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(54) **COMPOSITIONS DE PLAQUETTES ET D'HEMATIES NON IMMUNOGENES ET TOLERAGENES**

(54) **NON-IMMUNOGENIC AND TOLERAGENIC PLATELET AND RED BLOOD CELL COMPOSITIONS**

(57) L'invention concerne des compositions de plaquettes et d'hématies non immunogènes et toléragènes, sensiblement dépourvues de leucocytes. Elle concerne également des procédés de fabrication de ces compositions à l'aide de diverses combinaisons de filtration, centrifugation et irradiation U.V.

(57) Non-immunogenic and/or toleragenic platelet and red blood cell compositions are provided. Such compositions are substantially free of white blood cells. Methods for preparing such compositions using various combinations of filtration, centrifugation and UV irradiation are also provided.

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(54) Title: NON-IMMUNOGENIC AND TOLERAGENIC PLATELET AND RED BLOOD CELL COMPOSITIONS

(57) Abstract

Non-immunogenic and/or toleragenic platelet and red blood cell compositions are provided. Such compositions are substantially free of white blood cells. Methods for preparing such compositions using various combinations of filtration, centrifugation and UV irradiation are also provided.

Description

NON-IMMUNOGENIC AND TOLERAGENIC PLATELET AND RED BLOOD CELL COMPOSITIONS

Technical Field

The present invention relates generally to blood products, and more specifically to non-immunogenic and toleragenic platelet and red blood cell compositions, and methods of producing the same.

Background of the Invention

Patients with a variety of disorders receive intermittent or chronic transfusion support or require tissue grafting to replace a defective organ. Individuals requiring transfusion support may have either a genetic or acquired deficiency of one or more blood components that require replacement therapy. Many different products prepared from blood are available for transfusion, including both cellular and plasma components. However, repeated exposure to blood products often results in recipient recognition of the foreign transfused antigens. For instance, alloimmune platelet refractoriness occurs in up to 30%-60% of patients requiring repeated platelet transfusions. Such immune recognition of the foreign antigens results in a failure to achieve a benefit from the transfusion and in some circumstances may even cause a transfusion reaction with adverse consequences to the recipient.

Several approaches have been used to either prevent or delay alloimmunization. The majority of the techniques involve giving immunosuppressive therapy to the transfusion recipient to prevent recognition of the transfused foreign antigens. Such immunosuppressive therapy is often inadequate to suppress the recognition process resulting in alloimmunization in spite of the treatment. Furthermore, the immunosuppressive therapy may have undesirable side effects including organ toxicity and immunosuppression of desirable responses such as recognition and destruction of pathogenic bacteria.

Once an immune response to foreign antigens has occurred, there is little evidence that any immunosuppressive therapy is beneficial. Continued adequate transfusion support is possible only if antigen matching between donor and recipient is achieved. Often a matched donor is not available or for some transfusion products so little is known about the antigen systems involved in the immune response that laboratory methods are not available to appropriately select a matched donor.

An alternative approach to preventing alloimmunization, other than immunosuppressing the recipient, is to reduce the immunogenicity of the transfused product. As all transfused blood products are immunogenic and will eventually induce an immune response in most transfused recipients, any procedure that can prevent or at least delay immunization is beneficial. Selecting only antigen compatible donors beginning with the first transfusion is possible in some circumstances, but for the majority of patients not enough donors are available to continue this process or a matching procedure does not exist.

For organ grafting, because there is persistent exposure to foreign tissue antigens, eventual rejection of the grafted tissue occurs. To prevent graft rejection several approaches have been used: recipient immunosuppression, matching tissue antigens of donor and recipient, reducing the immunogenicity of the grafted tissue, or inducing a state of tolerance in the recipient to the foreign antigens of the graft. Furthermore, depending on the tissue being grafted different approaches may be required to achieve a successful graft and combined therapies may be additive in their beneficial effects. For example, in bone marrow transplantation massive doses of chemo-radiotherapy are given to the recipient to destroy the recipient's autologous marrow and to induce immunosuppression to allow engraftment of the donor marrow. Even better results are obtained if marrow donor and recipient are related and well-matched for the major histocompatibility antigen system (HLA). Although post-marrow grafting immunosuppression is usually given, it is for only a limited time.

In contrast, for kidney grafting lesser degrees of immunosuppressive therapy are required to avoid unacceptable marrow and gastrointestinal toxicity. Furthermore, continuous post-grafting immunosuppression is required. Often a related

kidney donor is not available, and lesser degrees of HLA matching between donor and recipient are more often accepted than for bone marrow transplantation.

Another major difference between these two types of tissue grafting is the effect of prior transfusions on engraftment. For kidney graft recipients, prior transfusions, particularly from the intended kidney donor, are beneficial apparently by inducing some degree of tolerance to the subsequent kidney graft. However, prior blood transfusions before marrow grafting, especially if the blood has come from the intended marrow donor, markedly increase the risk of graft rejection. Thus, although there are similarities in procedures to enhance organ grafts (immunosuppression and donor-recipient HLA matching) there are clear differences in (1) the amounts, type, and duration of immunosuppression required; (2) the acceptance of non-HLA identity between organ donor and recipient; and, (3) the effects of prior transfusions on enhancing or impairing a subsequent organ graft. Furthermore, even the best combined therapies are not always successful in ensuring a successful organ graft, and there may be substantial toxicities associated with the therapies being used.

Besides using HLA matching and recipient immunosuppression, efforts to directly reduce immunogenicity of the engrafted tissue or to induce tolerance in the recipient, other than by prior blood transfusions in kidney recipients, have been limited. In bone marrow transplantation efforts to purify or enrich the marrow graft for stem cells and eliminate T-lymphocytes that may be responsible for graft vs. host disease (a post-grafting complication) have often resulted in a transplanted marrow that has failed to engraft. Some investigators have stored or cultured the graft *in vitro* prior to transplantation (skin grafting) to facilitate engraftment. Most of these latter methods to enhance organ grafting have had limited success.

It would be advantageous to avoid immune recognition by the recipient of incompatible donor antigens and the consequent destruction of allogeneic tissue following transfusion or transplantation. The present invention fulfills this need and further provides other related advantages.

Summary of the Invention

The present invention provides non-immunogenic and/or toleragenic platelet and/or red blood cell compositions. Such compositions are substantially free of white blood cells.

In a related aspect of the present invention, methods of preparing such non-immunogenic and toleragenic platelet compositions are provided, comprising centrifuging a platelet-rich blood product one or more times and recovering the supernatant; and filtering the supernatant and recovering the filtrate to produce the platelet composition. The step of centrifuging may comprise one or two soft spins, with or without a hard spin performed at any point in the platelet preparation process. The step of filtration may be performed after the step of centrifugation or between the first and second soft spins. Alternatively, the step of filtration may be performed prior to the step of centrifugation.

Within another related aspect, a method of preparing a non-immunogenic and toleragenic platelet composition comprises: (a) performing a first soft spin on a platelet-rich blood product and recovering a supernatant platelet composition; (b) performing a second soft spin on the platelet composition and recovering a supernatant platelet composition; (c) performing a hard spin on the platelet composition and recovering a supernatant platelet composition; and (d) filtering the platelet composition and recovering a filtrate platelet composition; wherein step (a) is performed prior to steps (b), (c) and (d) and wherein steps (b), (c) and (d) may be performed in any order.

In yet another related aspect, a method of preparing a non-immunogenic and toleragenic platelet composition comprises: (a) performing a hard spin on a platelet-rich blood product and recovering a supernatant platelet composition; (b) performing a soft spin on the platelet composition and recovering a supernatant platelet composition; and (c) filtering the platelet composition and recovering a filtrate platelet composition; wherein step (a) is performed prior to steps (b) and (c) and wherein steps (b) and (c) may be performed in either order.

In another aspect of the present invention, a non-immunogenic platelet composition is provided. The composition may be produced by either (a) centrifuging a

platelet-rich blood product, recovering the supernatant and then exposing the composition to UV irradiation; or by (b) filtering the platelet-rich blood product, recovering the filtrate and then exposing the filtrate to UV irradiation. Any of UV-A, UV-B or UV-C irradiation may be used with or without a photoadditive agent (*e.g.*, psoralin), although UV-B is preferred. It will be evident that the steps of centrifugation or filtration and UV irradiation may be performed in either order.

In any of the methods described above, suitable platelet-rich blood products include whole blood, platelet-rich plasma, platelet concentrates, apheresis platelets and buffy-coat prepared platelets.

In another aspect, the present invention provides a method of preparing a non-immunogenic and/or toleragenic red blood cell composition, comprising: (a) centrifuging a red blood cell-rich blood product and recovering the lower layer of red cells; and (b) subsequently filtering the lower layer and recovering the filtrate to produce a non-immunogenic and toleragenic red blood cell composition. Alternatively, the filtration can be performed first, followed by the centrifugation step.

In further aspects of the present invention, methods are provided for inducing immunologic tolerance in a patient to transfused or transplanted tissue. In one such aspect, the methods comprise administering to a patient a non-immunogenic and toleragenic platelet composition, said composition being substantially free of white blood cells, in an amount sufficient to induce immunologic tolerance to subsequently transfused or transplanted tissue. In another such aspect, the methods comprise administering to a patient a non-immunogenic and toleragenic red blood cell composition, said composition being substantially free of white blood cells, in an amount sufficient to induce immunologic tolerance to subsequently transfused or transplanted tissue. In various embodiments, the transfused or transplanted tissue may be derived from bone marrow, blood, skin, pancreas, bone, liver, heart, lung, kidney or cornea. Within preferred embodiments, the transfused or transplanted tissue is blood.

These and other aspects will become apparent upon reference to the following detailed description. All references disclosed herein are hereby incorporated by reference in their entirety as if each was incorporated individually.

Detailed Description of the Invention

Prior to setting forth the invention, it may be helpful to the understanding thereof to set forth definitions of certain terms to be used hereinafter.

"Non-immunogenic" as used herein refers to a characteristic of compositions which, when administered to a human, are not associated with either the development of a humoral (antibody) or cellular immune response.

"Toleragenic" as used herein refers to the capacity of a composition to generate an immunologic response consisting of specific non-reactivity of the immune system to a given antigen that, in other circumstances, can induce cell-mediated or humoral immunity.

"Substantially free" as used herein refers to a composition that contains at least a two-three log reduction in the number of cells of a given type (*e.g.*, white blood cells) from baseline, preferably to a residual level of 10^5 per transfusion (as determined by manual counting using a Neubauer chamber with propidium-iodide staining).

As noted above, the present invention provides non-immunogenic and/or toleragenic platelet compositions, the compositions being substantially free of white blood cells. Critical to achieving such compositions is the use of a combination of filtration and centrifugation steps, which may be performed in either order. In a preferred embodiment, one or more centrifugation steps are performed prior to a filtration step. In addition, as noted above, the present invention provides non-immunogenic platelet compositions. Such compositions are produced through either a combination of centrifugation and UV irradiation or filtration and UV irradiation, which steps can be performed in either order. The present invention also provides non-immunogenic and/or toleragenic red blood cell compositions, which may be produced through a combination of centrifugation and filtration steps (which may be performed in either order).

Within the context of the present invention, platelet compositions may be generated from any platelet-rich product, such as whole blood, platelet-rich plasma,

platelet concentrates, apheresis platelets or buffy-coat prepared platelet concentrates. Similarly, red blood cell compositions may be prepared from any red blood cell-rich product, such as whole blood, packed red blood cells or aphoresis red cells. It will be apparent to those of ordinary skill in the art that it may be advantageous to perform one or more steps prior to centrifugation and/or filtration, depending on the starting material employed. For example, whole blood may be diluted (*e.g.*, 1:1) with Ringer's Citrate Dextrose (RCD) prior to centrifugation and filtration to enhance separation of the platelets from red blood cells. RCD may be prepared by adding 20ml of 0.2micron filtered 1M sodium citrate in sterile, non-pyrogenic, injectable water to a 500ml bag of 5% Dextrose in Ringer's Injection solution.

It has been found within the context of the present invention that, except when starting with buffy coat prepared platelet concentrates, both centrifugation and filtration are required to generate a non-immunogenic and toleragenic product. For buffy coat platelets, it has been found that filtration as described herein is sufficient to generate a non-immunogenic and toleragenic product. Without wishing to be bound by any particular theory, it is believed that white blood cell contamination is a significant source of immunogenicity in blood-derived products. Within the present invention, it has been found that blood contains two distinct populations of white blood cells, and that a combination of centrifugation and filtration steps is necessary to render the product substantially free of white blood cells.

For generating a non-immunogenic and toleragenic product, at least one centrifugation step is preferably performed prior to filtration. A first step of centrifugation as described herein is performed to separate red blood cells from platelets, and to remove some of the white blood cells. Such separation may generally be achieved using one or more soft spins and/or a hard spin. As used herein, the term "soft spin" refers to centrifugation performed at 180 to 2000 xg, depending on the volume of blood being processed. Preferably, centrifugation is at about 250 xg for a blood volume of 30 to 60 ml. Centrifugation times of 4 to 30 minutes are sufficient, depending on the blood volume being processed and the g force used. For a blood volume of 30 to 60 ml, 10 minutes at 250 xg is preferred. The term "hard spin" refers to

centrifugation at 500 to 6000 xg for 4 to 40 minutes, depending upon the volume being processed, preferably about 900 xg for about 10 minutes for a volume of 30 to 60 ml. Any of a variety of commercially available centrifuges, such as a swinging bucket rotor centrifuge (IEC, Needham Heights, MA), may be employed for the centrifugation step(s).

The initial step of centrifugation may be a soft spin or a hard spin. In one preferred embodiment, an initial soft spin is followed by a second soft spin, a hard spin and a filtration step, which may be performed in any order. In another preferred embodiment, an initial hard spin is followed by a soft spin and a filtration step, which may be performed in either order. The order of steps in these preferred embodiments is provided in Table I, below.

Table I

Order of Steps Within Preferred Methods for Preparing Non-immunogenic and/or Toleragenic Platelet Compositions

Step	Representative Method					
1	soft spin	soft spin	soft spin	soft spin	soft spin	soft spin
2	hard spin	soft spin	hard spin	soft spin	filtration	filtration
3	soft spin	hard spin	filtration	filtration	soft spin	hard spin
4	filtration	filtration	soft spin	hard spin	hard spin	soft spin
1	hard spin	hard spin				
2	soft spin	filtration				
3	filtration	soft spin				

While these steps are generally sufficient to generate a non-immunogenic and toleragenic platelet composition, it will be apparent that other centrifugation and/or filtration steps may also be performed at any point within a series of steps provided above.

For example, to prepare a non-immunogenic and toleragenic platelet composition, an initial soft spin may be performed as described above to separate red blood cells from platelets, and to remove some of the white blood cells. A soft spin performed on whole blood results in a lower layer containing red blood cells, an upper

layer containing primarily platelets and plasma, and an interface containing white and red blood cells. For preparing a non-immunogenic and toleragenic red blood cell composition, the upper layer is removed, and it may be filtered directly, as described below.

A hard spin may then be performed to concentrate the platelets. Preferably, the hard spin is performed after the first soft spin and filtration, resulting in the generation of a lower layer of platelet concentrate, and allowing the removal of plasma. The platelet concentrate may then be resuspended and subjected to a second soft spin after filtration for more complete removal of white blood cells. However, the second soft spin to remove additional white blood cells may also be performed on the platelet-rich plasma prior to filtration, after filtration prior to the hard spin, or as described above after both the filtration and hard spin have been performed.

Alternatively, a platelet preparation (a buffy coat platelet concentrate) may be prepared by first performing a hard spin on the whole blood. A hard spin separates the whole blood into an upper layer containing mainly plasma, a lower layer containing red blood cells, and an interface containing white cells, red cells, and platelets. The plasma is removed and the interface containing white cells, platelets and red cells is removed and subjected to a soft centrifugation. Either individual units are subjected to the soft spin, or the soft spin is performed after pooling of individual buffy coats prepared from other blood donations. This soft centrifugation removes white and red blood cells. These buffy coat platelets are then subjected to filtration.

To prepare non-immunogenic and toleragenic red blood cells, at least one centrifugation step (either a hard spin or a soft spin) and a filtration step may be performed. In a preferred embodiment, the lower layer of red cells obtained either following a soft or hard centrifugation of the whole blood is removed and filtered. Alternatively, a red blood cell-rich product such as whole blood can be filtered and then subjected to a hard or soft centrifugation with removal of the lower layer of red blood cells. Filtration may be performed using any of a variety of apparatus and procedures known to those of ordinary skill in the art. For example, suitable filters include Pall filters BPF-4 and RC-10 (Pall Biomedical Products, East Hills, NY).

Filtration step(s) as described herein are performed to further reduce the number of white blood cells. Filtration may be performed using any of a variety of apparatus and procedures known to those of ordinary skill in the art. For example, suitable filters include Pall filters (Pall Biomedical Products, East Hills, NY), such as PLF-1, PLF-10, PL-50 and PL-100, platelet filter TF*IG500 (Terumo, Somerset, NJ) and platelet filter 4C2477 (Fenwal/Baxter, Chicago, IL), with PLF-10 being preferred.

To prepare a platelet composition, if one or more centrifugation steps are performed prior to filtration, the collected supernatant is passed through the filtration apparatus. Within the various embodiments, the step of filtration may be performed a second time, with an expectation of a slightly greater degree of leuko-reduction. However, to achieve the advantages described herein, it is not necessary to filter the product more than once. As noted above, filtration may be performed prior to centrifugation. In such embodiments, the filtrate is then subjected to centrifugation as described above.

The centrifugation and filtration steps described above are sufficient to generate a non-immunogenic and toleragenic product. A centrifuged and/or filtered platelet composition may, however, be further treated by exposure to UV irradiation. Although any of UV-A, UV-B or UV-C irradiation may be used, UV-B is preferred. UV-A represents a wavelength of 320-400 nm, UV-B represents a wavelength of 290-320 nm, and UV-C represents a wavelength of 200-290 nm. The total dose of UV-B irradiation generally results in an exposure of between approximately 600 and 1632 mJ/cm^2, with approximately 612 mJ/cm^2 being preferred. The total dose of UV-C irradiation is preferably between 12 and 36 mJ/cm^2. The intensity of the irradiation (and therefore a determination of the total exposure) may be obtained through use of a radiation detector (International Light, Inc. Newburyport, MA).

The UV-A, UV-B or UV-C irradiation (with or without photoadditive agents (*e.g.*, psoralin)) may be performed at any time in the preparation of the platelet and/or red blood cell composition. It will be evident that a wide variety of apparatus may be used to provide the UV exposure described herein. Such apparatus include a germicidal lamp (General Electric) and a Haemonetics (Braintree, MA) UV-B

irradiation device. Upon preparation of a UV-irradiated platelet or red blood cell composition, one can confirm its non-immunogenic and toleragenic properties *in vitro* by performing mixed lymphocyte culture (MLC) experiments and determining that the residual lymphocytes are no longer able to stimulate or respond in MLC.

As noted above, the non-immunogenic and toleragenic platelet and red blood cell compositions described herein may be used for tolerance induction. Methods of administration of the compositions for this purpose will be evident to those skilled in the art. Tolerance is induced by administering transfusions, generally repeated transfusions, of a platelet or red cell composition to a recipient. Transfusions are usually given on a weekly basis, but other schedules are suitable. Generally, from one to eight transfusions are provided, with at least three transfusions being preferred.

To determine whether alloimmune platelet refractoriness has occurred, both platelet responses and antibody measurements are useful. Platelet responses are measured by determining pre- and post-transfusion platelet counts and calculating platelet increments, % platelet recovery, or corrected count increments. Antibody measurements are made using a variety of tests including platelet and/or lymphocytotoxic antibody tests. Such tests are well known to those skilled in the art.

Alternatively, to determine whether only alloimmunization has occurred, antibody measurements are used. Antibody measurements are made using a variety of tests including platelet and/or lymphocytotoxic antibody tests.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1

Preparation of Platelet Compositions

This Example illustrates the preparation of non-immunogenic and/or toleragenic platelet compositions.

Twenty-seven milliliters of dog blood is drawn via a jugular vein puncture with an 18G 11/2" needle (Becton-Dickinson Immunocytometry Systems, San Jose, CA) into a 30ml syringe (Becton-Dickinson) containing 3ml of Acid Citrate Dextrose formula A (ACD-A anti-coagulant solution). The blood is then mixed with the ACD-A (Fenwal/Baxter, Chicago, IL) by inverting the syringe three times. The anti-coagulated blood is expressed into a 150ml transfer pack (Baxter) to which 30ml of Ringer's Citrate Dextrose (RCD) has been added. RCD is prepared by adding 20ml of 0.2micron filtered (Gelman Sciences, Ann Arbor, MI) 1M sodium citrate (Mallinckrodt, Inc., Paris, KY) in sterile, non-pyrogenic, injectable water (Baxter) to a 500ml bag of 5% Dextrose in Ringer's Injection solution (Baxter). The 150ml pack is inverted three times to mix the ACD blood and RCD and placed in a swinging bucket rotor centrifuge (IEC) for 10 minutes at 250 xg. Platelet rich plasma (PRP) is expressed along with the buffy coat down to the red cell layer via a plasma press (Fenwal) into a second 150ml transfer pack. The PRP is filtered via gravity flow through a PLF-1 filter (Pall) connected to a third 150ml transfer pack. The third transfer pack containing the leucopoor PRP cells is centrifuged in a swinging bucket rotor centrifuge at 900 xg for 10 minutes. Plasma/RCD is expressed into a fourth 150ml transfer pack via a plasma press and saved. The remaining cell pellet is gently massaged in the third transfer pack to resuspend it in the residual solution left by the plasma press. 300μCi of sterile, liquid sodium ^{51}chromate (Amersham) is added and the bag is gently inverted five times to mix the solution. The bag is left to incubate at room temperature for 60 minutes. The unbound 51CR is washed away by transferring the set aside plasma/RCD in the 150ml transfer pack into the 150ml transfer pack containing the 51CR labeled platelets. The components are mixed by inverting three times, and the radioactive platelets pelleted by

spinning for 10 minutes at 900 xg in a swinging bucket rotor centrifuge. The radioactive plasma is decanted and 6ml of RCD is added to the 150ml pack. The pelleted platelets are resuspend by gentle massage, decanted into a 13ml sterile snap cap tube (Falcon #1054) and centrifuged in a swinging bucket for five minutes at 180 xg. The supernatant is aspirated to within 5 mm of the RBC/WBC pellet into a 10ml syringe using a 10cm long piece of transfer pack tubing attached to the end. Five milliliters is used for injection.

This method of preparation was used to prepare the control platelets (*i.e.*, without filtration or UV irradiation, but leuko-reduced by centrifugation) (row 1, Table III), the leuko-reduced composition prepared by centrifugation and UV irradiation (the filtration step described above was not performed, but rather UV irradiation was performed as described in Example 2) (row 4, Table III), and the composition leuko-reduced by both filtration and centrifugation (row 5, Table III).

Example 2

Preparation of Platelet Compositions

This Example illustrates the preparation of further non-immunogenic and/or toleragenic platelet compositions.

Twenty-seven milliliters of dog blood is drawn via a jugular vein puncture with an 18G 11/2" needle (Becton-Dickinson) into a 30ml syringe (Becton-Dickinson) containing 3ml of Acid Citrate Dextrose formula A (ACD-A anti-coagulant solution). The blood is then mixed with the ACD-A (Fenwal/Baxter) by inverting the syringe three times. The anti-coagulated blood is expressed into a 150ml transfer pack (Baxter) to which 30ml of Ringer's Citrate Dextrose (RCD) has been added. RCD is prepared by adding 20ml of 0.2micron filtered (Gelman) 1M sodium citrate (Mallinckrodt) in sterile, non-pyrogenic, injectable water (Baxter) to a 500ml bag of 5% Dextrose in Ringer's Injection solution (Baxter). The 150ml pack is inverted three times to mix the ACD blood and RCD and placed in a swinging bucket rotor centrifuge (IEC) for 10 minutes at 250 xg. Platelet rich plasma (PRP) is expressed along with the buffy coat down to the red cell layer via a plasma press (Fenwal) into a second 150ml

transfer pack. The PRP is filtered via gravity flow through a PLF-1 filter (Pall) connected to another 150ml transfer pack. If the product is to be UV-irradiated, the leucopoor PRP is transferred to a 300ml uv transmissible bag (Stericell by Terumo, Somerset, NJ) and illuminated with a monitored dose (International Light Inc., Newburyport, MA) of 612mJ/cm^2 uvB with agitation on a Haemonetics Platelet Treatment System (Braintree, MA). The UV irradiated leucopoor PRP is transferred to a 150ml pack in which the cells are pelleted in a swinging bucket rotor centrifuge at 900 xg for 10 minutes. Plasma/RCD is expressed into a 150ml transfer pack via a plasma press and saved for later. The remaining cell pellet is gently massaged in the transfer pack to resuspend it in the residual solution left by the plasma press. The platelet suspension is decanted into a 13ml sterile snap cap centrifuge tube (Falcon #2054) and two sequential 3ml rinses of the pack with RCD in a 3ml syringe (Becton-Dickinson) are added to the snap cap tube. This tube is spun in a swinging bucket (IEC) at 180xg for five minutes.

The PRP is removed with a transfer pipette (Globe Scientific, Inc., Paramus, NJ) to within 5mm of the RBC/WBC pellet into another snap cap tube, while the tube containing the RBC/WBC pellet is saved. 300uCi of sterile, liquid sodium 51chromate (Amersham) is added and gently vortexed for one second. The tube is left to incubate at room temperature for 60 minutes. The unbound 51CR is washed away by transferring the 1CR/PRP back to the 150ml pack containing plasma/RCD. The components are mixed by inverting three times, and the radioactive platelets pelleted by spinning for 10 minutes at 900 xg. in a swinging bucket rotor centrifuge. The radioactive plasma is decanted and 6ml of RCD added to the 150ml pack. The pelleted platelets are resuspended by gentle massage, decanted into a 13ml sterile snap cap tube and centrifuged in a swinging bucket for five minutes at 180 xg. Using a transfer pipette, the platelets are transferred into the saved 13ml snap cap tube containing the pelleted RBC/WBC. The platelets and RBC/WBC are mixed gently by squeezing and releasing the bulb of the transfer pipette 5 to 10 times and aspirated into a 10ml syringe using a 10cm long piece of transfer pack tubing attached to the end. Five milliliters is used for injection.

This method of preparation was used to prepare the leuko-reduced composition prepared by filtration (row 2, Table III) and the composition prepared by filtration and UV irradiation (row 3, Table III).

Example 3

Prevention of Platelet Alloimmunization

This Example illustrates the ability of representative non-immunogenic and/or toleragenic platelet compositions to prevent platelet alloimmunization.

Pairs of dogs were selected as donor and recipient. Each recipient's response to transfused radiolabeled donor platelets was determined according to a specified transfusion program. In order to detect immunization to the primary donor, up to 8 weeks of treated platelet transfusions were given form the primary donor (1°). In order to evaluate tolerance induction to the primary donor, up to 8 additional weeks of control platelet transfusions from the primary donor were given, if the dog had not become refractory to their 8 weeks of treated platelet transfusions. In order to evaluate tolerance induction to other donors, up to 8 weeks of control platelet transfusions were given from a secondary (2°) and a tertiary donor (3°). For each of these transfusion schedules, 51Chromium radiolabeled donor platelets were given every week for up to 8 weeks or until the recipient became refractory, defined as less than 5% recovery of donor platelets in the transfused recipient at 24 hours post-injection on 2 sequential occasions. At time of refractoriness, transfusions from the next donor were initiated. In addition to determining platelet refractoriness by radiolabeled platelet survival measurements, some of the transfused recipients also had measurements performed for recipient antibodies against donor platelets, lymphocytes, and red cells by flow cytometry techniques using a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Table II depicts the results of the cell counts done on the platelet products (per dose) prior to injection as prepared by the techniques outlined under Examples 1 and 2.

Table II
Range of Total Cell Counts of Transfusion Products

	Control Platelets	Treated Platelets				
	LR by CENT	LR by Fx1	LR by Fx2	LR by Fx1 & UVI	LR by Cent & UVI	LR by Fx1 *plus* LR & ER by CENT
PLAT*	$1.2 - 3.4 \times 10^9$	$1.8 - 4.7 \times 10^9$	$1.0 - 3.0 \times 10^9$	$1.0 - 2.2 \times 10^9$	$1.3 - 2.8 \times 10^9$	$0.8 - 4.0 \times 10^9$
WBC**	$<3 \times 10^3$*** to 1×10^4	$<3 \times 10^3$ to 1×10^5	$<3 \times 10^3$	$<3 \times 10^3$ to 1×10^5	$<3 \times 10^3$ to 1×10^4	$<3 \times 10^3$

* Determined by automated cell counter (Coulter).

** Determined by manual counting (Neubauer chamber with Propidium-Iodide staining).

*** Limit of sensitivity by the method used for WBC counting

Table III depicts the results of the transfusion experiments. Using modified platelet transfusions from the primary donor, neither the control platelet products that were white cell reduced by centrifugation nor the filtered products--whether filtration was performed either once or twice-- were able to prevent platelet alloimmunization, compared to the products that received more than one type of modification; *i.e.,* platelets that were white cell reduced by centrifugation and then either UV irradiated or filtered, or leukocyte-reduced by filtration and then UV irradiation. Within the group of doubly-modified products, none of these products were significantly different from each other in their ability to prevent primary alloimmunization, and they were equally efficacious in maintaining tolerance to control platelet products from their primary donors. However, only the platelet products that were white cell reduced by centrifugation and also further leukocyte-reduced by filtration were able to induce tolerance to control platelet transfusions from the 2° and 3° donors to whom they had never previously been exposed ($p<0.001$, compared to all

other platelet products given). In other experiments, if plasma free red cells that had been leuko-reduced by centrifugation and filtration or plasma that had been leuko-reduced by centrifugation and filtration were added to the primary donor dog's platelets that had been leuko-reduced by centrifugation and filtration, these platelet products were still non-immunogenic. RBCs for these experiments were prepared by filtering packed red cells (platelet-rich plasma removed) through two BPF-4 filters, centrifuging the filtered RBCs at 1300 xg for 15 minutes and removing the bottom 1-2 cc of RBCs. The RBCs were then washed with saline twice. To prepare the plasma, whole blood was spun at 1300 xg for 10 minutes. The supernatant plasma was removed and filtered through a PLF-1 filter.

Table III

Transfusion Outcomes

Platelets Given	Recip-ient Dogs	Recipients Non-Refractory To Rx Platelets From 1° Donor		Recipients Tolerant To Non-Rx Platelets*** From 1° Donor		Recipients Tolerant To Non-Rx Platelets*** From 2° & 3° Donors	
Control (LR by CENT)*	21	3	(14%)	1	(5%)	ND	
LR by F x1 or x2**	13	4	(31%)	3	(23%)	1	(8%)
LR by F x1 & UVI	14	10	(71%)	8	(51%)	1	(10%)
LR by CENT & UVI	11	6	(55%)	6	(55%)	3	(27%)
LR by F x1 *plus* LR by CENT	10	9	(90%)	9	(90%)	9	(90%)

p=0.05; p=0.004; NS; NS

* Historic controls. No *p* values are given for comparison with current Rx arms.

** Of the 8 dogs who received platelets filtered x1, only 3 were non-refractory (38%), and, of the 5 dogs who received platelets filtered x2, only 1 (20%) was non-refractory. Therefore, as there was no difference between the data for filtered x1 and x2, the data have been combined.

*** All non-Rx platelets from 1°, 2°, and 3° donors are prepared like the control platelets; i.e., they are LR by CENT.

ND Not done.

Example 4

Induction of Immunologic Tolerance

This Example illustrates the use of platelet compositions described in Examples 1 and 2 to induce immunologic tolerance. Of the subgroup of 29 animals in Table III (lines 2-5) which did not become refractory to treated platelets from their primary donors, only 3 (10%) subsequently became refractory to their primary donor's platelets which were leukocyte-reduced by centrifugation. This rate of refractoriness is much lower than the 86% platelet refractory rate seen when this product was given as the initial platelet treatment program (Table III, line 1), suggesting tolerance induction by prior treatment with filtered, filtered-UVI, centrifuged-UVI, and filtered-centrifuged products. In subsequent experiments, it has been determined that animals which have received doubly modified products are tolerant to unmodified platelet transfusions from their primary donor. Such doubly modified platelet and red blood cell products may also be used to induce tolerance to other transfused and transplanted tissues, particularly the centrifuged and filtered leukocyte-reduced platelet and red blood cell products.

Claims

1. A non-immunogenic and/or toleragenic platelet composition, said composition being substantially free of white blood cells.

2. A method of preparing a non-immunogenic and toleragenic platelet composition, comprising:

(a) centrifuging a platelet-rich blood product one or more times and recovering the supernatant; and

(b) filtering the supernatant and recovering the filtrate to produce a non-immunogenic and toleragenic platelet composition.

3. A method according to claim 2, wherein the step of centrifuging comprises a first soft spin and a second soft spin.

4. A method according to claim 3, wherein the step of centrifuging further comprises a hard spin to produce a platelet concentrate, and wherein said hard spin is performed prior to the second soft spin.

5. A method according to claim 2 wherein the step of centrifuging comprises at least one soft spin and a hard spin, and wherein said soft and hard spins are performed prior to the step of filtering.

6. A method according to claim 3 wherein the step of filtering is performed after the first soft spin and before the second soft spin.

7. A method of preparing a non-immunogenic and toleragenic platelet composition, comprising:

(a) performing a first soft spin on a platelet-rich blood product and recovering a supernatant platelet composition, and thereafter, in any order, performing the following steps:

(1) performing a second soft spin on the platelet composition and recovering a supernatant platelet composition;

(2) performing a hard spin on the platelet composition and recovering a supernatant platelet composition; and

(3) filtering the platelet composition and recovering a filtrate platelet composition;

thereby producing a non-immunogenic and toleragenic platelet composition.

8. A method of preparing a non-immunogenic and toleragenic platelet composition, comprising:

(a) performing a hard spin on a platelet-rich blood product and recovering a supernatant platelet composition, and thereafter, in any order, performing the following steps:

(1) performing a soft spin on the platelet composition and recovering a supernatant platelet composition; and

(2) filtering the platelet composition and recovering a filtrate platelet composition;

thereby producing a non-immunogenic and toleragenic platelet composition.

9. A method of preparing a non-immunogenic and toleragenic platelet composition, comprising:

(a) filtering a platelet-rich blood product and recovering the filtrate; and

(b) subsequently centrifuging the filtrate and recovering the supernatant, to produce a non-immunogenic and toleragenic platelet composition.

10. A method according to any one of claims 2, 7, 8 or 9, wherein the platelet-rich blood product is selected from the group consisting of whole blood, platelet-rich plasma, platelet concentrates, apheresis platelets and buffy-coat platelet preparations.

11. A method of inducing immunologic tolerance in a patient to transfused or transplanted tissue, comprising:

administering to a patient a non-immunogenic and toleragenic platelet composition, said composition being substantially free of white blood cells, in an amount sufficient to induce immunologic tolerance to subsequently transfused or transplanted tissue.

12. A method according to claim 11 wherein the transfused or transplanted tissue is selected from the group consisting of bone marrow, skin, pancreas, bone, liver, heart, lung, kidney and cornea.

13. A method according to claim 11 wherein the transfused or transplanted tissue is blood.

14. A non-immunogenic platelet composition, said composition being substantially free of white blood cells.

15. A method of preparing a non-immunogenic platelet composition, comprising:

(a) centrifuging a platelet-rich blood product and recovering the supernatant; and

(b) exposing the supernatant to UV irradiation.

16. A method of preparing a non-immunogenic platelet composition, comprising:

(a) filtering a platelet-rich blood product and recovering the filtrate; and

(b) exposing the filtrate to UV irradiation.

17. A method according to claim 15 or claim 16 wherein the UV irradiation is UV-B irradiation.

18. A method according to claim 17 wherein the UV-B irradiation results in an exposure of between approximately 600 and 1632 mJ/cm^2.

19. A method according to claim 15 or claim 16 wherein the platelet-rich blood product is selected from the group consisting of whole blood, platelet-rich plasma, platelet concentrates, apheresis platelets and buffy-coat platelet preparations.

20. A non-immunogenic and/or toleragenic red blood cell composition, said composition being substantially free of white blood cells.

21. A method of preparing a non-immunogenic and toleragenic red blood cell composition, comprising:

(a) centrifuging a red blood cell-rich blood product and recovering the lower layer ; and

(b) subsequently filtering the lower layer and recovering the filtrate to produce a non-immunogenic and toleragenic red blood cell composition.

22. A method according to claim 21, wherein the step of centrifuging comprises a hard spin.

23. A method of preparing a non-immunogenic and toleragenic red blood cell composition, comprising:

(a) filtering a red blood cell-rich blood product and recovering the filtrate; and

(b) subsequently centrifuging the filtrate and recovering the lower layer to produce a non-immunogenic and toleragenic red blood cell composition.

24. A method according to claim 23, wherein the step of centrifuging comprises a hard spin.

25. A method of inducing immunologic tolerance in a patient to transfused or transplanted tissue, comprising:

administering to a patient a non-immunogenic and toleragenic red blood cell composition, said composition being substantially free of white blood cells, in an amount sufficient to induce immunologic tolerance to subsequently transfused or transplanted tissue.

26. A method according to claim 25 wherein the transfused or transplanted tissue is selected from the group consisting of bone marrow, skin, pancreas, bone, liver, heart, lung, kidney and cornea.

27. A method according to claim 25 wherein the transfused or transplanted tissue is blood.