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

Loren D. Fast, Gilbert DiLeone, Gene Cardarelli, Junzhi Li, and Raymond Goodrich

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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TRANSFUSION 2006;46:1553-1560.

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 ($62.9 \pm 8.3\%$) were compared to treated groups ($64.3 \pm 10.6\%$). 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