Platelets, the smallest cellular components of blood, are critically involved at each step of the haemostatic response, from the initial sealing of damaged endothelium to supporting coagulation reactions and finally, retraction of the fibrin clot which enhances fibrinolysis and wound healing. Consequently, if the platelet concentration is decreased and/or the platelet function is abnormal, the risk of haemorrhage is increased.

Ever since Duke's report in 1910 of the first use of platelet-containing fresh whole blood to treat three bleeding thrombocytopenic patients, platelet transfusions have gained value in medicine and nowadays they are essential for the management of patients with primary thrombocytopenia, or for support of those treated with intensive chemo/radiotherapeutic regimens associated with prolonged periods of bone marrow aplasia. Unfortunately, the supply of platelets for clinical use is hampered by an increasing demand and our limited capacity to preserve this blood component under in vitro conditions. Thus, platelet shortage occurs frequently and, consequently, the development of platelet products with longer shelf-life and/or of platelet substitutes is a major goal in modern transfusion medicine.

This overview will briefly discuss the approaches that have been taken over the past few years to develop alternative products to the current 22°C liquid-stored platelet concentrates (PCs).

Drawbacks of conventional 22°C liquid stored PCs

Conventional liquid PCs consisting of platelets with a distinct number of contaminating white cells, re-suspended in autologous plasma, are routinely prepared by fractionation of whole blood units by either the platelet-rich-plasma (PRP) or the buffy coat methods, or directly harvested from blood circulation by apheresis. Although each procedure has advantages and disadvantages, there is a tendency in Western countries to increasing use of single donor PCs obtained by apheresis. Currently, all types of liquid PCs are stored in highly permeable plastic bags at a controlled temperature of 22±2°C, and with constant mild agitation to facilitate gas exchange through the plastic container. However, these storage conditions are by no means optimal, and transfusion of conventional 22°C liquid stored PCs has several drawbacks. First, the transmission of an infectious agent is a major concern for the transfusion of any blood product, and only recently have approaches been developed to attempt inactivation of infectious pathogens - viruses, bacteria, and protozoa - in PCs. Particularly, septic reactions associated with transfusion of bacterial contaminated PCs are a recognised and still unsolved problem of relevant dimensions (approximately 1 in 1,500 transfusions), with potentially fatal consequences for recipients. This risk is higher for PCs than for other blood components stored under refrigeration, such as red cells, since storage at room temperature facilitates the propagation of a potentially present bacterial load. In fact, the increased risk of bacterial growth associated with the 22°C storage temperature is the primary reason for the current 5-day shelf-life of PCs.

In addition, alloimmunisation against histocompatibility antigens occurs in many patients receiving multiple transfusions of random donor platelets. Alloimmunised patients who, in recent experience, may reach as many as 25-35% of newly diagnosed subjects with acute myeloid leukaemia, become refractory and can be extremely difficult to treat with platelet transfusions. Histocompatible donors are often not available, and as many as 50% of apparently histocompatible platelet transfusions administered to alloimmunised patients do not achieve good post-transfusion recovery. Thus, the cost and difficulty of platelet transfusion therapy in these alloimmunised patients are high, and often they remain at risk of haemorrhagic morbidity and mortality. Fortunately, there is now substantial evidence suggesting that the leukocytes contaminating platelet preparations are the primary stimulus for alloimmunisation. Thus, the use of leukodepleted PCs is becoming a routine in transfusion medicine that could drastically reduce the incidence of alloimmunisation in the near future. Additional benefits of leukodepletion of PCs would be the lower incidence and severity of febrile non-haemolytic transfusion reactions mediated by bioactive substances released by passenger leukocytes during storage, and the decreased risks of immunomodulatory effects and of graft-versus-host disease.
Finally, it is well established that during storage of PCs at 22°C serial deleterious changes in the platelet properties occur, collectively referred to as the platelet storage lesion. Thus we, and other groups, have demonstrated that storage promotes platelet shape change, surface expression of activation proteins, loss of GP Ib, and impaired response to agonists. Whether these in vitro observed changes are reversible upon transfusion or significantly affect the in vivo viability and function of platelets remains unclear, but the awareness of the platelet storage lesion is an additional reason for the current 5-day restriction of conventional 22°C liquid-stored PCs. It seems clear that the short shelf-life of PCs under current storage methods results in complex inventory management, loss of units due to outdatedness, difficulty in providing platelets far from the production place, and frequent platelet shortages.

For all the above reasons, much effort has been made in academic and commercial settings to improve the current platelet storage methods, and to develop novel platelet substitutes.

The challenge of designing and evaluating novel platelet products

As mentioned above, platelets have many complex functions (adhesion and spreading on injured vessel, support for coagulant activity, modulation of fibrinolysis, and others). Therefore, an important question in the quest for platelet substitutes is, what function(s) of platelets is needed to be mimicked by the platelet product or substitute? For example, products retaining the platelet procoagulant activity may be sufficient to reduce the risk of venous haemorrhage. By contrast, maintenance of haemostasis in the arterial circulation, where a high shear stress exists, might require most, if not all, of the platelet functions. Thus, we may speculate that in the future it would be possible to tailor and produce specific platelet products for the management of different clinical situations (dilutional thrombocytopenia, immune thrombocytopenic purpura, chemotherapy-induced thrombocytopenia, or a dysfunctional platelet disease).

In the short term, however, we can only hope that alternative platelet products have a few desirable properties. Some of these properties relate to practical aspects such as, need of no specialised storage conditions, prolonged shelf-life compared to that of current PCs, and minimal manipulation before transfusion. Others relate to safety and clinical effects on recipients, such as being sterile or suitable for sterilisation, haemostatically effective during long intervals, and having no major thrombogenic or immunogenic effects. Appropriate demonstration that proposed platelet products accomplish these requirements of safety and efficacy would be a difficult task, because it would need: 1) the performance of in vitro assays valuable for testing platelet function; 2) the carrying out of pre-clinical studies in animal models; and 3) clinical trials in volunteers and/or in patients. In addition, this task would be hampered by 1) the lack of consensus regarding the in vitro tests that best reflect the viability and function of platelets; 2) the controversy about what are valid species for pre-clinical studies regarding generalisation of the data obtained to humans, and about the correct way to induce the animals' thrombocytopenia; and 3) the lack of validated end points or surrogate measurements for demonstration of clinical benefit to the patient population included in trials.

Snapshot of advances in platelet products and substitutes

The research performed in this field over the past few years can be classified in five general areas.

1. Storage of liquid PCs under refrigeration

Early attempts to store platelets were performed, as for whole blood or red cells, at 4°C. However, refrigerated storage was soon abandoned due to the finding that cold temperatures significantly and irreversibly affect platelets. Advances in understanding the cold-induced platelet responses, a process termed cold activation, have shown similarities with physiological agonist-induced platelet activation (shape change, calcium mobilisation, actin filament assembly, aggregation, etc.). This knowledge, and awareness of the risk of bacteriemia associated with transfusion of room temperature-stored PCs, have renewed the interest for development of platelet cold storage.

Several physical methods (increased atmospheric pressures, temperature cycling), and biochemical strategies (cytoskeletal stabilisers, antifreeze glycoproteins, signal transduction inhibitors), for prevention of cold-induced platelet activation have been explored. The microtubule stabiliser agent taxol was used in early attempts to prevent platelet changes induced by chilling, but discoid shape and physiological responses were not fully preserved in treated platelets. Most recently, platelets cooled to 4°C in the presence of the drug cytochalasin, an inhibitor of actin filament assembly, in combination with a cytoplasmic calcium chelator (Quin 2) have been shown to remain discoid and responsive to glass and thrombin activation. Whether this treatment preserves other platelet properties is not known.

Another interesting approach to the cold storage of platelets is the use of antifreeze glycoproteins isolated from polar fish. It has been shown that these proteins prevent, in a dose-dependent manner, the cold-induced platelet shape change and surface expression of activation proteins (LAMP, CD63), and preserve the thrombin responsiveness of cold-stored platelets. The underlying mechanism of these actions is unclear, but some evidence suggests that antifreeze glycoproteins could protect membrane phospholipids from a phase transition change during platelet chilling, thus preventing damage to the membrane.
We, and others, have recently evaluated the storage at 4°C of PCs supplemented with a combination of signal transduction inhibitors.6,7 This cocktail, named ThromboSol, is made up of agents that enhance the platelet cAMP/cGMP concentrations (adenosine, sodium nitroprusside and dipyridamole), amiloride, ticlopidine, and quinacrine. In these studies, ThromboSol protected platelets from morphological changes and spontaneous aggregation during cold storage. Moreover, treated platelets stored-refrigerated for 9 days displayed responses to agonists (ADP, collagen, thrombin) comparable to those of platelets stored at 22°C for 5 days. The protective effect of this additive solution was related to the ability of its components to sustain high levels of cAMP and to inhibit TXA2 production during the entire storage period at 4°C. An additional benefit of cold storage of PCs treated with ThromboSol was impaired production of cytokines (IL6, IL8) compared to that found in PCs conventionally stored at 22°C.  

2. Cryopreservation of platelets  
As for many cell types, freezing has been long considered an alternative for platelet preservation, with the major advantage of extending the storage period from a few days to years. Unfortunately, current methods for platelet cryopreservation require expertise, are labour-intensive, and involve the use of cryoprotectant agents, potentially harmful for recipients, and thus requiring wash-out before infusion. Thus, frozen storage of platelets is not routinely considered and is scarcely used.  

Although it has been shown that cryopreserved platelets display several metabolic and functional changes, they are claimed to exert haemostatic properties when infused in vivo. In fact, several studies have proven the usefulness of frozen platelets in the prophylaxis of bleeding in different clinical settings (cardiopulmonary bypass, onco-haematological patients undergoing high-dose chemotherapy and/or haematopoietic progenitor cell transplantation).8,9  

So far, the most widely used cryoprotectant agent for platelet freezing is dimethyl sulfoxide (DMSO) (at 5 to 10%). Other substances sporadically being employed are glycerol based solutions, hydroxyethylstarch, trehalose, or propylene-1,4-diol. Since DMSO has well recognised adverse effects, a reduction of its concentration in platelet freezing or substitution by less toxic cryoprotectant regimen is desirable to allow direct infusion of frozen-thawed platelets. In this regard, PCs frozen with a solution containing 20% polyvinyl-pyrolidone, 10% mannitol, 5% glycerol, and a mixture of salts, appear to function well when infused to thrombocytopenic rabbits without post-thaw washing of the cryoprotectants. Also, a reduced DMSO concentration (2%) combined with a modified ThromboSol mixture (amiloride, sodium nitroprusside and adenosine) is being tested as an alternative cryopreserving solution. Other authors and our group, have observed that platelets frozen with this solution display in vitro properties similar to those cells cryopreserved with the standard 6% concentration of DMSO. Moreover, platelets cryopreserved in this manner retain their haemostatic function in rabbits, and it has recently been shown that their recovery and survival when re-infused to autologous human volunteers is superior to that of 6% frozen platelets, and comparable to that of platelets stored at 22°C for five days.9  

3. Preservation of platelets in a freeze-dried state  
Freeze-dried platelets are seen as a potentially durable platelet product, lightweight, and easier to transport and store than liquid or cryopreserved PCs. Early attempts in the 50’s to use lyophilised platelets in animal models and in humans were unsuccessful, and this technology was abandoned for many years. However, the strategy has emerged with great vigour thanks to the work of Read et al.9 These authors developed a lyophilisation procedure in which platelets are first stabilised with 1.8% paraformaldehyde for 1 hour, washed, re-suspended in 5% albumin, and then freeze-dried for 24 hours at -20 to -40°C. When re-hydrated, the platelets in the lyophilised product appear to have normal morphology, surface expression of receptors, adhere appropriately to thrombogenic surfaces, and have procoagulant activity. Studies in vivo in rats, rabbits; and dogs have shown that lyophilised platelets correct the bleeding time. In addition, the platelet treatment with 1.8% paraformaldehyde is virucidal and bactericidally effective, providing a 5-7 log reduction in infectious material (including HIV).4,5 Other recent works have also shown the presence of GP receptors Ia and IIa in lyophilised platelets, and have demonstrated that these freeze-dried cells retain their ability to interact with exposed subendothelium in a perfusion model. The safety and efficacy of these lyophilised platelet products in humans remain to be elucidated in clinical trials.  

4. Use of platelet fragments or microparticles  
Activation of platelets is known to induce shedding of platelet membranes or microparticles with procoagulant properties that support the haemostatic function of intact platelets. This microparticle formation also occurs during conventional storage of PCs, and increases with platelet chilling. Recently, human infusible microparticles have been developed and studied.4,5 The manufacturing process involves repeated freezing/thawing of platelets, high speed centrifugation to isolate particulate material, wet heat viral inactivation, and lyophilisation. The resulting product has a phospholipid content similar to that of platelets, and retains varying quantities of platelet membrane receptors and procoagulant activity. It appears to have reduced class I HLA expression, and has a shelf life of three years. These platelet microparticles were first found to shorten the bleeding time with no evidence of toxicity in a rabbit model of busulfan-induced
thrombocytopenia. In phase I studies, human volunteers tolerated the treatment well with platelet membranes, which were not associated with adverse changes in biochemical or coagulation indices. In addition, no signs of immunogenicity (antibodies reactive to normal platelets or lymphocytotoxic HLA antibodies) were observed in fifteen normal subjects injected twice with microparticles for up to two months after the first exposure. There is also a phase II clinical study of microparticles in thrombocytopenic patients with non-life threatening active mucosal bleeding. Sixty-five per cent of patients infused with microparticles achieved improvement or cessation of bleeding. More importantly, 58% of patients refractory to normal platelets did respond to microparticle treatment. No patients experienced serious adverse events attributable to microparticle infusion. These are promising data, but routine bleeding prophylaxis with platelet microparticles awaits definitive confirmation of safety and efficacy in prospective randomised controlled trials.

5. Synthetic or semi-synthetic platelet substitutes

There have been several investigative approaches to preparing synthetic or semi-synthetic products that could sustain some of the properties of functional platelets.1-5

Agam and Livne10 explored the haemostatic properties of red cells coated with fibrinogen. They found that these coated erythrocytes enhance platelet aggregation induced by agonists, and shorten by 4-fold the tail bleeding times in thrombocytopenic rats. Dr. Barry Coller and co-workers11 have developed and evaluated thromboerythrocytes, which are red cells that are covalently coupled with RGD-containing peptides. This Arg-Gly-Asp (RGD) sequence is present in fibrinogen and other ligands that bind to the GP Ib/IIa receptor. Thromboerythrocytes bind to platelets adhered to collagen under static or low shear conditions. They also co-aggregate with platelets stimulated with ADP. However, discrepant results have been found concerning the capacity of thromboerythrocytes to reduce prolonged bleeding time in animal models.

More artificial products are thrombospheres and liposome-based agents. The former are spheres of cross-linked human albumin with fibrinogen covalently bound to the surface. They are small (1.2 μm), thus circulate freely through small vessels with appropriate haemorrhheologic characteristics. These microspheres do not spontaneously clump, but co-aggregate with platelets in the presence of a platelet agonist. When infused as a single bolus to thrombocytopenic rabbits, thrombospheres shorten ear bleeding time and reduce 51Cr-blood loss, while they do not seem to have apparent thrombogenicity. Their safety and efficacy in humans have not yet been investigated.

The haemostatic efficacy of a liposome-like product, namely Plateletsome, has been suggested. This product consists of a deoxycholate extract of platelet membranes, including major glycoprotein receptors, incorporated into unilamellar lipid vesicles. Plateletsomes have no in vitro effect on platelet aggregation, but decrease the tail bleeding time in thrombocytopenic rats by 67%. No evidence of intravascular coagulation is observed upon intravenous infusion of Plateletsomes to rabbits, and no pathologic thrombi were detected on post-mortem examination of treated rats. Procoagulant liposomes have also been explored. In one study, infusion of a combination of phosphatidylinositol (PE)/phosphatidylethanolamine (PC) vesicles and factor Xa normalised the bleeding time in haemophilic dogs. However, this preparation was toxic in dogs and baboons, inducing a decrease in factor V and VIII as well as in the platelet count and haematocrit.4,5 Recently, Galán et al.12 described liposomal preparations that under in vitro conditions of flow, fulfill the procoagulant function of platelets. By using the Baumgartner perfusion system, these authors demonstrated that addition of vesicles made of PC, phosphatidylinositol (PI), phosphatidylethanolamine (PE)/PC (1:1), and (PS/PC) (3:1) to platelet and white cell depleted blood, significantly increased fibrin formation on exposed subendothelium, and increased post-perfusion levels of F1+2, suggesting thrombin generation. These in vitro results further support the value of phospholipid preparations as potential platelet substitutes for treatment of thrombocytopenic patients. However, issues such as toxicity, optimal lipid composition, concentration, size, and stability of these procoagulant liposomes need to be determined.

Concluding remarks

The technologies to produce many kinds of platelet products are in place, and should be encouraged in order to avoid shortages of this blood component, and the problems associated with transfusion of conventional PCs. The major goal now is to develop appropriate ways to assess the safety and efficacy of these products before they are used in the clinical setting.

References

4. Alving BM, Reid TJ, Fratantoni JC, Finlayson JS. Frozen platelets and platelet substitutes in transfusion med-