REVIEW

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

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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 $1\,\mu M$, 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 $0.3\,\mu M$. 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

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 proto-zoal 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

Methylene blue homologues

Fig. 1. MB and its photodegradation products.

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
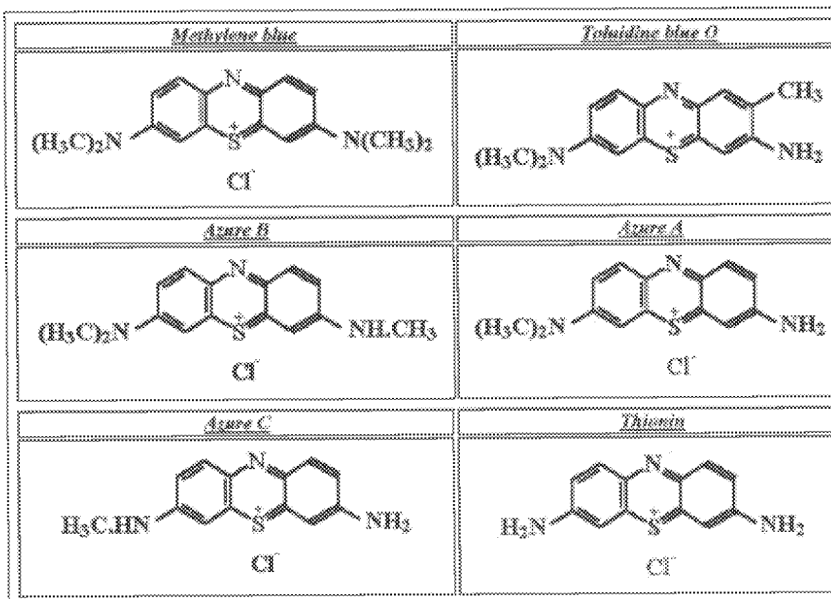
TABLE 1. Virus reduction			
Lipid enveloped		Non-lipid enveloped	
Virus	log reduction factor	Virus	log reduction factor
HIV	>5.5	HAV	0.0
Bovine viral diarrhea	>6.2	Encephalomyocarditis	0.0
Duck HBV	3.9	Porcine parvovirus	0.0
Influenza	5.1	Polio	0.0
Pseudorabies	5.4	SV40	4.3
Herpes simplex	>6.5	Adenovirus	4.0
Vesicular stomatitis	>4.9	Human parvovirus B19	≥4.0
West Nile virus	>6.5	Calicivirus (HEV)	>3.9

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 sero-converted 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.

The MACO PHARMA Plasma Membrane filtration Methylene Blue Illumination and MB Depletion Set
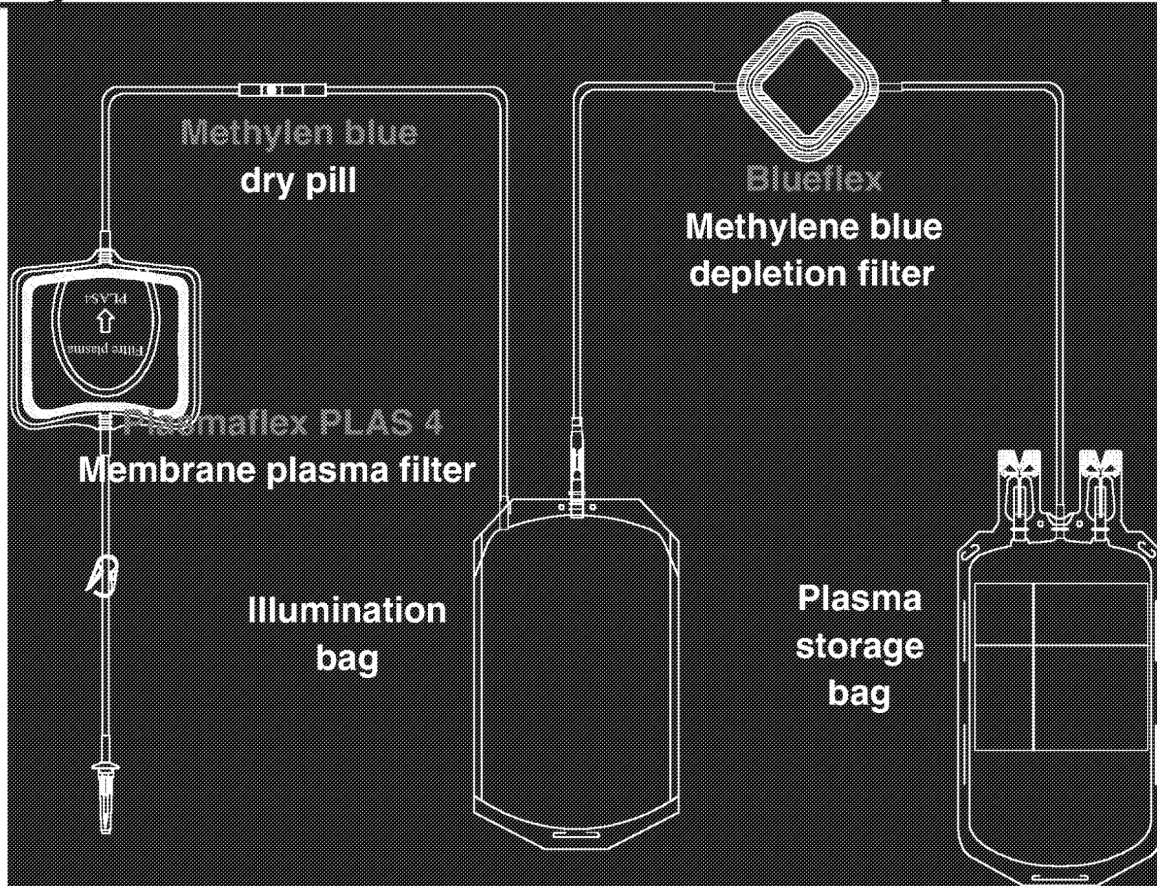

Fig. 2. Schematic representation of the closed bag system for MB treatment of fresh-frozen plasma.

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]
α₁-antitrypsin (U/mL)	→[16]	155 mg/dL
Plasminogen (U/mL)	→[10,16]	0.90,[10] 0.98[16]
α₂-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 $1 \mu M$ 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 $\times 10$, to a final concentration of 0.1 to 0.3 μM. 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 alpha₂-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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