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Leukocytes and pathogen inactivation methods

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Summary

Transfusion of blood components with residual leukocytes may lead to a number of adverse reactions based on two separate immune-mediated mechanisms. Such complications include transfusion associated — graft versus host disease (TA-GvHD), febrile nonhemolytic transfusion reactions (FNHTRs), which are caused by release of cytokines and recipient alloimmunization with HLA antigens. This leads to anti-HLA antibody formation in the recipient and may induce refractory response to the transfused platelet concentrate (PC). To reduce the risk of transferring residual leukocytes with transfused blood components, for many years now various preparation methods have been applied, which contribute to reduction of white blood cell (WBC) count or to leukocyte inactivation. These include: leukoreduction, gamma irradiation and pathogen inactivation methods. Advancement of research on pathogen inactivation in blood components has demonstrated that some of the methods used for the routine pathogen inactivation in plasma and platelet concentrate (Mirasol®PRT system and Intercept) may also be effective for inactivation of T lymphocytes. These methods may therefore serve as alternative to irradiation. Also the inactivation method with UVC light when implemented into routine use, may replace the commonly used irradiator. Inactivation methods were found not only effective for protection against TA-GvHD, but also for inhibiting cytokine synthesis, primarily responsible for febrile nonhemolytic transfusion reactions.

Key words: leukocytes, pathogen inactivation methods, serious adverse reactions

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Serious adverse reactions

Transfusion of leukocyte-contaminated blood components may induce adverse reactions based on two separate immune-mediated mechanisms. One of these mechanisms occurs when leukocytes present in the transfused blood components stimulate the recipient's cells and tissues to induce acute post transfusion reactions such as Transfusion Associated-Graft versus Host Diseases (TA-GvHD) or Febrile Non Hemolytic Transfusion Reactions (FNHTRs) caused by cytokine release. In the other mechanism, recipient's T cells recognize foreign HLA antigens on donor leukocytes. In the presence of the molecules of the major histocompatibility complex (MHC) on donor antigen presenting cells (APC) (direct pathway) or recipient antigen presenting cells (indirect pathway) donor T cells recognize unknown HLA antigens. Recognition of foreign HLA donor antigen by recipient's T cells lead to anti-HLA antibody formation in the recipient, which may induce the patient's refractoriness to transfused platelet concentrate (PC).

To reduce the risk of transferring residual leukocytes with transfused blood components, for many years now various preparation methods have been applied the result of which is either reduc-

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Table 1. Serious adverse reactions after transfusion of blood components

Adverse reactions	Responsible factor
Transfusion Associated-Graft versus Host Disease, TA-GvHD	Immunocompetent donor T cells
Alloimmunization with HLA antigens	Donor leukocytes
Febrile nonhemolytic transfusion reaction, FNTHR	Cytokines released from donor leukocytes
Transfusion related acute lung injury, TRALI	Donor/recipient antibodies (anti-HLA class I or class II and anti-granulocytic antibodies)
Pathogen transmision	Infectious agents associated with leukocytes (e.g. CMV, HTLV 1/2)

tion of white blood cell (WBC) count or leukocyte inactivation. These methods serve to protect blood component recipients against alloimmunization with HLA antigens, FNHTRs or TA-GvHD. Haemovigilance data (HV, haemovigilance) at both national and European level demonstrates that even small numbers of leukocytes in the transfused blood components may be sufficient to induce severe adverse post-transfusion reactions in the recipient (Table 1) [1, 2].

Transfusion-associated graft versus host disease

Transfusion-associated graft versus host disease (TA-GvHD) is one of the most severe adverse reactions which may result either from transfusion of immunocompetent donor T cells (which the recipient's immune system is unable to destroy) or else the donor is homozygous for one of the recipient's HLA haplotypes. TA-GvHD occurs when the recipient's tissues are damaged as result of the interaction between donor and recipient cells of the immune system. Various lymphocyte subsets such as interleukins, pro-inflammatory cytokines and adhesion particles are involved in the process of host-cell damage following transfusion of lymphocyte contaminated blood components. First, the recipient's dendritic cells and macrophages, which function as APC cells, present HLA antigens to transfused T lymphocytes. Interleukin-1 (IL-1) secreted by macrophages and the thymocyte-stimulating factor activate helper CD4 + T cells to secrete interleukin-2 (IL-2) which in turn stimulates CD 8+ lymphocytes. Activation of T lymphocytes stimulates the release of cytokines from various cells, resulting in clonal proliferation of T lymphocytes, which differentiate into cytolytic cells or lymphokine secreting cells. At this stage, there occurs an uncontrolled robust generation of cytokines with direct impact on recipient's target cells, or indirect impact through stimulation of cells of the hematopoietic line, such as B lymphocytes, cytotoxic lymphocytes or macrophages. These cells act as secondary affectors and, together with cytokines, are responsible for damage and death of the recipient's target cell [3, 4].

According to the reports of Serious Hazards of Transfusion (SHOT), TA-GvHD is relatively rare with the incidence rate of approximately 0.1–1% of all adverse reactions and mostly observed in patients with haematopoietic hyperplasia and lymphoproliferative disorders. The complication is a serious problem in clinical practice because there is no effective treatment and the mortality rate is high (> 90%). Moreover, even new generation leukocyte-removal filters offer no protection against development of TA-GvHD because the residual leukocyte count in blood components ($< 1 \times 10^6$) is sufficient to induce the reaction. So far, the only effective method of protecting at risk-patients against development of TA-GvHD was to subject cellular blood components to gamma or X-ray irradiation (Cs^{137}, Co^{60} or X) [5–9].

Advancement of research on pathogen inactivation in blood components demonstrated that some of the methods used for the purpose may also be effective for inactivation of T lymphocytes. These methods may therefore serve as alternative to irradiation [10]. The principle is to induce irreversible changes in the nucleic acids of viruses, bacteria, parasites and leukocytes by inhibiting their multiplication. In world literature there are reports on the effectiveness of white blood cell inactivation in PCs with routinely used pathogen inactivation systems (Intercept and Mirasol) and therefore the possibility of replacing irradiated PC with PC inactivated with one of the above-mentioned systems has also been confirmed. Some countries (France, Spain, Austria, Luxembourg, Qatar, Poland) use Mirasol®PRT system or Intercept systems for inactivation of pathogens in PCs dedicated for patients at risk of

Table 2. Patients at risk of TA-GvHD broken down into probability of occurrence

Proven risk	Possible risk	Unspecified risk
• Recipients of allo- and autologous bone marrow transplants • Persons with congenital immune deficiencies • Intrauterine transfusion • Persons receiving blood components from related persons (1st and 2nd degree) • Persons with Hodgkin's disease	• Premature babies • Persons with haematological diseases other than Hodgkin's disease • Persons with solid tumors • persons after organ transplantation	• AIDS • Neonates born on time

TA-GvHD. Irradiation of already inactivated PCs is not recommended [11].

Results of numerous studies confirm the methods of pathogen inactivation for red blood cells or whole blood (PEN 110, S-303, riboflavin) — under development or in course of clinical trials — to be equally effective for inactivation of white blood cells [12]. If implemented for routine use, these methods could replace the currently used gamma or X-ray irradiation. Pursuant to the guidelines of the National Atomic Energy Agency (PAA) and in view of the mounting threat of terroristic attacks the operation and management of radiators requires continuous monitoring of this radioactive source. In addition, gamma irradiation reduces the functional inactivation of red blood cells (leakage of intracellular potassium into extracellular space, hemolysis), which intensify with time. It is therefore recommended to irradiate (radiators) blood components for patients at risk of developing TA-GvHD immediately before transfusion (Table 2). Some study results on inactivation of leukocytes either with routinely used systems or with methods still under trial suggest that — apart from preventing TA-GvHD — these methods are also effective for prevention of alloimmunization with HLA antigens as well as febrile nonhemolytic transfusion reactions (FNHTR) [13, 14].

Alloimmunization

The TRAP (Trial to Reduce Alloimmunization to Platelets) reports no platelet recovery in the recipient's circulation both after 1 and 24 hours of PC transfusion which meant that the frequency of transfusions had to be higher. There were attempts to explain the phenomenon by the appearance of antibodies in the recipient, but the results of lymphocytotoxicity tests demonstrated that reduced platelet recovery occurs both in the presence and absence of anti-leukocyte antibodies. Repeat analysis of data demonstrated that the differences in the number of recipient platelet cells depended on the type of PC transfused. Higher platelet recovery was observed following transfusion of leukoreduced or UV-irradiated PC than after transfusing standard PC. Therefore leukocyte-contaminated PCs were considered the most likely cause of alloimmunization with HLA antigens responsible for resistance to transfused PCs which occurs in patients subjected to multiple PC transfusions. Numerous studies have however confirmed, that gamma irradiation is no protection against alloantibody formation. One of the management procedures currently used is transfusion of leukoreduced PCs or HLA compatible PCs. These procedures however, are not in routine use [15–17].

Febrile nonhemolytic transfusion reaction

Febrile nonhemolytic transfusion reactions (FNTHR) following PC transfusion are most often caused by the presence of leukocytes in the transfused blood component and anti-leukocyte antibodies detected in recepients. Recipient's macrophages are activated as a result of the antigen-antibody-complement reaction. These interactions induce cytokine secretion at concentrations proportional to the number of leukocytes in PC as well as duration of storage prior to transfusion. Heddle demonstrated that elevated levels of interleukin-1beta (IL-1β) and interleukin-6 (IL-6) in stored PCs are the most common causes of FNTHR. The adverse reaction can be prevented by leukocyte removal no later than 6 hours of PC preparation [18].

The incidence rate for FNHTR following transfusion of non-leukoreduced red blood cells is estimated at 0.12 to 0.5%, while for non-leukoreduced PCs it is significantly higher, from 1.7 to 31%. The relationship between high content of IL-1α, IL-6

and TNF-α and that of white blood cells in PC above 3×10^6/l was also confirmed. Although filtration or plasma removal significantly contributes to reduction of cytokine concentration and therefore reduction of the incidence rate for FNTHR, adverse reactions due to leukocyte contamination sporadically occur despite leukoreduced PCs. Muylle et al. performed analyses of transfusion procedures in 45 patients who were transfused with leukoreduced PC and yet 13% of them presented a feverish reaction due to significant increase in TNF-αl and IL-6 in UKKP [2, 19, 20].

Apart from secretion of leukocyte-derived cytokins during PC storage there also occurs the secretion of alpha granules and granular platelet chemokine cells that induce allergic reactions. These include platelet-derived: CCL3 (MIP-1, macrophage inflammatory protein), CCL5 (RANTES, regulated upon activation and normal T cell expressed and secreted), CXCL4 (PF4, platelet factor4), TGF-beta1-transforming growth factor beta 1. Some of these cytokines, e.g. CCL5 (RANTES) are involved in non-haemolytic, allergic or pro-inflammatory reactions following transfusion of leukoreduced PCs induced — among others — by their ability to attract and stimulate human eosinophils and to induce histamine secretion from human basophils. For allergic reactions, the RANTES range was estimated at 200–1000 ng/ml [21–23].

Transfusion related acute lung injury

Transfusion related acute lung injury (TRALI) is a very severe adverse reaction characterized by breath shortage (pulmonary edema with hypoxia). It may occur during transfusion or up to 72 hours of the procedure. Untreated TRALI leads to death in approximately 25% of patients. The incidence rate for TRALI is estimated at about 1/1500–5000 transfusions although the exact data is unavailable. At exceptionally high risk are patients in critical condition (6–8%). TRALI syndrome has been reported after transfusion of all types of blood components as well as intravenous administration of immunoglobulins. TRALI pathogenesis is not fully recognized. In most cases, the syndrome is induced by leukocyte antibodies, most often anti-HLA class I and II or specific anti-human neutrophil antigen (anti-HNA-1a, 1b, 2a, 3a) antibodies. Not only anti-leukocyte antibodies are responsible for neutrophil activation in transfused blood components but also biologically active lipids, lysophosphatidylcholine (L-PC) or cytokines that accumulate during storage of blood components [24–26].

Leukocyte contamination and methods of pathogen inactivation

Leukocyte inactivation with the use of the Mirasol®PRT system

Results of numerous studies have confirmed that the inactivation method used in the Mirasol®PRT system is also effective for leukocytes, including T lymphocytes, the transfusion of which (via blood components) puts the recipient at risk of TA-GvHD. The degree of T cell inactivation was determined using — among others — the limiting dilution assay (LDA) or by measurement of CD69 antigen expression, an early marker of T cell activation involved in transfer of the activation signal, which leads to the synthesis of various cytokines (including IL-2, INF-γ).

The LDA method revealed that following inactivation with the Mirasol®PRT system the number of viable T cells decreased by over 6 logs. It was also determined that the Mirasol®PRT system is effective for inhibiting T lymphocyte activation, as demonstrated by complete lack of CD69 expression on cells inactivated with this system (1.7 ± 1.3%) as compared to CD69 expression on control cells (64.4 ± 15.6%) [27].

Numerous *in vitro* studies also demonstrate that leukocytes inactivated in the Mirasol®PRT system are not capable of stimulating or binding allogeneic cells. Unlike leukocytes exposed to gamma irradiation, leukocytes inactivated in the Mirasol®PRT system were observed to lose their ability to act as antigen presenting cells (APCs). This is most likely due to significant reduction in the expression of some surface receptors such as HLA-DR, ICAM 1-3, CD80, and CD86 responsible for activation and adhesion of T lymphocytes. A slight reduction in the expression of HLA class II surface antigens and co-stimulatory molecules was observed in leukocytes inactivated with riboflavin and UV light as well as a significant reduction in expression of numerous adhesion molecules in antigen presenting cells. Almost complete loss of immunogenicity has also been observed [28].

Studies were performed to verify whether the Mirasol®PRT system is effective for reducing the risk of febrile nonhemolytic transfusion reactions (FNTHR). A significant inhibition of the synthesis of cytokines: IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ, IL-1β, IL-12p70 was observed in mononuclear cells inactivated in the Mirasol® PRT system as compared to control mononuclear cells and mononuclear cells subjected to irradiation [29].

Inactivation of leukocytes using the Intercept system

Even the first results of *in vitro* and *in vivo* studies have already confirmed the method based on the use of amotosalen hydrochloride and UVA to be effective also for inactivation of leukocytes, mononuclear cells (MNCs), T lymphocytes in particular. Following LDA in PC samples collected after pathogen inactivation with the Intercept system (150 μM S-59 and 3 J/cm^2), a 5.4 log decrease in the number of viable T lymphocytes was observed and no T lymphocyte colony-formation was confirmed. Additional studies with lower doses of amotosalen hydrochloride (S-59) were performed in order to determine the safety margin for the photochemical method with regard to T cells. The studies have demonstrated exceptional sensitivity of T lymphocytes to the photochemical method. Amotosalen hydrochloride at a dose 1500–3000 fold lower than normally used in the Intercept system and 2 fold lower UV dose than the virucidal dose were sufficient to inactivate T lymphocytes to limit of detection.

Like the Mirasol®PRT system, the Intercept system, inhibits CD 69 expression. Fiebig et al. determined CD69 antigen expression in control PC samples, in PC samples exposed to gamma irradiation and samples inactivated in the Intercept system. Immediate reduction of CD69 expression was observed in T lymphocytes exposed to gamma irradiation and those inactivated in the Intercept system to 21% and 12% respectively. In the control group, the average expression was 82%) [30, 31].

In vitro studies also confirmed the Intercept system to be effective for inhibition of cytokine synthesis. Interleukin-8 (IL-8) concentration was compared in 3 types of PCs stored for up to 7 days (control PC, PC exposed to gamma irradiation and PC inactivated in the Intercept system). A significantly higher IL-8 concentration was reported for control PCs and in PCs subjected to irradiation. In their studies Hei et al. confirmed the Intercept system to inhibit the synthesis of IL-8, IL-1β, TNF-α and IL-6. It should be emphasized, that also bacteria contribute to cytokine secretion and inactivation of bacteria with the Intercept system prior to PC storage is an additional preventive measure against hemolytic febrile transfusion reactions. Research from numerous scientific centers has confirmed the Intercept system to completely inhibit the synthesis of cytokines and — unlike gamma irradiation which reduces the synthesis of cytokines by only 40% — it provides additional protection against the development of FNTHR [22, 23, 32, 33].

Leukocyte Inactivation with other pathogen inactivation methods

Theraflex UV-Platelets based on UVC light is a method not as yet implemented for routine use but already evaluated and found effective for inactivation of white blood cells. Results of the first studies performed on xenogenic mouse models indicate that UVC used in the Theraflex MB Plasma UV-Platelets system is as effective as the routine gamma irradiation. Even half the routine dose of UVC has been found to inactivate T lymphocytes. Paralel *in vitro* studies demonstrated no proliferation of mononuclear white blood cells (following PHA, anti CD3 and anti CD28 stimulation in mixed lymphocyte culture) when 0.1 J/cm^2 and 0.2 J/cm^2 were used (the latter dose is routinely used in Theraflex UVC system). This indicates that the lower dose of 0.1 J/cm^2 is absolutely sufficient to inhibit the proliferative capacity of mononucleated white blood cells, in contrast to mononuclear white blood cells isolated from PCs exposed to gamma irradiation. It can therefore be assumed that UVC irradiation prevents proliferation of T lymphocytes responsible for the development of TA-GvHD [34, 35].

It also turned out that UVC light, even at a dose of 0.1 J/cm^2, protects antigen presenting cells against stimulation with allogeneic cells. It seems likely therefore, that the UVC light-based method offers protection against alloimmunity. Deterioration of direct antigen presentation by antigen presenting cells (APCs) may result either from the decrease in the expression of critical surface receptors for T lymphocyte activation and adhesion (characteristic for prevention of alloimmunization in the Mirasol®PRT system) or from defect in antigen processing [28].

UVC irradiation has also been found effective for significant IL-1β and IL-6 cytokines reduction. Strong inhibition of cytokine secretion already occurred after application of the UVC light at a dose — 0.1 J/cm^2 (which is half the routine dose of 0.2 J/cm^2) in PCs subjected to inactivation. Synthesis of IL-8, a cytokine secreted during PC storage, was also significantly inhibited after application of UVC light [18].

The Mirasol®PRT system developed for pathogen inactivation in whole blood has also been found effective with regard to leukocytes. Using 33 or 44 J/ml $_{RBC}$ energy, inactivation of

mononucleated cells (isolated from whole blood) was found. Human T cells previously inactivated in the Mirasol®PRT system were transplanted into mice which did not develop GVHD. This indicates that the Mirasol®PRT system may be an alternative to whole-blood irradiation.

Summary

Results from various research centers dealing with pathogen inactivation methods in blood components which are presented in this review confirm that most of these methods have also been proved effective for inactivation of leukocytes responsible for many serious adverse reactions. The methods used in Mirasol®PRT system and Intercept systems currently in routine use for pathogen inactivation in plasma and PC have also been found effective for inactivation of T lymphocytes. These methods therefore may be regarded as an alternative to irradiation. Once the method of inactivation based on the use of UVC light is implemented for routine use, it will also replace a commonly used irradiation method. It was also demonstrated that inactivation methods not only protect against TA-GvHD, but also inhibit the synthesis of cytokines, which are primarily responsible for transfusion related non hemolytic febrile reactions.

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