Peripheral Blood Stem Cell Collection: from Mobilization to Infusion

Hematopoietic progenitor cells are capable of unlimited renewal and differentiation into multiple cell lineages including erythrocytes, leukocytes, lymphocytes and platelets. Progenitor cells in human blood were identified in 1971 and transplantation with peripheral blood progenitor cells (PBPC) was first successfully performed in 1985. PBPC collection and transplantation have been described in previous lab news articles (see 3/1995, 2/1996 and 12/1997), therefore this review aims to provide an overview and update on current practices in the collection and use of PBPC for the purpose of bone marrow transplantation.

Stem Cell Rescue with PBPCs

Currently, hematopoietic progenitor cell transplantation is mostly used to overcome myeloablation following administration of high-dose chemotherapy for resistant hematopoietic cancers. These progenitor cells can be obtained from bone marrow, peripheral blood or increasingly from umbilical cord blood. Transplant with progenitor cells from all three sites have been successfully used to rescue aplastic bone marrow, but PBPC transplantation results in a faster hematological recovery and involves a less invasive collection procedure with the opportunity for multiple collections if more PBPC are needed. Therefore, PBPC are now used more commonly for hematopoietic rescue than bone marrow. Umbilical cord progenitor cell collection is the least invasive procedure of the three options but offers the additional advantage of a ready source; however, the engraftment time can be even longer than bone marrow or PBPC transplantation and multiple cord blood donors are typically needed for a single adult transplant.

Indications and Types of PBPC Transplantation

The major indications for PBPC transplantation are presented in Table 1. In general, PBPC can be obtained either from the patient (autologous transplant) or a donor (allogeneic transplant). In the latter case, the recipient and donor are usually genetically distinct except at the Human Leukocyte Antigen (HLA) site, which typically requires a greater than six out of ten loci match for graft acceptance. Autologous transplants are effective for certain diseases including some lymphomas and solid tumors and obviate the need for post-transplant immunosuppression. Allogeneic transplants are used in diseases that benefit from a graft-versus-leukemia (GVL) effect (e.g., chronic myelogenous leukemia), for diseases that primarily affect the bone marrow (e.g., leukemia) or in those patients with aplastic anemia. Major disadvantages of allogeneic transplant include the potential to develop graft-versus-host-disease (GVHD) and the infections that result from the immunosuppression required to prevent GVHD.

Mobilization

Progenitor cells need to be mobilized (released from the bone marrow into the blood) prior to collection because they are normally found at relatively low levels in the peripheral blood. Using flow cytometry for CD34 expressing cells, peripheral blood generally has 0 – 5 CD34+ cells/μL. Allogeneic PBPC donors (or autologous donors in remission) are typically mobilized with 10 μg/kg granulocyte-colony stimulating factor (G-CSF) daily for 3 – 4 days. Common side effects from G-CSF (filgrastism or Neupogen) include bone pain, headache, fatigue and very rarely splenic rupture. GM-CSF is another growth factor that mobilizes stem cells but has been associated with a higher incidence of side effects and is therefore not routinely used at YNHH.

For autologous donation, the most effective mobilization protocol utilizes both G-CSF and chemotherapy and can be done in an outpatient setting. The chemotherapy regimen is tailored to the type of malignancy being treated and is delivered at non-myeloablative doses three to four weeks following the final cycle of debulking therapy. These regimens often involve one or more alkylating agents (i.e., cyclophosphamide). Approximately 1 - 3 days after starting the chemotherapy regimen, G-CSF is added to enhance mobilization. Once the WBC count begins to recover (usually 7-10 days after starting G-CSF), daily flow cytometric analysis of the peripheral blood CD34+ cell count is attained.

CD34 is a transmembrane protein present on nearly all early hematopoietic progenitor cells; its function remains...
unknown. It is also present on some committed progenitor cells and endothelium, and is not typically present on malignant cells (except for some forms of AML). Approximately 1-5% of bone marrow and mobilized peripheral blood mononuclear cells are CD34+. The flow cytometry lab at YNHH detects CD34 on the surface of the harvested cells with a fluorescent monoclonal antibody (Fig. 1). A cut-off of greater than 20 CD34+ cells/μL of blood is used to start the apheresis collection process, although certain patients (depending on age, diagnosis, previous chemotherapy/radiation regimens and prior mobilization) are not likely to attain this level and may be started at a lower cut-off.

Cytapheresis

The process of cytapheresis involves the continuous centrifugation of blood in a sterile closed-loop apheresis machine resulting in cell layer separation according to density. Blood removal and reinfusion is achieved via either peripheral venous access (16 to 18-gauge needles) or central venous access with a Quinton-Mahurkar dual-lumen large bore catheter. Using a Cobe Spectra or Baxter Amicus instrument, the mononuclear cell fraction is selected, with 1-5% of these cells usually representing CD34+ stem and progenitor cells, and the remaining blood constituents (RBCs, platelets and plasma) are returned to the patient.

Apheresis continues until an adequate number of PBPC are harvested for either 1-3 transplants (usually 2.5 - 5 x 10^6 CD34+ cells/kg per transplant). Collection of enough PBPC for one transplant in a typical patient will usually take 2-3 consecutive days of apheresis. Each collection usually takes 3-5 hours to process 3-5 blood volumes.

Anticoagulation of the blood in the apheresis machine is achieved with citrate (ACD-A) in adults and heparin and/or ACD-A in children. Citrate chelates free calcium in the blood and prevents the clotting cascade from proceeding during the extracorporeal flow of blood through the apheresis device, but does not generally anticoagulate the patient because it is metabolized in vivo. Citrate toxicity, however, can be a major concern during the apheresis procedure, which manifests with symptoms of hypocalcemia (paresthesias, nausea, tetany, seizures and arrhythmias) and is usually prevented with prophylactic IV infusion of calcium gluconate. Potassium and magnesium are also reduced during the procedure and are routinely checked. In addition, patients may experience a vasovagal-like response. As a hematocrit above 28% and a platelet count adequate to avoid bleeding at the catheter site are advised, a daily blood count is attained and appropriate transfusions are administered as necessary prior to initiating the apheresis procedure.

PBPC Processing

Each bag of PBPCs goes through rigorous processing in the Department of Laboratory Medicine’s Frisbee cell-processing laboratory at YNHH that ultimately preserves the cells for future transplantation and assures the quality, quantity and safety of cells. Each collection bag is first evaluated for absolute CD34+ cell count, an aliquot is cultured to assess bacterial or fungal contamination, and the ABO blood type is confirmed. Cells from allogeneic donors are further tested for the presence of allogeneic RBC antibodies. If there is a blood group mismatch between donor and recipient, RBC and/or plasma depletion is performed as needed on the progenitor cell collection prior to transplantation. Other processing options exist that can be used to either reduce potentially contaminating tumor cells (in an autologous transplant) or minimize graft-versus-host-disease (in an allogeneic transplant) including positive selection of CD34+ cells or negative selection of CD3+ cells (T lymphocytes). All stem cell products then undergo any needed volume reduction and addition of DMSO prior to being placed in a controlled-rate liquid nitrogen freezer followed by transfer to a storage freezer in which cells are stored at -196°C. Lastly, an aliquot of the frozen PBPC is tested for viability post-thaw to validate the freezing and thawing protocol.

Transplantation

Prior to infusion of the PBPC product, the patient undergoes high dose chemotherapy with or without radiation to ideally eradicate any residual malignant cells. The cryopreserved cells are then thawed in a 37-degree water bath at the patient’s bedside and infused within 30 minutes to avoid potential cell toxicity from DMSO. During infusion, the DMSO may produce mild side effects including nausea and vomiting.

In an autologous PBPC transplantation, granulocytes usually recover within 8-14 days (defined as an absolute neutrophil count greater than 500/μL) and platelets usually reach 20,000/μL independent of transfusions around day 10. Engraftment may be slower for allogeneic transplants. Prior to leucocyte engraftment, the patients are at increased risk for opportunistic bacterial and fungal infections as well as viral reactivation. In addition, these patients may require transfusion support (irradiated, leukoreduced cellular products) to avoid anemia or thrombocytopenia during the first few weeks following transplantation.

Graft-versus-host-disease (GVHD) is a major complication of allogeneic bone marrow transplants. Immunocompetent T cells from the donor PBSC product target antigens on recipient tissue that are disparate between donor and recipient (often in the skin, liver or GI tract). Immunosuppression with agents such as cyclosporine A or methotrexate is required to prevent GVHD.

Future Directions

The characterization and use of PBPC is clearly an area of intense research. Other mobilization agents under investigation include CXCR4 chemokine receptor blockers (AMD-3100) and other recombinant cytokines and hormones. New protocols trying to limit the extent of GVHD but maximize any GVL effect are under investigation and involve further
selection techniques and processing of the PBPCs. To reduce the potential for tumor contamination of PBPC in patients with B cell tumors, Rituximab has been used prior to harvest to deplete B cells (and therefore tumor cells) from the collection product.

REFERENCES

Table 1. Common Indications for Hematopoietic Stem Cell Transplant at Yale

<table>
<thead>
<tr>
<th>Autologous</th>
<th>Either</th>
<th>Allogeneic</th>
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<tbody>
<tr>
<td><strong>Solid Tumors</strong></td>
<td><strong>Leukemia</strong></td>
<td><strong>Leukemia</strong></td>
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<tr>
<td>Neuroblastoma</td>
<td>AML</td>
<td>CML</td>
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<tr>
<td>Ewing’s Sarcoma</td>
<td>ALL</td>
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<tr>
<td>Rhabdomyosarcoma</td>
<td><strong>Lymphoma</strong></td>
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<tr>
<td>Testicular Cancer</td>
<td>Non-Hodgkin’s Lymphoma</td>
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<tr>
<td></td>
<td>Hodgkin’s Disease</td>
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<td></td>
<td>Multiple Myeloma</td>
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Chronic Myelogenous Leukemia (CML); Acute Lymphoblastic Leukemia (ALL); Acute Myelogenous Leukemia (AML)

Figure 1. Flow cytometric quantitation of CD34+ stem cells in the peripheral blood (A) or in the collection product (B). The gate to the right of “R4” demarcates the CD34+ cells.
JAK2 Gene V617F Mutation Test by PCR

The Molecular Diagnostics Laboratory in the Department of Laboratory Medicine now tests for Janus kinase 2 (JAK2) mutations in conjunction with the diagnosis and classification of some myeloproliferative disorders (Campbell and Green, 2006).

Janus kinase 2 (JAK2) is a cytoplasmic tyrosine kinase, an important regulatory protein of the JAK-STAT signaling pathway, the gene for which is located on chromosome 9. The protein is known to bind to cytokine receptors such as the erythropoietin receptor and affect STAT binding through tyrosine phosphorylation. JAK2 is made up of several domains, including the JH1 kinase domain, which is regulated by the adjacent JH2 pseudokinase domain. Recently, five research groups have discovered that a mutation in the JAK2 gene is associated with certain chronic myeloproliferative diseases (MPDs) (Baxter et al., 2005, James et al., 2005a, Levine et al., 2005, Zhao et al., 2005, Kralovics et al., 2005).

Previously it was demonstrated that MPDs had abnormal responses to numerous cytokines. This was clearly seen in polycythemia vera (PV) patients where erythroid progenitor cells proliferate and differentiate without erythropoietin [endogenous erythroid colony (EEC) formation] and formed the basis of a diagnostic test for PV. When Gleevec, an inhibitor of the BCR-ABL tyrosine kinase fusion protein, came into use, it was found that Gleevec also inhibited EEC formation in PV patients, suggesting that a tyrosine kinase was involved. In addition, it had been shown earlier that 30% of PV patients had a 9pLOH lesion. These three observations were variously exploited by the five groups to find the JAK2 mutation, either through (a) analysis of genes lost in the 9pLOH region, (b) demonstration that inhibitors of JAK2 blocked EEC formation, or (c) screening for tyrosine kinase mutations using high throughput DNA sequencing. The single mutation found is a G to T nucleotide change located in the JH2 region which causes replacement of valine with phenylalanine at amino acid position 617 (V617F) and facilitates a “gain of function” mutation where JAK2 becomes constitutively phosphorylated and active.

Myeloproliferative disorders are clinically diverse but derived from the clonal proliferation of myeloid stem cells. In the case of polycythemia vera (PV), the JAK2 V617F mutation is present in multipotent hematopoietic stem cells and their myeloid-restricted progeny (Jamison et al., 2006). Another study found the JAK2 V617F mutation in B and T lymphocytes in a subset of PV patients (Ishi et al., 2006). Although the currently available data clearly demonstrate that the JAK2 V617F mutation participates in the pathogenesis of the MPDs, it is generally agreed that the exact role for activated JAK2 signaling in the pathogenesis of these MPDs remains uncertain.

The association between the JAK2 V617F mutation and various hematologic diseases has been studied by several methods. Analysis of the various studies that use allele-specific PCR, which is the most sensitive method, has shown the frequency of the JAK2 V617F mutation in PV patients to be 97%, 49-57% in patients with essential thrombocytopenia (ET), and for chronic idiopathic myelofibrosis (CIMF) patients, it is 57% (Michiels et al., 2007). The JAK2 V617F mutation also occurs in a homozygous state in 25% to 30% of PV patients and 2% to 4% with ET (Baxter et al., 2005, James et al., 2005a, Levine et al., 2005, Zhao et al., 2005, Kralovics et al., 2005). In addition, the JAK2 V617F mutation can occasionally be detected in unclassified MPDs, myelodysplastic syndromes, chronic myelomonocytic leukemia, systemic mastocytosis, chronic neutrophilic leukemia, and hypereosinophilic syndromes (James et al., 2005b).

The Molecular Diagnostics Laboratory in the Department of Laboratory Medicine currently performs testing for the JAK2 V617F mutation. This PCR amplification reaction contains primers (common forward and control specific reverse primers) for an internal control region flanking the JAK2 V617F mutation and a reverse primer which recognizes the JAK2 V617F mutation (Baxter et al., 2005). After amplification the PCR products are electrophoresed on an agarose gel. A successful negative result is the presence of the internal control band (364bp), but absence of the JAK2 V617F-specific mutation band (208bp), while a successful positive result is the presence of both internal control and JAK2 V617F-specific mutation bands. The current method is sufficiently sensitive to detect the presence of a heterozygous mutation in as few as 5-10% mutant cells in the background of normal cells. The JAK2 V617F mutation is not detected in normal individuals. At present, it is not recommended that the assay be made to be more sensitive because of the possibility of a false positive result. A negative result does not completely rule out the presence of the JAK2 V617F mutation in a small number of cells or the possibility of a diagnosis of PV, ET or CIMF.

The usefulness of the JAK2 V617F mutation test has been recently highlighted in a publication describing changes to the 2001 WHO classification of MPDs (Tefferi et al, 2007). Most notable is the inclusion of the presence of a JAK2 V617F mutation in the WHO criteria for diagnosing PV, ET, and CIMF. This has led to a significant simplification of the WHO criteria especially for PV where the WHO 2007 major criteria are only erythrocytosis and the presence of the JAK2 V617F mutation. The WHO 2001 PV major criteria included erythrocytosis, lack of secondary erythrocytosis, splenomegaly, evidence of a clonal abnormality other than BCR-ABL and presence of endogenous erythroid colony formation.

There has been some question as to whether patients who are heterozygous for the JAK2 V617F mutation have a milder disease than patients that are homozygous for the mutation. Recent work has not shown this to be true for patients with PV; however, there does appear to be a difference for patients with ET (Vannucchi et al, 2007). ET patients homozygous for the JAK2 V617F mutation were found to have a significantly higher risk of cardiovascular events than either normal or heterozygous mutant patients. This observation, if confirmed, as well as the possibility for future development of targeted
new therapeutics may necessitate the development of a quantitative JAK2 V617F mutation test in the future to better follow outcomes. As noted, the current JAK2 V617F mutation test, which is not quantitative, is performed at YNHH Laboratory Medicine, and the preferred specimen is 3 ml of whole blood in an EDTA (lavender top) tube.

REFERENCES


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John Greg Howe PhD

Testing for Helicobacter pylori

*Helicobacter pylori* is a gram-negative spiral or comma-shaped bacterium that infects as much as 50 percent of the world population. Its role in the causation of gastritis and peptic ulcer disease was originally established by Robin Warren and Barry Marshall in 1983 for which they subsequently won the Nobel Prize in Medicine in 2005. It has also been shown to be associated with other gastrointestinal diseases including gastric carcinoma, and gastric mucosal-associated lymphoid tissue (MALT) lymphoma.

Age, socioeconomic status, and ethnic group appear to influence the rate of infection. In the United States, the prevalence rate is 26% in Caucasians compared to 50% and 60% in African-Americans and Mexican-Americans respectively. In developing countries the prevalence at age 50 is 90% compared to 50% for developed countries. In most patients, the organism is acquired by fecal-oral transmission in childhood and the rate of acquisition is positively associated with lack of available public health sanitation. In areas where sanitation has improved, rates of infection have decreased.

The organism appears to be restricted to the duodenum and stomach and can be seen in a patchy distribution. Patients who are found to have the organism are divided into two groups. The first group has no signs or symptoms of gastrointestinal disease and these are thought to be “colonized”. The second group is considered “infected” because of the presence of signs and symptoms.

The indications for *H. pylori* testing are shown in the table 1. Current guidelines for *H. pylori* testing are for those patients that are “infected” but fail to address those that are “colonized.” As yet, asymptomatic routine screening has not become standard of care even though many individuals (50% in the US) harbor the organism and can transmit it to others. In order to eradicate *H. pylori*, future testing and treatment must address those that are “colonized”.
ammonia and carbon dioxide. The patient is given 13C or 14C isotopically labeled urea, as well as specialized equipment for detection of the labeled CO₂. The clinical microbiology laboratory performs the newer version of the stool antigen test- the ImmunoCard STAT!® HpSA®. This test is a rapid in vitro qualitative immunoassay that uses a monoclonal anti-*H. pylori* antibody to detect *Helicobacter pylori*-specific antigens in stool. It is approved for use in the initial diagnosis of *H. pylori* infection as well as response to treatment with post-treatment testing after an interval of 4-8 weeks.² ⁵ ⁶

In summary the Clinical Microbiology Laboratory in the Department of Laboratory Medicine offers a sensitive stool antigen assay for the identification of *H. pylori* antigen. This test appears to have good sensitivity, specificity, and predictive values in adults and children for use in the diagnosis of suspected cases and follow-up of antibiotic treatment in *H. pylori* infection, albeit with several caveats noted above. Although other alternative diagnostic methodologies are available and a single "best" approach is not defined, among non-invasive tests, the stool assay has advantages of simplicity and does not require radiolabeled isotopes.

### Table 1. Indications for Helicobacter pylori Testing

<table>
<thead>
<tr>
<th>Definite Indication</th>
<th>Controversial</th>
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<tbody>
<tr>
<td>Gastric MALT lymphoma</td>
<td>Non-ulcer dyspepsia</td>
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<tr>
<td>Current or past ulcer (not previously treated)</td>
<td>Chronic NSAID or PPI drug therapy</td>
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<tr>
<td>Previous early gastric cancer resection</td>
<td>Gastroesophageal reflux disease</td>
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<tr>
<td>Uninvestigated dyspepsia</td>
<td>Populations at higher risk for gastric cancer</td>
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<td></td>
<td>Unexplained iron deficiency anemia</td>
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Several diagnostic options are available including invasive and non-invasive methods. Invasive methods require endoscopy and include biopsy with histology (giemsa stain), culture of biopsy tissue, and rapid urease testing of biopsy tissue. Advantages to these tests include their ability to detect active infection, and their high specificity and positive predictive value. On the other hand, these tests are expensive, uncomfortable to the patient, and carry a small risk secondary to the endoscopic procedure. Sampling is also another limitation, as the organism is found in patches that might be missed by biopsy.

Non-invasive tests such as serology, the urea breath test (UBT), and the relatively new *H. pylori* stool antigen test (SAT) have become popular methods utilized in patients not requiring biopsy. These methods do not require endoscopy and are relatively simple to perform. Inexpensive serological tests are based on the detection of IgG against *H. pylori* and have been used for screening of patients with uncomplicated infections. They do not differentiate between current or past infection, but are useful in certain populations depending on the rate of infection, and they also have a high negative predictive value. The urea breath test detects urease that is produced by the organism. This enzyme splits urea into ammonia and carbon dioxide. The patient is given ¹³C or ¹⁴C labeled urea and the ¹³CO₂ or ¹⁴CO₂ diffuses from the superficial gastric epithelium into the bloodstream where it is exhaled, captured by a CO₂ trapping agent, and detected with a scintillation counter (¹³CO₂) or mass spectrometer (¹⁴CO₂).⁴ This test is highly sensitive and specific, but has several drawbacks in that it is time consuming, and requires ingestion of isotopically labeled urea, as well as specialized equipment for detection of the labeled CO₂. In comparison to the urea breath test, the stool antigen test performs better in patients that have had previous gastrectomy or have end stage renal disease, but worse in cirrhotic patients. Also, studies suggest that acute GI bleeding, has a negative impact on the sensitivity, specificity and predictive values for detecting the *H. pylori* infection.

Two important caveats of the organism-based (culture, histology, rapid urease, urea breath test, and stool antigen) tests must be addressed. Concurrent or recent administration of proton pump inhibitors, antibiotics, or bismuth derivatives can result in a false negative result due to inhibition of the organism.¹⁰ These drugs do not affect *H. pylori* antibody testing. Secondly, the stool antigen test should be repeated no earlier than 4 weeks after discontinuing antibiotic therapy.² ³ ¹⁰

In general, studies have shown that the stool antigen test produces results that are comparable to that of the urea breath test.⁴ ⁵ ⁶ Both are recommended for use in diagnostic screening within the context of a "test and treat approach" in patients under 55 with no alarm signs indicative of severe disease.⁶ ⁷ A meta analysis of 89 studies (10,858 patients) evaluating a stool antigen test showed an overall sensitivity of 91%, specificity of 93%, positive predictive value of 92%, and negative predictive value of 87%.⁵ When the analysis was restricted to monoclonal antibody assays, the sensitivity was 96%, specificity was 97%, positive predictive value was 96%, and negative predictive value of 97%. Further, 39 studies assessed the presence of *H. pylori* after therapy with antibiotics and showed a sensitivity of 86%, specificity of 92%, positive predictive value of 76% and a negative predictive value of 93%. These findings improved when the analysis was limited to six studies that utilized a monoclonal antibody.

In contrast to the urea breath test, the stool antigen test performs better in patients that have had previous gastrectomy or have end stage renal disease, but worse in cirrhotic patients. Also, studies suggest that acute GI bleeding, has a negative impact on the sensitivity, specificity and predictive values for detecting the *H. pylori* infection.
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