB removal	After MB removal	Before MB removal	After MB removal
Number	11		11	
PT (ratio)	1.17 (1.11-1.34)	1.07 (1.03-1.24)*	1.23 (1.13-1.33)	1.22 (1.13-1.32)*
APTT (ratio)	1.11 (1.05-1.28)	1.13 (1.06-1.30)†	1.25 (1.17-1.39)	1.27 (1.19-1.43)*
Fibrinogen (g/L)	1.88 (1.30-2.13)	1.93 (1.28-2.27)*	2.04 (1.37-2.13)	1.96 (1.28-2.32)
FII (IU/mL)	0.96 (0.77-1.04)	1.00 (0.78-1.04)†	0.95 (0.77-1.04)	0.97 (0.77-1.04)
FV (U/mL)	0.78 (0.55-0.86)	0.80 (0.56-0.91)*	0.76 (0.58-0.97)	0.76 (0.58-0.88)
FVII (IU/mL)	0.99 (0.79-1.43)	1.02 (0.83-1.55)*	1.01 (0.77-1.40)	1.00 (0.78-1.45)
FVIII (IU/mL)	0.63 (0.47-0.89)	0.62 (0.48-0.86)	0.61 (0.48-0.79)	0.61 (0.46-0.76)
F IX (IU/mL)	0.96 (0.78-1.06)	0.96 (0.83-1.11)		
FX (IU/mL)	0.95 (0.70-1.12)	1.02 (0.75-1.15)*	0.95 (0.72-1.09)	0.96 (0.73-1.07)
FXI (U/mL)	0.77 (0.57-0.99)	0.77 (0.59-0.99)	0.75 (0.55-0.99)	0.71 (0.58-0.78)
FXII (U/mL)	0.88 (0.41-1.07)	0.88 (0.40-1.09)		
VWF:Ag (IU/mL)	0.99 (0.74-1.19)	0.98 (0.75-1.18)	0.92 (0.70-1.09)	0.93 (0.70-1.09)
VWF:CB (U/mL)	0.60 (0.47-0.68)	0.73 (0.42-0.85)	0.70 (0.59-0.87)	0.74 (0.56-0.82)
FXIIa (ng/mL)	1.50 (0.40-2.50)	1.80 (0.70-2.90)*	1.25 (0.75-2.00)	1.50 (1.00-2.25)*
Prothrombin *F*1 + 2 (nM)	0.96 (0.61-1.75)	0.88 (0.76-1.83)	0.74 (0.58-1.19)	0.75 (0.52-1.25)
C3a des arg (ng/mL)	438 (207-1928)	304 (107-431)*	337 (225-1909)	302 (226-1713)

* $p < 0.01$ refers to statistical significance from the Wilcoxon rank sum test between plasma before and after MB removal.
† $p < 0.05$.

TABLE 3. Final levels of coagulation factors and activation markers in freeze-thawed MB-photoinactivated plasma after MB removal

Factor	Final level in plasma*	Reference range†	Units in range (%)
Number	22	66	
PT (ratio)	1.16 (1.03-1.32)	1.05 (0.95-1.16)‡	50
APTT (ratio)	1.24 (1.06-1.43)	1.09 (0.86-1.36)‡	95
Fibrinogen (g/L)	1.95 (1.28-2.32)	1.10-4.30	100
FII (IU/mL)	0.98 (0.77-1.04)	0.70-1.20	100
FV (U/mL)	0.79 (0.56-0.91)	0.50-1.40	100
FVII (IU/mL)	1.01 (0.78-1.55)	0.60-1.40	100
FVIII (IU/mL)	0.62 (0.46-0.86)	0.40-1.60	100
F IX (IU/mL)	0.96 (0.83-1.11)§	0.60-1.40	100
FX (IU/mL)	1.01 (0.73-1.15)	0.70-1.30	100
FXI (U/mL)	0.75 (0.58-0.99)	0.60-1.30	91
FXII (U/mL)	0.88 (0.40-1.09)§	0.40-1.50	100
VWF:Ag (IU/mL)	0.96 (0.70-1.18)	0.60-1.65	100
VWF:CB (U/mL)	0.74 (0.42-0.85)	0.50-1.50	91
FXIIa (ng/mL)	1.50 (0.40-2.90)	0.50-5.00	100
Prothrombin *F*1 + 2 (n*M*)	0.85 (0.52-1.83)	0.20-1.10	77
C3a des arg (ng/mL)	303 (107-1713)	1117 (0-12,330)	100

* Data (n = 22) are represented by the median (range) from plasma MB-removed by MB1 filter (n = 11) and Blueflex filter (n = 11).
† Reference range of normal plasmas is defined as the mean ± 2 SD for normally distributed data and the geometric mean with 95-percent CI for skewed data based on WBC-reduced FFP. Percentage of units in range is defined as above the lower limit for coagulation factors and below the upper limit for PT, APTT, and activation markers.
‡ n = 100.
§ n = 11, using MB1 filter only.

1.70 [1.24-2.31 g/L] non-WBC reduced) in plasma subsequently MB treated and removed using the MB1 filter. VWF cleaving protease (VWF:CP) activity was measured in four MB-inactivated plasmas, which were WBC reduced before freezing and MB-removed using the MB1 removal filter. VWF:CP results ranged from 0.81 to 1.00 (normal range, 0.80-1.20 in citrated plasma). We have not assessed VWF:CP activity in plasma MB-depleted using the Blueflex filter. Quality-monitoring data from routinely processed units (n = 225), WBC-reduced before freezing, MB-treated, and MB-removed using the MB1 filters, showed a mean volume of 242 mL (SD = 19) and FVIII content of 0.79 IU per mL (SD = 0.25). These results comply with UK specifications for MB-treated FFP.[20]

Effect on cellular content of plasma

When platelets were spiked into plasma, there was no consistent difference between levels measured before and after freeze-thawing when measured by hematology analyzer, but levels were consistently lower after thawing when measured by flow cytometry (Table 4). When WBCs were spiked into plasma, there appeared to be a trend for lower WBC counts and higher levels of α_1-proteinase inhibitor: PMN elastase complexes in plasma after freezing (Table 4). However, in the absence of platelets, levels of LDH did not increase substantially. Freeze-thawing of plasma resulted in an increase in levels of platelet microparticles as well as microparticles characterized by the binding of purified annexin V (Table 4). Platelet microparticles were reduced to levels observed in fresh WBC-reduced plasma or below after WBC reduction of frozen plasma with the PLAS 4 filter. However, a significant proportion (~30%) of microparticles characterized by annexin V binding were not removed by the WBC-reduction or the MB1 removal step. These also appeared to be derived solely from plate-

lets because levels after WBC reduction in samples spiked with WBCs in the absence of platelets were not different from WBC-reduced plasma alone. However, even at an added platelet count of 30×10^9 per L (Sample D, the current UK specification), the number of annexin V-positive microparticles in frozen-thawed plasma subsequently filtered using the PLAS 4 filter (43×10^9/L) is similar to that seen in our current routine WBC-reduced non-MB-treated plasma product (mean residual platelet count of 3 $\times 10^9$/L).

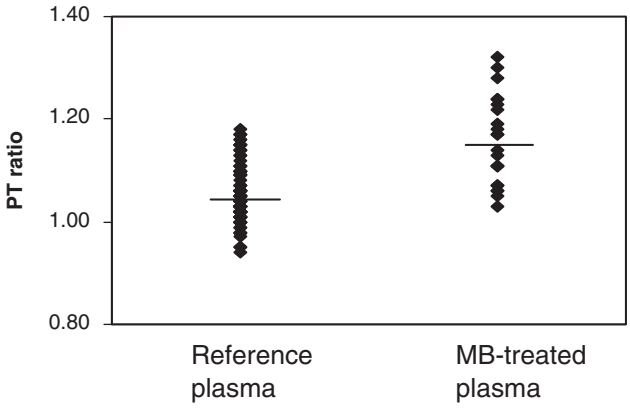

Fig. 2. PT ratio in MB-treated (n = 22) or reference plasma (n = 100). For MB-treated plasma, MB was removed by MB1 filter (n = 11) and Blueflex filter (n = 11). Reference plasma is historical data from WBC-reduced FFP. Horizontal bar represents the median value. The PT is expressed as a ratio to the geometric mean result of 20 normal citrated plasmas.

Before freezing, levels of RBC microparticles were 8 (5-12 $\times$ 10^9/L), increasing by 33 percent after freeze-thawing. This was not related to platelet or WBC content, and levels after WBC reduction were below the detection of the assay system used (data not shown).

DISCUSSION

MB treatment of plasma has been shown to inactivate 4 to 6 logs of transfusion-transmitted viruses, including HIV, HBV, parvovirus B19, and West Nile virus.[5,21,22] Original studies on MB inactivation were reported on plasma freeze-thawed before treatment.[5,7] Later, work on other systems (Baxter Pathinact and Maco Pharma Theraflex systems) was performed on fresh plasma.[10-12] However, there are no data available on the difference between using fresh or freeze-thawed plasma as a starting component for MB treatment. In our study, freeze-thawing of plasma resulted in a small loss of FXII and VWF:CB activity

TABLE 4. The effect of freeze-thawing, WBC reduction, and MB removal on cellular components of plasma

	Spike*									
	A	B	C	D	E	F	G	H	I	J
Platelets – HA† (10^9/L)										
Before freeze	<3	<3	10	31	108	<3	<3	<3	<3	<3
After thaw	<3	6	10	32	97	<3	<3	<3	<3	<3
Platelets – FC‡ (10^9/L)										
Before freeze	<0.5	5.9	10.8	32.0	125.1	<0.5	<0.5	<0.5	<0.5	<0.5
After thaw	<0.5	4.8	9.0	24.6	92.2	<0.5	<0.5	<0.5	<0.5	<0.5
WBCs (10^6/L)										
Before freeze	<1	8.9	29.6	90.7	233.8	<1	1.8	14.1	50.6	92.9
After thaw	<1	9.1	28.1	94.0	210.9	<1	1.6	13.2	43.4	89.5
PMN elastase (μg/L)										
Before freeze	46.4	39.7	37.5	46.3	57.0	20.6	23.7	24.0	25.3	25.7
After thaw	37.5	35.8	42.2	53.5	79.8	22.8	23.1	30.4	43.2	58.8
After WBC reduction	34.5	35.3	40.8	57.4	77.4	21.1	20.5	27.0	41.2	53.5
After MB1 filter	35.1	37.0	45.1	58.2	71.9	18.1	21.3	24.8	37.9	57.9
Platelet microparticles (10^9/L)										
Before freeze	0.2	0.2	0.4	0.5	1.0	0.3	0.5	0.4	0.3	0.9
After thaw	0.1	0.5	0.7	2.6	10.1	0.1	0.1	0.0	0.3	0.0
After WBC reduction	0.0	0	0	0.1	0.2	0	0	0	0.1	0
After MB1 filter	0.1	0.0	0.1	0.1	0.2	0.1	0.0	0.1	0.0	0.3
Annexin V +ve microparticles (10^9/L)										
Before freeze	6	7	8	10	13	4	4	4	5	4
After thaw	12	31	54	138	574	9	9	9	13	13
After WBC reduction	3	10	20	46	198	5	2	3	5	5
After MB1 filter	3	8	15	43	196	4	4	4	4	8
LDH (U/mL)										
Before freeze	393	400	423	476	732	390	381	388	376	382
After thaw	384	404	434	523	796	388	386	385	393	395

* WBC-reduced plasma units were spiked with WBCs alone (Samples G-J) or WBCs and platelets (Samples B-E) to the concentrations shown in the before freeze rows. Samples A and F were not spiked. Plasma was frozen-thawed, WBC reduced using the PLAS 4 filer; MB added and MB removed using the MB1 filter. Results are from a single experiment. Platelets and WBCs were not detectable following WBC reduction.
† FC-flow cytometry.
‡ HA-haematology analyser.

as well as a minor prolongation of PT and APTT. Interestingly, there was an apparent increase in activity of FVII and FXI on freeze-thawing, presumably reflecting small changes in the activation status of these factors. However, other factors studied remained unchanged. This agrees with the work of Zeiler et al.,[7] who showed that the loss of coagulation activity in MB-treated plasma was mainly attributable to the MB photoinactivation step rather than freeze-thawing of plasma. The loss of activity observed in frozen-thawed units due to MB photoinactivation in our study was similar to that previously reported.[5,7] For the variables we studied, with the exception of FVII, loss of coagulation factor activity due to the MB-inactivation step (including the WBC reduction filter) was 8-percent higher when frozen-thawed plasma units were used rather than fresh. In addition, the increase in prothrombin *F*1 + 2 levels after MB inactivation and WBC reduction was higher in units frozen-thawed compared with fresh plasma, indicating a higher degree of thrombin generation. This was not associated with an increase in FXIIa. Because our MB process includes an integral WBC-reduction step, we cannot determine whether the differences seen between fresh and frozen-thawed plasma are attributable to the WBC-reduction or MB process. The WBC-reduction filter used in the MB packs has previously been shown to have minimal effect on coagulation activity (unpublished data) using fresh plasma, but this could be different for frozen-thawed plasma.

We also sought to compare the effect of two different types of MB removal filters (Maco Pharma Blueflex or Pall MB1 filter) on plasma factor activity. Neither filter resulted in loss of any variable studied. Both MB removal filters resulted in a small increase in the APTT ratio, which might be attributable to contact activation of plasma with the filter. This is difficult to assess because although both filters increased FXIIa antigen, this assay is influenced by MB. However, levels of FXIIa antigen in the final MB-removed component were not higher than untreated plasma units. The filtration times for one filter (Maco Pharma Blueflex) were longer compared with the other (Pall MB1) (5-11 vs. 2-5 min, respectively), but the loss of plasma was equivalent for both filters. However, there did not appear to be any difference between the two filters in terms of activation of the contact system or thrombin generation as evidenced by the generation of FXIIa or prothrombin *F*1 + 2. Our results using the MB1 filter compare well with that previously published,[14] showing minimal loss of clotting factor activity. However, there was an apparent increase in levels of fibrinogen and PT-derived coagulation factors after filtration with the MB1 filter, which was associated with a small decrease in the PT ratio. We cannot explain these results because these assays do not appear to be influenced by the presence of MB, but they could possibly be a result of small increases in the activation state of coagulation factors. The changes in coagulation factors observed after filtration with either MB-removal filter appear to be clinically insignificant. Neither MB-removal filter resulted in generation of C3a des arg, a marker of complement activation. However, levels after filtration were reduced using the Pall MB1 filter. Whether this has any clinical benefit in terms of acute reactions is unclear.

As well as examining the loss of coagulation factors during MB photoinactivation, we compared residual levels in the final component with reference ranges based on previous studies of nontreated FFP in our laboratories. Despite the observed losses of coagulation factors due to MB treatment, final levels of all coagulation factors in frozen-thawed, MB-treated, and removed FFP were above the lower limit of the reference range in over 90 percent of units. However, over 50 percent of units were above the upper reference range for PT ratio. This presumably reflects the loss of fibrinogen and FII, FV, FVII, and FX because the PT is dependent upon these factors. However, the PT ratio of all units was less than 1.35. In addition, 23 percent of units had levels of prothrombin *F*1 + 2 higher than the upper limit of the reference range, reflecting the increase in prothrombin *F*1 + 2 seen due to the MB process. The clinical significance of increased *F*1 + 2 levels is unclear, but values higher than those observed in our study are seen in S/D-treated plasma.[23] We did not assess the effect of MB treatment or removal on plasma inhibitors of coagulation. However, MB treatment is reported to have minimal effect on levels of antithrombin, α_2-antiplasmin and protein C & S.[5,7,11] After MB removal (Pall MB1 filter), levels of antithrombin and protein C & S are within the normal range.[14]

We also evaluated the addition of a WBC-reduction step before freezing plasma, which did not appear to augment the loss of fibrinogen and FVIII activity. VWF:CP activity in plasma that was WBC reduced, MB inactivated, and removed using the MB1 filter was also within reference ranges established by other laboratories, suggesting that these processing steps do not have a major effect on VWF:CP using the techniques employed. These results are consistent with our previous findings on MB-treated fresh plasma[24] and others results on frozen-thawed plasma.[25] However, we cannot exclude small losses of activity given the relatively small number of samples used in this study.

In the UK, all blood components are WBC-reduced before storage. However, for logistical reasons we also wanted the flexibility to freeze non-WBC-reduced plasma intended for MB treatment, as a subsequent WBC-reduction step is integral to the Maco Pharma MB process. We were concerned that freeze-thawing may result in reduced cell removal by the WBC-reduction filter or cause fragmentation of cells with the potential for increasing the risk of transmission of vCJD. Most cellular prion protein in blood, used here as a surrogate marker for the potentially infective abnormal prion protein, is associated with