

CHAPTER 3

LABORATORY PROCEDURES

CORD BLOOD TRANSPLANTATION STUDY PROTOCOL

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3.1 HLA TYPING

Molecular HLA typing will be performed for all donor cord blood units and patients in the three reference laboratories identified for the COBLT study. The laboratories are led by Dr. LeeAnn Baxter-Lowe, University of California, San Francisco, Dr. Elaine Reed, University of California - Los Angeles, and Dr. Jennifer Ng, Navy Medical Research Institute.

Initial HLA typing will be done at low resolution for class I, HLA-A, -B, and at intermediate to high resolution for HLA-DRB1. Retrospective typing will be done for all donor-patient pairs. The retrospective typing will be performed at high resolution (corresponding to a single allele for most samples) for HLA-A, -B, -C, DRB1, and DQB1. The types used will be defined according to the current WHO Nomenclature Committee for Factors of the HLA System. These will be updated as necessary. Supplemental typing of HLA-DQA, DPB, and DPA may be determined at a later date.

The specimens for HLA typing will be: a) for the cord blood unit - frozen aliquots from the granulocyte/red cell-enriched pellets that remain after preparation of the cord blood unit, or appropriate sample if unit is obtained from a non-COBLT cord blood bank, b) patient - blood samples from the recipient.

Appropriate HLA typing reagents are described in the detailed protocols of each reference Laboratory including oligonucleotide sequences of PCR and sequencing primers. A list of these reagents will be maintained by the Laboratory in compliance with ASHI regulations. Reagents may be revised during the project as techniques and knowledge of HLA polymorphism improves. Historic records regarding reagents are maintained in accordance with ASHI regulations.

Samples will be typed according to the appropriate SOPs within the reference Laboratory. The final data will be interpreted with respect to a list of recognized HLA alleles that is maintained by the Medical Coordinating Center (MCC).

Each reference laboratory will send to the MCC a report and an electronic data file containing the following information for typings completed during the week:

- a. Specimen identification, including bar code label number
- b. Assigned type
- c. HLA alleles that are potentially present in the specimen
- d. Special notation for samples with unusual linkage
- e. Typing method
- f. Data required by the MCC to update typing assignments as knowledge of HLA polymorphism improves

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3.2 CHARACTERIZATION OF GRAFT

A list of the variables proposed for characterizing the cells present in umbilical cord blood units banked for transplant is shown in the table below. With the exception of CD3 each of these measures is in widespread use for characterization of both autologous and allogeneic hematopoietic grafts. CD34-positive cell assay and the colony forming cell assays (CFU-GM and BFU-E) are included as they have the potential to provide a measure of the hematopoietic engraftment potential of the umbilical cord blood unit. In addition, the colony assays provide a direct measure of the viability of hematopoietic progenitor cells. This parameter would otherwise be extrapolated from the viability of the population as a whole as determined by trypan blue dye exclusion. CD3 is included in this panel because the content of T-lymphocytes might have an impact on the incidence and severity of GVHD following transplant.

Variables Used in Graft Characterization

Following red cell and plasma depletion, but prior to addition of cryoprotectant:	
nucleated cell count	automated cell counter e.g.: Coulter MD II 8
leukocyte count	manual differential count
mononuclear cell count	manual differential count
viability	trypan blue dye exclusion
CD34-positive cell count	flow cytometry
CD3-positive cells count	flow cytometry
CFU-GM	colony-forming cell assay
BFU-E	colony-forming cell assay
At the time of thaw/transplant:	
nucleated cell count	automated cell counter e.g.: Coulter MD II 8
viability	trypan blue dye exclusion

To be defined as CD34-positive in this study a cell must exhibit each of the following properties.

- ! low to intermediate side scatter
- ! CD34-bright (high fluorescence intensity)
- ! CD 45-dim (clearly CD45-positive, but not as intensely positive as CD34-negative cells)

To be defined as CD3-positive in this study a cell must exhibit each of the following properties.

- ! low forward and side scatter
- ! CD3-bright (high fluorescence intensity)
- ! CD 45- bright (high fluorescence intensity)

The flow cytometric panel performed at the COBLT Cord Blood Banks for graft characterization prior to cryopreservation is shown in the table below. The CB34⁺ subsets (34⁺/61⁺, 34⁺/90⁺, and 34⁺/38⁻) will be discontinued when sufficient numbers of units have been evaluated as determined by the COBLT Bank Subcommittee.

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Tube	Marker	Stains
1	Procount (or equivalent)	Nucleic Acid Dye/34/45
2	Procount Control (or equivalent)	Nucleic Acid Dye/Gamma 1/45
3	34+/61+	CD61 FITC / CD34 PE / CD45 (Per-CP or equivalent)
4	34+/90+	CD90 FITC / CD34 PE / CD45 (Per-CP or equivalent)
5	34+/38-	CD38 FITC / CD34 PE / CD45 (Per-CP or equivalent)
6	34+/Control	IgG1 FITC Control / CD34 PE / CD45 (Per-CP or equivalent)
7	19+/16+ & 56+	CD19 FITC / CD16 & 56 PE / CD45 (Per-CP or equivalent)
8	Tri-test or equivalent	CD4 FITC / CD8 PE / CD3 (Per-CP or equivalent)

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3.3 IMMUNE RECONSTITUTION

3.3.1 Introduction and Objectives

Two of the principal problems that occur following allogeneic transplantation are graft versus host disease (GVHD) and post-transplant immunodeficiency. After marrow transplantation, the severity of acute GVHD increases as the difference in HLA antigens between donors and recipients increases. Recipients of unrelated-donor marrow have an immunodeficiency of greater severity compared to recipients of related marrow than can be explained by the increased severity and frequency of their acute GVHD. Thus, other factors beside GVHD may contribute to the prolonged immunodeficiency observed in recipients of unrelated marrow. Among the reasons hypothesized for the increased immunodeficiency are the effects of GVHD on the thymus (4), defects in differentiation of donor T cells in the recipient, and histo-incompatibility between donor-derived T cells and patient antigen presenting cells. It is of interest to determine the extent to which this prolonged immunodeficiency occurs following cord blood transplantation.

There is no permanent carryover of antigen specific T lymphocyte or B lymphocyte functions. Antigen specific immune function after transplantation depends on the development of new antigen specific T cells that differentiate through the recipient thymus, and on subsequent development and maturation of new functional B cells. Most of the patients in the post-transplant period are receiving IVIG. Therefore, development of B lymphocyte function can only be assessed by immunization of the patients with neo-antigens. For this reason, the objectives of this study are to characterize the regulation of the production of new T cells in the recipient, evaluate the antigen specific T cell response to infectious antigens (herpes simplex, HSV; varicella, VZV; cytomegalovirus, CMV) and following immunization (tetanus toxoid, TT), determine the role of cytokines (IL-2 and IL-7) in antigen specific function, and to investigate the capacity of recipient T cells to interact with B cells to induce specific antibody production. Data will be analyzed considering patient age at transplant, primary disease, and degree of HLA mismatch. Each assay will be performed in a single laboratory to eliminate inter-site variation.

3.3.2 Specific Aims

The three specific aims of the laboratory studies of immune reconstitution are listed below.

Specific Aim 1 - Production of New Lymphocytes:

The hypothesis for this aim is that following cord blood transplant, the patients without GVHD will develop antigen specific T lymphocytes within 9 months, and that the development is delayed in patients with GVHD.

Peripheral blood samples will be obtained to evaluate immunological responses according to the schedule in Table 3.3.3.1.

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Specific Aim 2 - Antigen Specific T Cell Function

The hypothesis for this aim is that cord blood transplant patients will develop a proliferative response to the mitogen PHA by three months post transplant, a response to tetanus toxoid immunization by six months, and a response to herpes viral antigens by 6 months. The secondary hypotheses that exogenous IL-2 and IL-7 will hasten the antigen specific responses and occurrence of GVHD will delay the antigen specific responses will be evaluated. The antigen specific response will be correlated with the appearance of T cells as determined in specific aim 1.

Specific Aim 3 - T and B Cell Cooperation:

The hypothesis for this aim is that cord blood transplant recipients produce antibodies to Φ X 174 antigen synchronously with development of expression of CD40 ligand.

3.3.3 Schedule and Samples

Immunizations will be performed and peripheral blood mononuclear cells isolated from transplant patients will be tested according to the schedule in Table 3.3.3.1. Procedures for immunization, antibody analysis and CD40 ligand induction are specified in chapter 9 of the MOP. Use of the phage antigen Φ X174 allows assessment of a specific antibody response in patients receiving immune globulin. Antibody isotype, avidity, and class switching will be studied.

For immunophenotyping, specimens will be prepared as described in MOP chapter 9 and analyzed by two color immunofluorescence using the monoclonal antibody combinations displayed in Table 3.3.3.2. Three color immunofluorescence will be used for CD4/CD45RA/CD45RO.

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**Table 3.3.3.1
SCHEDULE OF IMMUNE EVALUATION**

Forms	Months Post-Transplant																			
	1	2	3	4	5	6	7	8	9	10	11	12	15	18	21	24	30	36	42	48
Immunophenotyping ¹	X	X	X			X			X			X		X		X		X		X
PHA Response ²	X		X			X			X			X		X		X		X		X
Antigen Specific Blastogenesis ³			X			X			X			X		X		X		X		X
Tetanus Toxoid Immunization			X			X						X								
ΦX174 Stimulation												X				X		X		X
CD40 Ligand Expression ⁴												X				X		X		X

¹ CD3, 4, 8, 56, 19/20, 4/8, RA/RO/4

² 3 doses of PHA ± IL-2

³ Tetanus toxoid, varicella zoster, CMV, herpes simplex, ± IL-2, IL-7

⁴ After PHA/PMA stimulation

**Table 3.3.3.2
MONOCLONAL ANTIBODY PANEL FOR IMMUNOPHENOTYPING-
IMMUNE RECONSTITUTION**

FITC Antibody	PE Antibody	Detects
IgG1	IgG2a	Non-specific Binding
IgG2a	IgG1 ¹	Non-specific Binding
Anti-CD3 ²	Anti-CD4	Helper T Cell Subset
Anti-CD3	Anti-CD8	Cytotoxic T Cell Subset
Anti-CD4	Anti-CD8	Double Positive T Cells
Anti-CD45RA ³	Anti-CD45RO	Naive vs Memory T Cells
Anti-CD16	Anti-CD3	NK Cells
Anti-CD3	Anti-CD19	B Cells

¹ Plus additional isotype controls as required

² CD3+ cells will be characterized as CD3^{dim} or CD3^{bright}

³ Three-color immunofluorescence using CD4

3.4 CHIMERISM

TO BE COMPLETED