マコファルマ社回答

平成20年4月8日開催　血液事業部会運営委員会・安全技術調査会合同委員会後
追加質問事項2（各社共通）

1. EU主要国等、各社の不活化技術を導入しているそれぞれの国における供給の実態。輸血用血液製剤の全供給数（不活化技術を適用している製剤と適用していない製剤の合計）、そのうち不活化技術を適用している製剤の割合は何%か。また、供給はどのように行われているか（供給先、医師の希望により供給できるのか等）。

回答

国	血漿製剤不活化の義務化	マコファルマ製品により処理された血漿製剤（本数）	マコファルマ製品により処理された血漿製剤（%）	不活化処理された血漿製剤（%）
アルゼンチン	×	2,000	—	—
ベルギー	○	70,000	100	100
ブラジル	×	2,000	—	—
フランス	○（将来的に）	150,000（2008 年予測）	60（2008 年予測）	100（2008 年予測）
ギリシャ	×	8,000	5	—
イタリア	×	34,000	5	25
マレーシア	×	300	—	—
ロシア	×	17,000	1	1
シンガポール	×	1,000	—	—
スペイン	×	105,000	44	69
イギリス	×	9,000	2	2

ベルギー及びフランスにおいては国立輸血サービス（National Transfusion Service）が血漿のみの不活化製剤を提供している（ベルギーでは 2004 年から、フランスでは 2009 年までの移行が予定されている）。その他の全ての国においては、医師並びに病院が使用する血漿製剤（不活化製剤及び検疫製剤）を自由に選択することが可能である。しかしながら欧州においては、血漿製剤並びに血小板製剤の不活化傾向が顕著である。

2. 各社の不活化技術を適用する前の製剤に白血球除去を行った場合と、不活化技術を適用した製剤に白血球除去を行わなかった場合を比較して、GVHD等の副作用の推

移はどのようになっているか。

回答

欧州全ての国において赤血球製剤並びに血小板製剤の白血球除去は、長年に亘り義務化されている。白血球除去の導入後、輸血由来による熱発例数は著しく減少した(M. M. Mueller et al., "Clinical impact of leucocyte depletion – what is the evidence?"参照)。GVHD の予防に関して、輸血製剤は依然としてガンマ線照射されている。しかしながらマコファルマ社製 THERAFLEX UVC を含む血小板製剤不活化技術は、ガンマ線照射の代替になり得ると期待されている。

3. EU主要国における不活化技術導入後不要になった技術(白除、NAT等)

回答

不活化技術が採用されたいくつかの国においては、検疫保管期間が省略された。その結果、より良好な流通、保管スペースの節約及び迅速な血漿製剤供給につながった。メチレンブルー処理の導入に伴い白血球除去並びに既存の検査を省略した国は無い。一方でメチレンブルー処理の導入により新たな検査の追加及び既存の検査感度を向上させる措置(シングル NAT 等)は行われていない。

血漿製剤 :

欧州において、キットに採用されている 0.65μm のフィルターは血漿製剤用白血球除去フィルターより効率が良いため、THERAFLEX MB-Plasma 処理は血漿製剤の白血球除去の代替となる。

血小板製剤 :

THERAFLEX UVC-Platelet 処理により、将来的にガンマ線照射並びに細菌スクリーニング検査の省略が期待される。現在の段階では、ウイルス NAT 検査の省略は推奨しない。

4. EU主要国における不活化技術を適用している製剤の市販後調査の実態(調査数)

回答

市販後調査は、欧州各国に導入されているヘモビジランス及び輸血サービス機関(Blood Transfusion Services)により実施されており、イギリス並びにフランスが多くの経験を有している。全ての欧州諸国がメチレンブルー処理された血漿製剤を含む全ての血液製剤に対するヘモビジランスプログラムを実施している。更に、既報の通り追跡評価の実施(フランスにおけるメチレンブルー処理血漿製剤の四相臨床治験、等)あるいは Politis 等による臨床使用経験(Vox Sanguinis, [2007];Volume 92, Issue 4, Pages: 319-326)が発表されている。

(参考)マコファルマ社回答(英語)

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Tokyo, JAPAN, 100-8916

Your Reference: Your Message of: Our Reference: Dr.W./AH Date: April 30th, 2008

Committee questions from April 24th, 2008

Dear Sirs,

following you will find answers to the questions raised by you on the Theraflex MB Plasma on April 24th, 2008.

1. **Actual conditions of blood products supply in each country, where the inactivation has been implemented**
   (*Please specify the total number of blood products supply with breakdown of inactivation processed and non-processed blood products in %. Also, describe, whether all supplied blood products must be processed mandatory or user (physician or hospital) can chose one).*

   ANSWER:

   Supply of pathogen reduced blood products by countries:
   In our letter of 31.3.2008 to you, we indicated in Table 1) the list of countries, where the Theraflex MB Plasma has been registered and used in clinical routine. Below you will find a table of the countries with the actual quantities of plasmas treated in 2007 and the percentage of the total therapeutical plasma units treated by Theraflex and/or by other methods.

Geschäftsführer:
Peter Kremmers
Amtsgericht Offenbach HRB 33620
UST-ID-Nr. DE 119 109 470

Deutsche Bank AG
BLZ: 505 700 18 · KTO. 781 777 800
BIC: DEUTDEFF505
IBAN: DE46505700180781777800

Dresdner Bank
BLZ: 500 800 00 · KTO. 926 000 00
BIC: DRESDEFF
IBAN: DE71500800000092600000

Country	Inactivation for plasma mandatory	Quantity of plasmas treated with Theraflex MB plasma	% of Theraflex to therapeutical plasma	% of inactivated plasma to therapeutical plasma
Argentina	No	2000	-	-
Belgium	Yes	70000	100	100
Brazil	No	2000	-	-
France	Yes (in future)	150000 (in 2008)	60 (in 2008)	100 (in 2008)
Greece	No	8000	5	-
Italy	No	34000	5	25
Malaysia	No	300	-	-
Russia	No	17000	1	1
Singapore	No	1000	-	-
Spain	No	105000	44	69
UK	No	9000	2	2

In Belgium and France the National Transfusion Services offer only pathogen reduced plasma products (in Belgium since 2004, in France conversion is planned by 2009).
In all other countries the physicians and hospitals are still free to use their plasma (pathogen treated or quarantine plasma) by themselves. However, the trend in Europe goes clearly to pathogen reduced plasma products and pathogen reduced platelet products.

2. **Leukocyte reduction:**
The trend of blood transfusion related side effect occurrence rate, such as GVHD, with inactivation processed with leukocyte reduction and without leukocyte reduction.

ANSWER:
Please note, that leucodepletion of all red cells and platelets is mandatory in Europe for all countries since many years.
The number of febrile transfusion reactions has decreased since significantly after Introduction of universal leucodepletion (see f.ex. *M. M. Mueller et al.*, "Clinical impact of leucocyte depletion – what is the evidence?"; Science series (2008), 3: 85-90).
To avoid GvHD, blood products are still gamma-irradiated. However, it is expected, that platelet pathogen reduction technologies, including the THERAFLEX UVC-treatment by MacoPharma, will substitute gamma-irradiation of platelet concentrates.

3. **Test:**
The existing tests and/or processes, such as leukocyte filtration and NAT, which can be omitted after inactivation implementation.

ANSWER:

In the different countries where the process is implemented the quarantine storage is omitted. This leads to be,tter logistics, reduced storage space, and immediate provision of the plasma.
Neither leukocyte filtration nor any at the time of implementation of the MB procedure used test for infection markers was stopped in any country. Nevertheless, the implementation of new tests or tests with enhanced sensitivity, like single NAT was also not done after introduction of the MB procedure.

Plasma:
In Europe the THERAFLEX MB-Plasma Procedure substitutes leucodepletion of plasma, since the membrane 0.65 µm filter is more efficient than a plasma leucodepletion filter.

Platelets:
We expect, that the THERAFLEX UVC-Platelet Procedure will substitute in the future both gamma-irradiation as well as bacterial screening.
We do not recommend any substitution of viral NAT-Testing for the moment

4. **Post marketing surveillance:**
(*Specify the actual condition of the PMS including total number in each country):*

ANSWER:
Post market surveillance is done by Blood Transfusion Services and by Hemovigilance procedures installed in all European Countries with most experience available in the U.K. and in France.
All European countries have ongoing Hemovigilance programmes for all components, which includes MB-treated plasma.
Additional, we have follow-up studies (like Phase IV Clinical Study on MB-Plasma In France) or Publications on clinical use of *Politis et al.* [Vox Sanguinis, (2007); Volume 92,Issue 4, Pages: 319-326], as already mentioned in our last letter to you dated 31.3.08, under point 4.b).

We do hope to have answered clearly to all your questions arising on April 24th, 2008.
If something is still unclear, please do not hesitate to approach us.

Sincerely yours,

MacoPharma International GmbH

Dr. Wolfram H. Walker
- Scientific Director -

Dr. Stefan Reichenberg
- Project Manager Pathogen Inactivation -

Copy: - Mr. Gus Ribeiro, General Manager, MacoPharma Asian / Pacific Region;
- Mr. Hirotaka Nagase, AMCO Inc., Tokyo / Japan;
- Mr. Kenzo Watanabe, AMCO Inc., Tokyo / Japan

BCT Japan 株式会社回答

4月8日運営委員会・安全技術調査会合同委員会後

追加質問事項2(各社共通)

1. EU主要国等、各社の不活化技術を導入しているそれぞれの国における供給の実態。輸血用血液製剤の全供給数(不活化技術を適用している製剤と適用していない製剤の合計)、そのうち不活化技術を適用している製剤の割合は何%か。また、供給はどのように行われているか(供給先、医師の希望により供給できるのか等)。

さまざまな病原体不活化技術 Pathogen Reduction Technologies(PRT)が開発されており、ヨーロッパでは現時点で通常使用されたり、さまざまな評価段階であったりしています。

ー3 種類の血漿処理のできる PRT 処理方法が現在市場で販売されています。
Solvent Detergent 処理(1990 年代初期からいろいろな国で既に使用中)
Methylene Blue(2001 年以降市場に出て、フランス、スペイン、イタリア、英国で通常使用)
Intercept Blood System(2006 年以降 CE マーク取得:通常使用についてはは未確認)
Milasol PRT System は、2008 年に血漿処理で CE マークを取得予定です。

ー2 種類の血小板処理のできる PRT 法が現在販売されています。
Intercept Blood System(2002 年の CE マーク取得以降、主にフランス、スペイン、ベルギー、ノルウェーとスウェーデンの血液センターで限定的に使用中)
Mirasol PRT System(2007 年後半に CE マーク取得済み。現在アイルランド、スペイン、イタリアと中東諸国のいくつかの血液センターで通常使用に向けた評価試験を実施中)

全体として、ヨーロッパでは輸血に使用されるすべての血小板製剤の 5%に PRT 処理が施されており、これらの製剤への PRT の使用は明らかに増加傾向があると我々は考えます。とりわけ血小板製剤について高い関心および使用を予定している国々には、フランス、ベルギー、アイルランド、スペイン及び中東諸国があります。

PRT 処理済みの製剤の供給および配送に関しては、PRT 処理方法の違いにより物流方法も異なります。Mirasol PRT での血小板と血漿の処理は短時間(処理時間は 15 分以内)で、しかも簡単に処理ができることから、血液センターの通常の血液成分製剤の製造

工程の一部として組み込むことができます。 血小板の出荷直前でも PRT 処理が可能であり、血液センターは PRT 処理済みの製剤を医療機関からの需要に合わせて供給することが可能になります。

病原体不活化技術(PRT)の幅広い利益を考えますと、すべての患者様は PRT 処理済みの血液製剤から利益を享受するものと考えます。したがって、最終的にはすべての血液製剤が不活化処理されることでしょう。しかし、専門家の先生方によれば、各病院には患者様のために PRT 処理された製剤を求める緊急度、もしくは求める能力に違いがある場合があるようです。そのため各血液センターは、当面各病院のニーズに合わせて血小板の在庫の一定の部分にのみ PRT 処理を行う必要があるでしょう。

2. 各社の不活化技術を適用する前の製剤に白血球除去を行った場合と、不活化技術を適用した製剤に白血球除去を行わなかった場合を比較して、GVHD等の副作用の推移はどのようになっているか。

私達の知る限りでは、輸血後GVHD予防に関連して白血球除去対非白血球除去製剤の不活化技術の有効性について、直接比較したヒューマンデータはありません。Mirasolで不活化処理したヒトの白血球(単核球)を、遺伝的に処理した免疫不全マウス(輸血後GVHDのモデルとして)に注入した動物実験の結果は、白血球除去が施されていない血液製剤に適用された場合、輸血後GVHDの予防においてMirasol不活化処理が有効であることを間接的に示しています。
さらに、PRTの功績に関する最近の出版物の中で、Dr. H. Alter(米国 国立衛生研究所)は、PRTを実施する場合、白血球除去を行わないという選択はコスト削減の戦略の一つとなり得る、と示唆しています。白血球除去が施されていない製剤に適用されるMirasol不活化技術のさらなる確立に向けて、様々な研究が現在計画されています。

既に白血球除去が施された製剤にPRT病原体不活化技術が適用される場合の輸血後GVHDの予防という観点では、実際の臨床経験は既にInterceptの研究で示されており、Mirasol PRTにおいても同様に入手可能となりつつあります。MIRACLE(Mirasol臨床評価)、これはランダム比較臨床試験ですが、その一環で、Mirasol処理された製剤の大多数(160/168[4])は輸血前にガンマ線照射は施されておらず、輸血後GVHDの危険性があると見なされた患者グループでは、リファレンスの輸血製剤(Mirasol処理されていない製剤)の大多数(122/166)にガンマ線照射が施されています。どちらの患者グループからも輸血後GVHDは報告されておりません。MIRACLEトライアルの最終結果は、今年の後半に提示される予定です。

3. EU主要国における不活化技術導入後不要になった技術(白除、NAT等)

この 20 年間もしくはそれ以上の年月の間に、血液安全性を主導する技術は数多く導入されてきました。その技術とは、ガンマ線照射、白血球除去、新しいマーカーに対する血清学検査、現存のマーカーに対する改良された血清学検査、NAT、バクテリア・スクリーニングです。一度検査が導入されると、通常はその検査を撤廃することはできません。一つの例外は P24 抗原分析の撤廃で、HIV 検査用の NAT 導入に伴い FDA により許可されました。これは、大規模な多施設臨床試験での入念な分析により達成されました。

この 20 年余りの間に導入されたこれら技術の多く、あるいは幾つかの技術は、長い年月をかければ、PRT により置き換えることは可能と考えられます。血液の安全性および安定供給に関するアドバイザリーコミッティー(米国　保健社会福祉省)は、ガンマ線照射とバクテリア検査はその候補になり得ることを示唆しています。さらに、過去 10 年以上の間に、NAT 検査のプールサイズは著しく減少し、10 以下となりました。全ての血液製剤において PRT が一般的に導入されると、各国政府はコスト削減に関連して、NAT 検査のプールサイズを以前のような大きさに戻すことを考慮し始めるかもしれません。

技術の撤廃や修正を行っていくにあたり、市販後調査や臨床試験の実施は必要となりましょう。

4. EU主要国における不活化技術を適用している製剤の市販後調査の実態(調査数)

Navigant Biotechnologies LLC 社と Gambro BCT 社は、Mirasol PRT 処理を行った製品の輸血の安全性を継続的にモニターするという市販後調査の実施を約束しています(CE マークの承認の一部として)。2007 年 11 月にヨーロッパ、中東各国とアフリカで Mirasol PRT System が公式に導入されて以降、各施設で Mirasol PRT 処理を行った 100 以上の血小板製剤の輸血に関しての安全性データが収集されており、今後もこの活動は継続されます。さらに、輸血業務における Mirasol PRT 処理製剤の臨床使用に関する重要なデータを記録することを目的に、電子データ収集システムが開発されました。最後になりましたが、我々は Mirasol の不活化処理に関心の深い国々の政府機関と協力し、各国の必要に応じた適切な安全性のモニタリングが確実に実施されるよう作業をすすめとおります。

(参考)BCT Japan 株式会社回答(英語)、参考資料(文献2報)

Additional questions after the Joint Session of April 8 (same questions to each companies)

1. Update the current use status of each major EU nations where pathogen reduction technology is known to be available: Total quantity of blood products for transfusion (ratio of the total number of PRT-Pathogen Reduction Products- supplied vs. non PRT). How PRT products are supplied and distributed? (i.e. can they supply PRT based on the demands from physicians or end users?)

Various Pathogen Reduction Technologies (PRT) have been developed and are in various stages of evaluation and/or routine use in Europe.
- Three PRT methods are currently marketed for treatment of plasma: Solvent Detergent (in use in various countries since early 1990's[1]), Methylene Blue (marketed since 2001 and routinely used in France, Spain, Italy, UK), and the Intercept Blood System (CE marked since 2006; status of routine use unknown). The Mirasol PRT System is expected to receive CE mark for treatment of plasma in 2008.
- Two PRT methods are being marketed for treatment of platelet concentrates: the Intercept Blood System (CE Marked since 2002 and in routine use in a limited number of blood centers primarily in France, Spain, Belgium, Norway, and Sweden), and the Mirasol PRT System (CE Marked since late 2007 and currently under evaluation for routine implementation in several blood centers in Ireland, Spain, Italy, and the Middle East).

Overall, we believe that approximately 5% of all platelet products transfused in Europe are PRT-treated, with a clear trend towards an increasing use of PRT for these products. Countries with a high interest in and/or commitment to PRT for platelets include France, Belgium, Ireland, Spain, and various countries in the Middle-East.

In terms of supply and distribution of PRT-treated products, the logistics vary by PRT method. Because the Mirasol PRT procedure for platelets and plasma is quick (<15 min. total processing time) and easy to perform, the treatment may be conducted as part of the routine component manufacturing process in the blood center, or may be performed immediately prior to issue of platelets and so allow a blood center to supply PRT-treated components on demand. Given the broad benefits of PRT, all patient groups would benefit from receiving PRT-treated components, therefore, ultimately universal conversion to PRT is likely. However, expert opinion suggests that different hospitals may have a different sense of urgency and/or ability to request PRT treated products for their patients, hence blood centers may initially be required to perform PRT on a portion of their platelet inventory to meet various

[1] Pelletier et al., Best Practice and Research Clinical Haematology (Elsevier), Vol 19: 205-242, 2006

hospitals' specific needs.

2. Is there any difference in the frequency of the occurrence of the transfusion related side effects such as GVHD by the use of the pathogen reduced product manufacturing methods when leukocyte reduced products and non leukocyte reduced products are compared.

To our knowledge, no human data are available providing a direct comparison of the effectiveness of PRT treatment of leukoreduced vs. non-leukoreduced components in preventing transfusion-associated (TA-) GVHD. Results from an animal model in which human white blood cells (mononuclear cells) treated with the Mirasol PRT System were injected into genetically immune-deficient mice (as a model for TA-GVHD), indirectly support the effectiveness of the Mirasol PRT system in preventing GVHD when applied to non-leukoreduced blood components[2]. Additionally, in a recent publication[3] on the merits of PRT, Dr. H. Alter (National Institute of Health, US) indicated that discontinuation of leukoreduction was one of several potential cost-saving strategies when implementing PRT. Various studies are planned to further establish the Mirasol system performance when applied to non-leukoreduced components.

In terms of prevention of TA-GVHD when applying PRT to already leukoreduced components, actual clinical experience has been presented for the Intercept system, and is now becoming available for the Mirasol PRT system as well. As part of the MIRACLE (Mirasol Clinical Evaluation) trial, a randomized controlled clinical trial, the majority (160/168[4]) of Mirasol-treated products were not gamma-irradiated prior to transfusion, whereas the majority of reference (untreated) products (122/166) were treated with gamma-irradiation, confirming that the patient group studied was considered at risk for TA-GVHD. There were no reports of TA-GVHD in either patient group. Complete results from the MIRACLE trial will be presented later this year.

3. Is there any technology becomes obsolete after introduction of Pathogen Reduction Technology?

There are a number of blood safety initiatives that have been introduced over the last 20 or more years: Gamma irradiation, leukocyte reduction, serology testing for new markers, enhanced serology testing for existing markers, NAT testing, Bacterial testing. Typically, once a test is introduced it has not been allowed by regulators to be removed; one exception is the dropping of P24 antigen assay that was allowed by the FDA upon the introduction of NAT testing for HIV. This was achieved through careful analysis of a large multi-center clinical trial.

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[2] Fast, et al., *Transfusion* 2006; 46: 1553-1560.

[3] Alter, *Transfusion Medicine Reviews*, Vol 22(2): 97-102 (relevant statement on bottom of page 100)

[4] Data submitted to Notified Body (KEMA). Based on this data the Mirasol System was granted CE Mark for application to platelets on October 5, 2007.

Potentially many or some of these technologies introduced over the last 20 years could over time be replaced by PRT. The indications of the Advisory Committee on Blood Safety and Availability (Department of Health and Human Services, US)[5] suggested that Gamma irradiation and Bacterial testing were likely candidates. Additionally, over the last 10 years the pool size for NAT testing has decreased significantly down to < 10. With the general introduction of PRT for all components, countries may consider a return to larger pool sizes for NAT testing with the associated reduction in costs.

For any of the technologies to be dropped or their use to be modified it is likely that Post Market surveillance and clinical trials will need to be conducted.

4. The investigation status on the post market approval status on the Pathogen Reduced Blood Products in the major EU nations. Reality of the post market approval investigation (number of investigated cases).

Navigant Biotechnologies, LLC and Gambro BCT Inc. have a commitment (as part of the CE Mark certification) to conduct Post-Market Surveillance studies to continue to monitor the safety of Mirasol-treated products transfused on a routine basis. Since the Mirasol PRT system was officially launched in Europe, the Middle East and Africa in November 2007, safety data have been collected on over 100 routine transfusions of Mirasol-treated platelet products at multiple sites, and this activity continues. Additionally, an electronic data capture system has been developed to allow transfusion services to record important data regarding the clinical use of Mirasol-treated products. Finally, we will be working with the authorities of countries interested in adopting the Mirasol process to ensure adequate safety monitoring is in place according to local country needs.

Updated response to Question (4) from initial Q&A document:

(4) Reaction of the medical agent with other medical agent and its issues.

There have been no conclusive reports from in-vivo studies on drug interactions that would raise any concerns related to the use of Mirasol. The information initially provided to MHLW regarding a possible interaction of Riboflavin with Tetracycline and Trimethoprim-sulfamethoxazole was based only on a review of the literature in which primarily in-vitro studies suggested some potential effect with Riboflavin solutions. A detailed report on this matter, specific to the Mirasol application, is being prepared by independent toxicology experts and will be made available upon request.

[5] Thirty-third meeting of the ACSBA meeting, Washington, DC, January 9-10.

Mirasol PRT treatment of donor white blood cells prevents the development of xenogeneic graft-versus-host disease in Rag2$^{-/-}\gamma c^{-/-}$ double knockout mice

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BACKGROUND: Mirasol PRT (Navigant Biotechnologies) treatment utilizes exposure to light in the presence of riboflavin to introduce irreparable lesions to nucleic acids thereby inhibiting pathogen and WBC replication. The ability of Mirasol PRT–treated mononuclear cells (MNCs) to generate xenogeneic graft-versus-host disease (GVHD) responses was used to model transfusion-associated GVHD (TAGVHD).

STUDY DESIGN AND METHODS: Pairs of human MNCs from five different individual donors that had or had not received Mirasol PRT treatment and contained 30×10^6 CD3+ cells were injected intraperitoneally into sublethally irradiated (350 cGy) Rag2$^{-/-}\gamma c^{-/-}$ double-knockout mice. Recipient mice were weighed and observed regularly and euthanized when they exhibited symptoms of GVHD or at termination of the experiment. Recipient lymphoid compartments were collected and phenotyped for the presence of human lymphoid cells. The presence of human cytokines and/or immunoglobulins in the recipient plasma was also used to detect the presence of human cells.

RESULTS: Twelve of 14 mice injected with untreated cells developed xenogeneic GVHD, whereas 0 of 14 mice injected with Mirasol PRT–treated cells developed xenogeneic GVHD. End-stage xenogeneic GVHD in the recipients of untreated cells was characterized by the presence of splenic human cytolytic CD4+ and CD8+ cells, with high levels of interferon-γ, interleukin-10, and xenoreactive antibodies in the plasma.

CONCLUSION: Mirasol PRT treatment of the donor MNCs abolished xenogeneic GVHD responses, indicating that the use of Mirasol PRT treatment of blood products should prevent the development of TAGVHD.

The presence of white blood cells (WBCs) in transfused blood products is a significant contributor to the immunologic consequences of transfusion.[1,2] The immune responses resulting from transfusion include donor antirecipient responses such as transfusion-associated graft-versus-host disease (TAGVHD), graft-versus-tumor responses, and production of cytokines. Recipient antidonor responses can be induced by direct presentation of antigen by donor WBCs or indirectly after processing of the donor cells by recipient antigen-presenting cells.[3] These responses include elimination of donor cells, production of alloantibodies, and the induction of immunoregulatory immune responses that result in increased infection, increased risk of tumor relapse, and increased survival of transplanted organs. Because TAGVHD is almost always fatal, the emphasis has been placed in the development of protocols that prevent TAGVHD. The severity of TAGVHD has made it impossible to design clinical trials to test the ability of different protocols to inhibit the development of TAGVHD. As a result, models of human in vivo immune responses are being developed to test the effectiveness of various protocols in preventing TAGVHD.

ABBREVIATIONS: PBST = phosphate-buffered saline containing 0.5 percent Tween 20; TAGVHD = transfusion-associated graft-versus-host disease.

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Although a number of in vitro assays have been developed to measure the functionality of human WBCs, it would be useful to develop a model that more closely mimics the in vivo responses resulting in TAGVHD. Recent studies have utilized the xenogeneic GVHD responses of human cells when injected into immunodeficient murine recipients as a model of TAGVHD.[4,5] Initial studies found that human WBCs injected into SCID mice lacking both T and B lymphocytes were rejected by the murine NK cells and macrophages still present in these SCID mice especially following intravenous injection of the human mononuclear cells (MNCs).[6-9] Depletion of murine NK cells or macrophages from the SCID mice before injection of the human cells resulted in improved engraftment of intraperitoneally injected cells. If a sufficient number of cells were injected, the human lymphocytes were able to overcome the recipient mediated rejection and attack the recipient resulting in xenogeneic GVHD.[5] Recent studies have shown that the use of Rag2$^{-/-}\gamma c^{-/-}$ double-knockout mice that lack B, T, and NK cells as recipients accelerated the xenogeneic GVHD response as a result of increased human T-cell engraftment.[4]

Several different approaches are being developed for inactivation of pathogens that could be present in blood products. Mirasol PRT technology (Navigant Biotechnologies, Lakewood, CO) accomplishes pathogen inactivation by introducing irreparable nucleic acid lesions by exposure to light in the presence of riboflavin.[10] An initial study found that riboflavin plus light exposure functionally inactivated WBCs when the WBCs were tested with a panel of in vitro assays.[11] The ability to generate xenogeneic GVHD responses was utilized to test the functional ability of Mirasol PRT–treated WBCs in vivo and as a surrogate indicator for the efficacy of this treatment on TAGVHD prevention. Pairs of Mirasol PRT–treated or control untreated WBCs were injected into sublethally irradiated Rag2$^{-/-}\gamma c^{-/-}$ double-knockout mice, and the development of xenogeneic GVHD was monitored by regular observation of the mice and further immunologic analysis was conducted when the mice exhibited symptoms of GVHD or at the termination of the experiment.

MATERIALS AND METHODS

Preparation and characterization of human WBCs

WBCs were obtained from the leukoreduction chamber of an automated blood collection machine (Trima, Gambro BCT, Lakewood, CO) after standard apheresis collection of platelets (PLTs) from five different volunteer donors. The cells were separated into the MNC fraction with Ficoll-Hypaque (Pharmacia, Piscataway, NJ) discontinuous centrifugation and then placed equally into two PLT bags containing autologous plasma. The test cells received the Mirasol PRT treatment in 10 to 15 minutes after cell preparation as described.[11] Control cells received no treatment. The treated and untreated cells were then washed three times with phosphate-buffered saline (PBS) containing 0.1 percent fetal calf serum (FCS) and resuspended in RPMI 1640 containing 10 percent FCS. The cell populations were sent via overnight courier to Rhode Island Hospital. Upon receipt, the cell populations were assessed for number and viability with trypan blue staining (all cells were viable). The cells were also stained with a panel of antibodies to CD45, CD3, CD14, CD19, and CD56 (BD Biosciences, San Jose, CA) as previously described[11] to characterize the distribution of WBC subpopulations present in each of the treated and untreated donor populations.

In addition, the ability of the donor cells to proliferate in response to mitomycin C–treated allogeneic peripheral blood mononuclear cells (PBMNCs) as previously described[12] or to mitomycin C–treated xenogeneic C57BL/6 spleen cells was tested in triplicate. The xenogeneic MLC was set up by mixing 100 µL of donor MNCs at 4×10^6 MNCs per mL with 100 µL of mitomycin C–treated C57BL/6 spleen cells at 4×10^6 cells per mL in MLC medium.[13] The cells were cultured for 5 days, and then the cells were pulsed with 1 µCi of [^{3}H]thymidine for 4 hours before harvesting the wells to measure incorporation of the [^{3}H]thymidine as a measure of proliferation.

Mice

Six- to eight-week-old Rag2$^{-/-}\gamma c^{-/-}$ double-knockout mice were obtained from Taconic (Germantown, NY). These mice were housed in the special suite for immunodeficient mice in the Central Research Facility at Rhode Island Hospital. C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME).

Analysis of GVHD response

The recipient mice received 350 cGy gamma irradiation the night before the injection of cells. Each recipient mouse was injected intraperitoneally with a treated or untreated cell population containing 30×10^6 CD3+ cells from a single donor (three mice per group). Recipient mice were weighed twice per week and observed regularly. One recipient mouse in the untreated group developed an inner ear problem and was euthanized because it was unable to get its head off the bedding. One recipient mouse in the treated group died unexpectedly without any symptoms of GVHD as evidence by weight loss or splenomegaly when autopsied. There was no evidence suggesting the death was related to injection of treated cells. Recipient mice that demonstrated a dramatic weight loss (usually >20%) and exhibited lethargy, hunched posture, and ruffled fur were euthanized. Blood was collected by cardiac puncture with a heparinized syringe. The blood

was centrifuged, the hematocrit (Hct) level was recorded, and the plasma collected and stored at –20°C. The buffy-coat cells were collected, and the remaining RBCs were lysed with RBC lysis solution (Gentra, Minneapolis, MN). In addition, spleen cells were obtained by dissociating the spleen in PBS after weighing the spleen, marrow cells were obtained by flushing marrow from the femurs with PBS containing 1 percent BSA, and cells were obtained from intestinal lymphoid tissue by dissociation in PBS. The liver MNC population was obtained from the dissociated liver cells by centrifuging the cells over a Ficoll-Hypaque discontinuous gradient and collecting the MNCs at the interface and washing them twice with PBS. All mice that did not exhibit GVHD symptoms were euthanized by Day 63 and a similar analysis to that described above was conducted on these recipient mice.

Analysis of human cell chimerism

Cells from the various tissues were initially stained with PECy5 or PE anti-human CD45 or isotype controls (BD Biosciences) and then analyzed immediately for the presence of human CD45+ cells on a flow cytometer (FACScan, BD Biosciences). If the number of CD45+ cells present were equal to or less than the number of positive cells in the isotype control, the result was recorded as 0.0. If human CD45+ cells were detected and sufficient cells were present in a cell population, a second battery of staining was done in which the expression of WBC subpopulation markers including CD3, CD4, CD8, CD14, CD19, and CD56 (BD Biosciences) was measured. The populations containing T cells were also stained for the presence on CD107a on the cell surface, an indicator that the cells were experienced cytolytic cells because this marker is expressed on the cell surface after granule exocytosis.[14,15]

Measurement of cytokines

The level of cytokines in the plasma samples were measured with the CBA human TH1/TH2 kit I and the CBA human inflammation kit according to manufacturer's instructions (BD Biosciences).

Measurement of immunoglobulin levels

Ninety-six well flat-bottom microtest plates (BD Labware, Franklin Lakes, NJ) were coated with goat anti-human IgG-IgM-IgA-light chains(Biosource, Camarillo, CA) at 5 µg per mL in 0.1 mol per L bicarbonate buffer, pH 9.6. The plates were incubated for 4 hours at room temperature and then stored at 4°C until used in the assay. On the day of assay, the plate was washed three times with Dulbecco's PBS containing 0.5 percent Tween 20 (PBST). Human IgG and IgM standards were prepared from purified IgG and IgM (Calbiochem, La Jolla, CA). Serial twofold dilutions of the standards were prepared in PBST starting at 500 ng per mL and ending with 4 ng per mL. The standards or plasma diluted 1:4 or 1:8 were added to designated wells (100 µL) and incubated at room temperature for 2 hours. After the plate was washed three times with PBST, 100 µL of horseradish peroxidase–goat anti-human IgG or IgM (Biosource) diluted 1:16000 in PBST were added. The plates were incubated for 2 hours at room temperature. After washing 3× in PBST, 100 µL of 1× 2,2'-azinobis-(3-ethylbenzothiazoline sulfonate (Zymed, San Francisco, CA) diluted in 0.1 mol per L citrate buffer containing 0.3 percent hydrogen peroxide was added to all wells. The plates were read at 405 nm on a enzyme-linked immunosorbent assay (ELISA) plate reader after a 20-minute incubation at room temperature. The levels of IgG and IgM in the plasma samples were determined by comparison to the values obtained for the standard curves for IgG and IgM.

RESULTS

The paired Mirasol PRT–treated and control untreated donor MNCs were stained with trypan blue upon receipt to measure viability. All cell preparations were found to be 100 percent viable. The cells were then stained with a panel of antibodies to define the distribution of different subpopulations in these donor WBC populations. Although there was variation in the donor cell subset distribution from donor to donor, no differences in the number of CD3+ cells were observed when untreated (62.9 ± 8.3%) were compared to treated groups (64.3 ± 10.6%). The donor MNCs were also characterized by testing their ability to proliferate in response to allogeneic and xenogeneic murine stimulator cells. The results (Fig. 1) show that control donor cells that had not received Mirasol PRT treatment were able to proliferate in response to both allogeneic and xenogeneic stimulator cells and these proliferative responses as well as nonstimulated responses were completely abrogated by Mirasol PRT treatment.

Recipient Rag2$^{-/-}\gamma c^{-/-}$ double-knockout mice that had received sublethal irradiation (350 cGy) the evening before were injected intraperitoneally with a treated or untreated donor cell populations from individual donors (n = 5) that contained 30×10^6 CD3+ cells. Recipient mice were euthanized when they demonstrated symptoms of xenogeneic GVHD such as more than 20 percent weight loss (Fig. 2), hunched posture, and ruffled fur or when the experiment was terminated. Assessment of clinical parameters in recipient mice including spleen weight, Hct, and the presence of human CD45+ cells in various lymphoid compartments indicated that 12 of 14 recipient mice injected with untreated cells displayed symptoms of xenogeneic GVHD and 12 of 13 expressed varying degrees

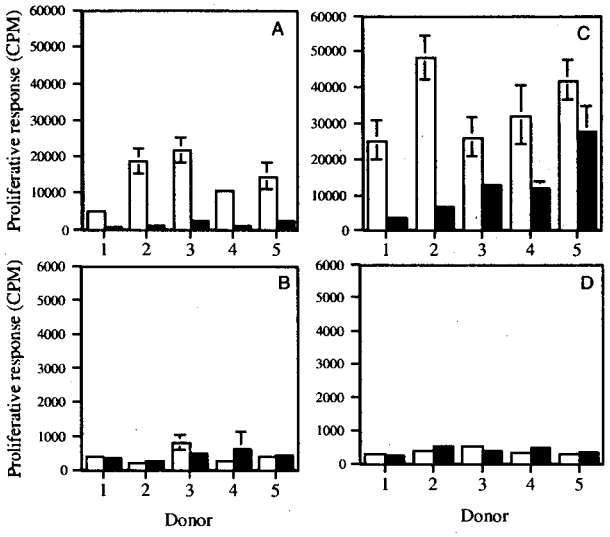

Fig. 1. Proliferative responses of Mirasol PRT–treated and untreated donor cells to allogeneic or xenogeneic stimulator cells. Five different paired sets of MNCs that had received Mirasol treatment (B and D) or no treatment (A and C) as responders were incubated with mitomycin C–treated allogeneic PBMNCs (A and B, □) or xenogeneic C57BL/6 spleen cells (C and D, □) or medium alone (■) for 5 days, and [^{3}H]thymidine incorporation was measured on Day 5.

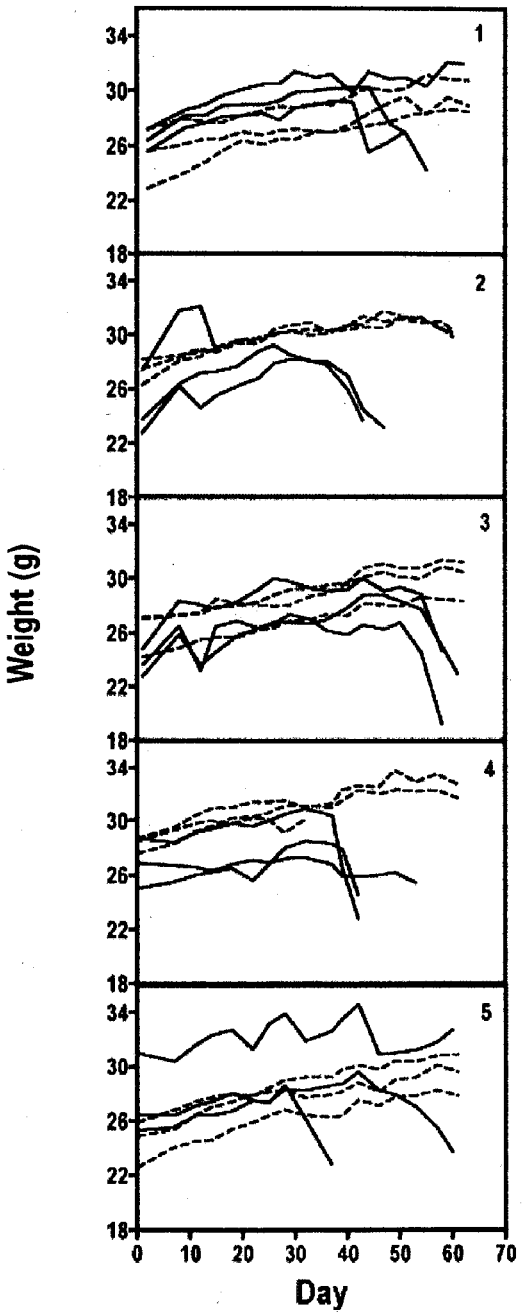

Fig. 2. The weight of recipient mice injected with untreated or Mirasol-treated donor cells. The weights (g) of recipient mice (3 mice per group) injected on Day 0 with Mirasol-treated (- - -) and untreated (—) cells from each of five donors (Panels 1-5).

of human cell chimerism (Table 1). In addition to exhibiting human cell chimerism, the recipients injected with untreated donor cells also exhibited increased spleen weight (splenomegaly) and significantly decreased Hct levels (Table 1).

In the cell populations from the recipient mice that contained sufficient numbers of human CD45+ cells to analyze, samples of cells were stained with a panel of antibodies to define the distribution of various WBC subpopulations in the lymphoid compartments. The results (Table 2) demonstrated that human T cells were the cells that were primarily present in the spleen, blood, and marrow. B cells were primarily found in the liver and intestinal lymphoid tissue. With the exception of one recipient mouse in which CD56+ cells were observed, no reconstitution with CD14+ cells (macrophages) or CD56+ cells (NK cells) was observed in any lymphoid compartment.[4] The cells were also stained with antibodies to CD4 and CD8 and the ratio of the percentage of T cells that were CD4+ to the percentage of T cells that were CD8+ T cells was determined. The CD4:CD8 ratios in the recipients of untreated cells from the different donors varied from 0.38 ± 0.08 to 1.82 ± 0.8 in a donor-dependent fashion.

Acute GVHD and especially TAGVHD are characterized by cytolytic responses. To determine whether the human T cells found in these mice exhibited properties of cytolytic cells, the T-cell subsets from a subset of recipients were dual-stained with anti-CD107a, a marker of cells that have previously mediated exocytosis of cytotoxic granules.[14,15] This staining procedure identified 41.1 ± 16.7 percent of human CD4+ cells (n = 8) and 45.5 ± 20.2 percent of human CD8+ cells (n = 8) as expressing CD107a. This would suggest that both CD4+ and CD8+

TABLE 1. Characterization of immune responses and reconstitution in mice injected with untreated or Mirasol PRT–treated MNCs

Parameter	Untreated donor cells	Treated donor cells
Number of mice with GVHD symptoms	12/14*	0/14*
Day euthanized	51.8 ± 8.2 days	Not applicable†
Number of mice with human cells	12/13‡	0/14
Spleen weight (gram)	0.27 ± 0.27	0.07 ± 0.07
Hct (%)	27.9 ± 16.9	53.9 ± 2.9
Percentage of CD45+ cells in		
Spleen	26.8 ± 19.8	0.0
Blood	4.6 ± 4.7	0.0
Marrow	4.9 ± 5.6	0.0
Intestinal lymphoid tissue	58.4 ± 28.4	0.0
Liver	20.3 ± 20.7	0.0

* One mouse was lost from a total of 15 recipient mice for unrelated reasons as detailed under Materials and Methods.
† None of the recipient mice in this group exhibited GVHD symptoms so mice were euthanized at the end of the experiment.
‡ One mouse that exhibited GVHD symptoms died before analysis could be conducted.

TABLE 2. The human lymphocyte subpopulations present in recipient lymphoid compartments

		Total number of human CD3+/CD19+ cells ($\times 10^6$)*				
Donor	Mouse	Spleen	Blood	Marrow	Liver	Intestinal
1	4	65.0/0.0	0.3/0.0	5.4/0.0	3.2/8.8	0.0/0.0
1	5	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0
1	6	0.4/0.2	0.01/0.06	0.03/0.4	1.5/6.1	0.7/0.2
2	10	2.8/0.02	0.04/0.1	0.1/0.2	0.2/1.1	0.1/0.5
2	12	1.5/0.4	0.2/0.6	0.7/0.7	0.3/1.2	0.7/5.3
3	16	0.08/0.4	0.06/0.07	0.1/0.2	0.2/2.1	0.0/0.0
3	17	0.09/0.4	0.04/0.06	0.1/0.1	0.5/2.7	0.04/0.4
3	18	0.04/0.01	0.02/0.1	0.08/0.1	0.1/0.5	0.0/0.0
4	22	1.3/0.4	0.1/0.02	0.2/0.1	2.0/6.6	0.4/0.01
4	23	4.2/0.3	0.2/0.01	0.2/0.01	0.0/0.0	0.3/0.01
5	28	5.7/0.7	0.5/0.5	0.9/0.09	5.5/5.3	0.5/0.6
5	29	3.5/0.2	0.2/0.05	1.8/1.0	0.0/0.0	0.1/1.0
5	30	4.3/0.09	0.3/0.0	0.4/0.0	0.4/1.1	0.0/0.0

* Each number is the product of the cell recovery in millions for each lymphoid compartment and the percentage of human CD3+ or CD19+ detected by flow cytometric analysis in each population.

cells have mediated cytolytic activity via granule exocytosis. CD4+CD25+ have been recently shown to contain granzymes and mediate cytolytic activity against autologous cells.[16,17] Staining of a limited number of samples with anti-CD25 found that less than 5 percent of the CD4+ cells expressed CD25, suggesting that the human CD4+ cells present in the spleen were not T regulatory cells.

Measurement of the levels of human cytokines in the plasma of the recipient mice was used as another approach to assess the possibility that human cells were present but not located in the lymphoid compartments. The plasma samples from recipient mice that had been injected with Mirasol PRT–treated cells did not contain any human cytokines. In contrast, the plasma samples of mice injected with untreated donor cells (n = 13) contained very high levels of interferon-γ (IFN-γ); high levels of interleukin (IL)-10; low levels of IL-5, IL-1β, tumor necrosis factor-α (TNF-α), IL-8, and IL-12p70; and no IL-2 or IL-4 (Fig. 3).

The measurement of human immunoglobulin levels in the plasma was a third approach to measure the presence of human lymphoid cell chimerism. An ELISA to measure the levels of human IgG and IgM in the plasma of the recipient found that human immunoglobulins were only detected in the plasma of recipients injected with untreated cells (Table 3). To test for the specificities exhibited by the human immunoglobulin, C57BL/6 spleen cells were stained with a 1:10 dilution of the recipients' plasma followed by fluorescein isothiocyanate–labeled anti-human IgM or IgG. Flow cytometric analysis of WBCs, RBCs, and PLTs was conducted by gating on the different sized populations. The results of this experiment (Table 3) indicated that there were high levels of IgM antibodies and lower levels of IgG antibodies binding to murine RBCs and PLTs with very low levels of antibodies binding to WBC.

DISCUSSION

Mirasol PRT is a novel technology for pathogen reduction that has been shown to effectively preserve PLT viability in a PLT recovery and survival clinical trial.[18] The validation clinical trial for its efficacy and safety in thrombocytopenic patients has just been initiated in Europe. During Mirasol PRT treatment, blood products such as PLT concentrates are exposed to light in the presence of riboflavin resulting in inactivation of a wide range of pathogens.[19-23] Because this process introduces irreparable lesions on nucleic acids,[20] the treatment was also expected to affect the function of WBCs. In a previous study, it was shown that Mirasol PRT treatment of WBCs prevented their ability to be activated, to proliferate in response to various stimuli, and to induce proliferation of normal PBMNCs.[11] The conclusion of these studies was that Mirasol PRT treatment caused a complete functional inactivation of WBCs. Several studies had previously shown that inhibition of in vitro proliferative responses correlated with an inability to generate GVHD.[13,24] To confirm the inactivation of WBC by Mirasol PRT treatment observed with in vitro assays also prevented in vivo GVHD responses, the ability of Mirasol PRT–treated cells to induce xenogeneic GVHD responses when injected into Rag2$^{-/-}\gamma c^{-/-}$ double-knockout recipient