CHAPTER H1

Human Vertebral Bodies as a Source of Bone Marrow for Cell Augmentation in Whole Organ Allografts

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Since 1959, cadaveric vertebral bodies (VB) have been recognized as a potential source of bone marrow for transplantation, when Ferrebee et al, reported for the first time a method of harvesting bone marrow cells directly from VB obtained during autopsy.[1] Their method, though crude, involved dissociating cells from small pieces of marrow by gentle chopping and mixing in a stainless steel chopper. This process would yield a single cell suspension which was subsequently collected into a reservoir following passage through a 30-mesh filter.[1] A more rapid, simple and economical method for obtaining large yields of fat-free, well dispersed hematopoietic cells from the VB was reported by Ray et al in 1964.[2] This procedure involved compression of surface-sensitized VB in a sterile pyrogen-free plastic container. The extruded marrow was then homogenized, filtered and the fat was removed by low speed centrifugation. Additionally, when this marrow was used to treat two patients with aplastic anemia, partial clinical and hematological remission was seen in only one patient who had received five concurrent bone marrow transfusions.[2]

However, it was not until 1970, that Thomas and Storb, by describing their novel technique for marrow recovery from the iliac crest of live related donors, established the basis for clinical bone marrow harvesting that remains in use today.[3] They addressed several important issues including anesthesia, sites of aspiration, and the preparation of the bone marrow cell suspension. A consequence of their pioneering work was the establishment of allogeneic bone marrow transplantation from living related donors as a basis for treating severe aplastic anemia and certain hematologic malignancies. Despite of obvious clinical implications, its therapeutic applications were limited since HLA matching was required to decrease the risks of rejection and graft-versus-host-disease (GVHD).[4,5] The interest in VB bone marrow harvesting was, however, rekindled in 1984, when Sharp et al described a unique method for surgical harvesting of cadaveric VB with subsequent bone marrow isolation.[6] They reaffirmed the importance of VB as a reliable source of nucleated bone marrow cells by emphasizing the higher cell yields and the relative ease of harvesting VB at the time of multi-organ procurement, thus making them more feasible than iliac crest as a potential source of bone marrow from cadaveric donors. Furthermore, they also postulated that marrow from cadaveric VB by containing fewer mature T cells, would be less likely to induce lethal GVHD, than that harvested from the iliac crest of live donors, which is routinely contaminated by blood-borne mature lymphocytes.[7,8]

The concept of actively acquired tolerance to whole organ allografts following donor bone marrow infusion was first conceived by Billingham, Brent and Medawar.[9] This idea was further extended by Slavin and Strober[10,11] who demonstrated stable chimerism with subsequent induction of donor-specific tolerance in rats, that were conditioned with fractionated total lymphoid irradiation (TLI) prior to bone marrow or whole organ transplantation. Sachs et al, using similar conditioning protocols, had

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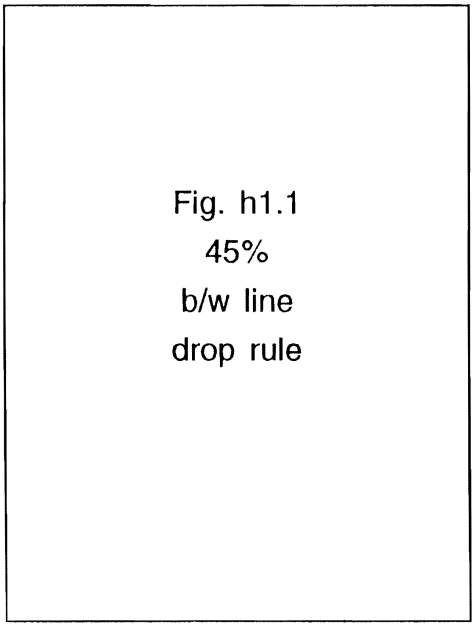
evidence of stable chimerism in rodents that were reconstituted with mixed allogeneic and xenogeneic bone marrow.[12,13,14] However, creation of "space" by prior conditioning of animals with radiation or other cytoreductive/cytoablative regimens was considered an obligatory step towards engraftment of donor bone marrow cells. Similar donor-specific hypo-responsiveness was also induced in large animals by infusion of bone marrow in irradiated recipients, prior to whole organ Tx.[15-18]

Instead of using host irradiation as an imperative prerequisite for subsequent donor bone marrow engraftment Monaco et al used anti-lymphocyte serum (ALS), for conditioning in recipients of combined bone marrow and whole organ transplants leading to induction of donor-specific tolerance.[19-22] Thomas et al,[23,24] using a five-day course of rabbit anti-thymocyte globulin [ATG], and ribs as a source of marrow, were also able to successfully induce transplantation tolerance in primates, which in their model was attributed to the presence of cells having "veto" function in donor bone marrow.[25]

However, the last 2 years have seen an explosion in the field of bone marrow and whole organ transplantation, triggered by a classical report in Lancet by Starzl et al,[26] which stressed for the first time the importance of donor bone marrow-derived cells in whole organ transplantation, and their pivotal role in the induction of donor-specific non-reactivity. The propriety of this paper hinged on a key observation that after whole organ transplantation, donor cells of hematolymphoid origin, migrate out of the graft and into the recipient, leading to the establishment of chimerism, which is the first step towards subsequent induction of donor-specific hypo-responsiveness.[27] This natural and universal phenomenon of donor cell migration after whole organ transplantation and its implications in the induction of donor-specific tolerance, has opened up new avenues in pre-clinical and clinical research.[28,29] Our experience is based on a trial of concomitant donor bone marrow and whole organ transplantation with infusion of donor bone marrow cells at the time of whole organ transplantation, without prior conditioning of the recipient. This chapter reviews the procurement of cadaveric VB from 50 donors and its use as a source of donor bone marrow. It also details the surface phenotyping of bone marrow cells isolated from cadaveric VB.

Fi. H1.1



PROCUREMENT OF CADAVERIC VERTEBRAL BODIES

All the donors were obtained through the Center for Organ Recovery and Education (CORE). The surgical technique for multi-organ harvesting has been described previously.[30] After all the solid organs have been retrieved, the surgical field is prepared for the resection of the vertebral column (VC) (Fig. H1.1). The protocol employed to harvest VB is a modification of an earlier technique described by Strong et al,[31] and all instruments and solutions utilized are listed in the Appendix I and II. The abdominal and thoracic cavities are aspirated and in order to expose the VC, bowel, mesentery, diaphragm, lungs, esophagus and other soft tissues are displaced. The psoas muscle is dissected free of bone by scalpel and the intervertebral discs are identified. A straight wide Lambotte osteotome is inserted using manual pressure between the L5-S1 interspace (Fig. H1.2), and the VC is retracted using the anterior face of S1

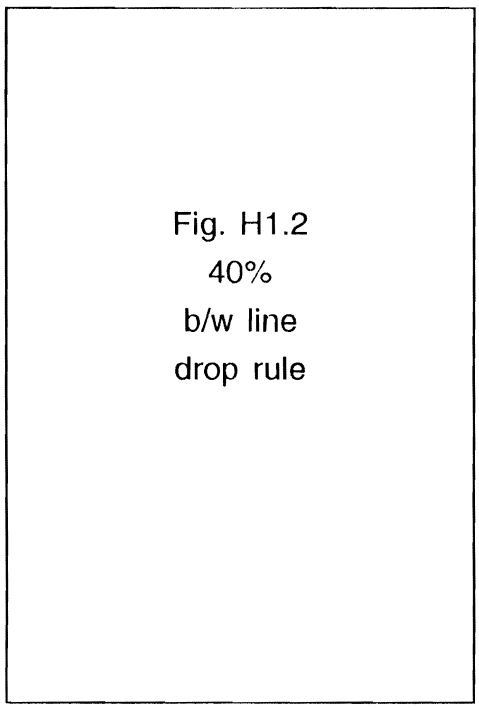


Fig. H1.2

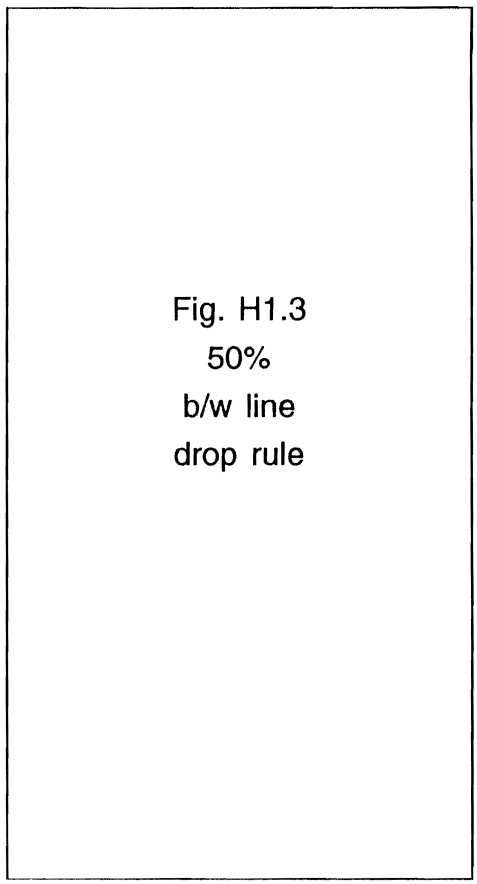
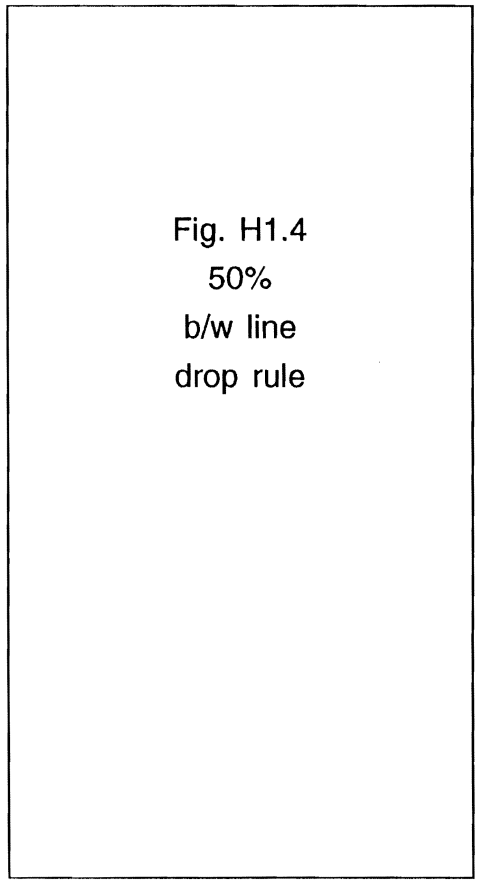


Fig. H1.3

as a fulcrum. The posterior intervertebral articulation is then dissociated using a curved osteotome and mallet. The paravertebral muscles are dissected free of the column, and kept on traction by means of an osteotome. The posterior interspinal ligaments are then severed and a cloth towel is inserted around S1, displacing the osteotome. Using the cloth towel for traction, the entire column is retrieved. and curved osteotome and mallet are used to separate the transverse processes and ribs from the VC (Fig. H1.3). The extent of VC resection however, depends on the number of bone marrow cells required. This procedure makes it possible to resect the entire thoracic and lumbar VC en-bloc (Fig. H1.4), in approximately 20 minutes.

The resected VC is then processed on a previously prepared back table, which contains among other things, one large basin, forceps, scalpels, scissors, mallet, osteotome and three plastic one-liter Nalgene jars. The VC is placed on a cloth towel, cleared of all the remaining soft tissue, and split through the intervertebral discs into three or four sections. It is then washed in 10% betadine followed by a final rinse in sterile saline solution, air dried and placed in Nalgene jars containing "harvesting solution" [see

Fig. H1.4



appendix II]. The processed VC is transported at room temperature back to the laboratory for bone marrow cell isolation.

ISOLATION OF BONE MARROW CELLS

Bone marrow cell isolation is carried out in a class-1000 clean room facility equipped with class-100 safety cabinets. The laboratory set-up for cell isolation is described in Appendix I. Employing aseptic techniques, the VC is separated into individual vertebra, divided along the sagittal cranio-caudal axis (Fig. H1.5), and placed in the processing medium [Appendix II]. Using large Rongeur, the soft tissue (intervertebral disc and the periosteum) is removed. The cancellous bone was chipped-off piecemeal by small Rongeur and placed in the processing medium (Fig. H1.6), for passive release of the majority of bone marrow cells. This cell suspension is then filtered through two consecutive stainless steel screens (450 and 180 μm respectively), centrifuged at 300g for 10 minutes, and the cell pellet is resuspended in 300 ml of "resuspension" solution. To release cells trapped within the trabecular framework of the marrow, cancellous chips retained on the filter are re-processed twice by gentle shaking in the processing medium for 30 minutes each (Fig. H1.7). The cell suspension is then centrifuged and processed as mentioned above. Cell count and viability are assessed by *trypan blue* exclusion, and the cells are finally resuspended at 10^8 cells ml^{-1}. Samples for progenitor cell assay (CFU), microbial surveillance and flow cytometric analysis are retained from the final cell preparation. The remaining bone marrow cells are then either used in clinical trials of simultaneous bone marrow and whole organ transplantation or cryopreserved for future use.

RESULTS

CELL YIELD

Vertebral bodies from 50 cadaveric donors have been harvested between March 1992 and October 1993. The average donor age was 30 years and on an average nine VB were recovered from each donor. Red blood cells [RBC] were lysed using 2% acetic acid, and viable cell count was determined by the trypan blue exclusion technique The average cell yield/donor was 5.1×10^{10}, giving an average cell yield/VB of 5.7×10^9 (Table H1.1).

FACS® ANALYSIS

Flow cytometric analysis of isolated bone marrow cells was performed using

Fig. H1.5

Fig. H1.5
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drop rule

Fig. H1.6

Fig. H1.6
45%
b/w line
drop rule

the following Phycoerythrin [PE] or Fluorescein isothiocyanate [FITC]-conjugated mouse-α-human primary monoclonal antibodies [mAb] directed against the following cell surface receptors: anti-leukocyte common antigen [IgG_1, HLe-1, CD45], anti-HLA-DR [IgG_{2a}, MHC class II], anti-Leu-M3 [IgG_{2b}, CD14], anti-Leu-M9 [IgG_1, CD33], anti-HPCA-1 [IgG_1, CD34], anti-Leu-14 [IgG_{2b}, CD22], anti-Leu-19 [IgG_1, CD56], anti-Leu-4 [IgG_1, CD3], anti-Leu-3a [IgG_1, CD4] and anti-Leu-2a [IgG_1, CD8]. Isotype-matched irrelevant mAb were used as negative controls. All antibodies were purchased from Becton Dickinson, Mountain View, Cal. Prior to staining, RBC were lysed by resuspending the cells in NH_4Cl solution [0.83% NH_4Cl and 0.1% $KHCO_3$ in H_2O]. 2 x 10^5 cells were placed in each well of a 96-well microtiter plate [Falcon, Lincoln Park, NJ], blocked with 10% goat serum for 15 min. and washed x2 with washing solution [WS; PBS + 1% FCS + 0.5% NaN_3] [Gibco, Grand Island, NY]. 20 µl of appropriate PE or FITC-conjugated primary mAb was added to each well, incubated for 30 minutes at 4°C, and then washed x2 with WS. The cells were finally fixed with 1% formaldehyde and stored at 4°C until analyzed.

The samples were analyzed using Consort 30 acquisition software on a Fluorescence Activated Cell Sorter [FACStar, Becton Dickinson, Mountain View, Cal.]. An analysis gate was established using the forward and orthogonal light scatter profile of unstained bone marrow cells. Markers to delineate positive staining were set based on staining of bone marrow cells with isotype matched irrelevant mAbs. Purity of the anlaysis gate was determined by staining of the bone marrow cells with FITC-conjugated anti-CD45 and PE-conjugated anti-CD14 (Leucogate™, Becton Dickinson) 50,000 events were acquired, and the expression of appropriate lineage marker evaluated. The phenotype of human bone marrow cell is detailed in Table H1.2.

THE CLONOGENIC CELL ASSAYS

To propagate colony forming units [CFU], duplicate samples of 2 x 10^5 bone

Flg. H1.7
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Fig. H1.7. Isolation of bone marrow from human vertebral bodies.

Table H1.1. Bone marrow cell yield from human vertebral bodies [VB]

	mean ± SE
Number of VB/donor	9 ± 0.34
Donor age [yr]	30 ± 3
Donor weight [kg]	74 ± 5.3
Cell yield/donor [x10^{10}]	5.1 ± 4.4
Cell yield/VB [x10^9]	5.7 ± 0.4
Cell yield/donor weight [x10^8/kg]	74 5.3

Table H1.2. FACS® analysis of the cell surface phenotype of human vertebral bone marrow

	Positive Events (%)	
Antigen*	Total [mean]	CD45-gated [mean ±1 SD]
Pan leukocyte		
CD45	38.4	
Progenitor cells		
CD33	–	1.5 ± 0.6
CD34	–	1.8 ± 0.4
Monocytes		
CD14	–	4.2 ± 1.6
B cells		
CD22	–	3.9 ± 1.6
NK cells		
CD56	–	3.6 ± 1.6
T cells		
CD3	–	14.7 ± 4.4
CD4	–	6.3 ± 2.9
CD8	–	8 ± 4.2
MHC		
HLA-DR	–	10 ± 1.1

Table H1.3. Clonogenic progenitor numbers in normal bone marrow from cadaveric vertebral bodies

Colonies	mean ±SE [per 2 x 105 cells]
BFU-E	92 ± 8.8
DFU-GM	46.4 ± 3.4
CFU-GEMM	5.9 ± 0.9

Table H1.4. Transplantation groups; combined donor bone marrow and whole organ

Organs Tx	with TLI [n]	without TLI [n]
Liver	2*	5
Liver + Islets	—	1
Kidney	—	7
Kidney + Islets	—	2
Heart	—	1
Small bowel	—	1
TOTAL	2	17

* each patient received a single dose of 500 and 550 cGy of TLI respectively

marrow cells were plated in 35 mm petri dishes in Terry Fox medium [Terry Fox Laboratory, Vancouver, B.C., Canada], and incubated for 14 days at 37°C in a humidified atmosphere of 5% CO_2 in air. At the end of this incubation period, colonies were scored using an inverted microscope.

Under appropriate culture conditions [see above], 2 x 10^5 nucleated bone marrow cells yield 92±8.8 burst-forming-unit erythroid (BFU-E), 46.4 ± 3.4 colony-forming-unit granulocyte and macrophage (CFU-GM), and 5.9 ± 0.9 colony-forming-unit granulocyte, erythrocyte, macrophage and megakaryocyte (CFU-GEMM) (Table H1.3).

CLINICAL TRANSPLANTATION

A total of 19 patients have received simultaneous donor bone marrow and whole organ transplantation between June 1992 and October 1993 at the University of Pittsburgh Medical Center (Table H1.4). The first two liver recipients were conditioned with a single dose [500 and 550 cGy respectively] of total lymphoid irradiation (TLI) prior to transplantation. Due to an unfavorable clinical outcome in one of these patients, prior TLI conditioning regimen was abandoned. All subsequent transplants were done without any cytoreductive or cytoablative conditioning of the recipients. Autologous bone marrow was harvested from the iliac crest of all recipients prior to transplantation and cryopreserved, in the event that host immunohematopoietic reconstitution would be required. Patients transplanted with donor bone marrow and various whole organs, without prior conditioning include; liver [n=5], liver + islets [n=1], kidney [n=7], kidney + islets [n=2], heart [n=1] and small bowel [n=1]. Immediately after whole organ transplantation, 3 x 10^8 donor bone marrow cells/kg body weight of the recipient, were infused through a central intravenous line. All recipients received routine immunosuppression with FK506 and Prednisone. The presence of donor cells [chimerism] was evaluated in the recipient's peripheral blood lymphocytes [PBL], weekly in the first month and monthly thereafter. For immunohistochemical identification of donor cells [by FACS® and cytospins], donor and recipient-specific anti-MHC class I monoclonal antibodies were used, whereas for PCR, donor and recipient-specific DR-probes were used. In occasional male to female organ recipients [n=4], the presence of Y chromosome in the recipient was also analyzed by PCR and in situ hybridization. The pre-transplant and monthly in vitro immune status of the recipient was monitored by mixed lymphocyte reaction [MLR] and cell mediated lymphocytotoxicity [CML] assays. Recipients of whole organ transplants alone were also monitored as controls.

Bone marrow infusion was uneventful in all patients, although both of the patients who received TLI conditioning prior to bone marrow and liver transplantation had a very turbulent post-operative course. One of them developed Grade III graft-versus-host-disease

[GVHD], which necessitated re-infusion of cryopreserved autologous bone marrow.[32] He subsequently lost all graft function and had to rescued with a re-transplant. He eventually succumbed from complications of intestinal perforation. All patients in the non-TLI group are doing fine and are chimeric. On the other hand, donor cells are barely detectable in patients in the control group [organ transplant alone] 4 weeks after transplantation. A variable degree of immunomodulation was also seen in patients in the study group, with some losing all donor-specific reactivity weeks after transplantation, and others maintaining proliferative response both against the donor and third party stimulators months after transplantation. Two patients in the non-TLI group developed mild asymptomatic skin rash [both recipients of liver allografts], shown on skin biopsy to be a GVH reaction. The rash was gone in 2-3 weeks without additional treatment in one patient, and an increase in coticosteroids in another patient. Eight out of 17 patients in the study group also developed mild to moderate acute cellular rejection, which was treated by an increase in the dose of their routine immunosuppression.

CONCLUSIONS

Vertebral bodies from cadaveric donors are a reliable source of bone marrow,[7,8] used routinely at our center for cell augmentation studies. Their harvesting is compatible with multi-organ procurement, providing a higher cell yield. Furthermore, bone marrow obtained from the VB has a much lower blood-borne mature lymphocyte contamination[33-37] and the number of progenitor cells isolated are comparable to that from the iliac crest.[38-41] Its use in 19 patients who received combined donor bone marrow and whole organ transplantation was uneventful, with evidence of engraftment without the necessity of prior cytoreduction.[42,43]

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APPENDIX I

A: Equipment Required For Human Vertebral Body Harvesting

- Straight Osteotome [1.25" wide]
- Curved Osteotome [1.25" wide]
- Mallet
- Scissors
- Forceps
- Procurement Solution [1L]
- Betadine
- Normal Saline [3L]
- Sterile Jars [Nalgene]
- Protective Face Shield
- Organ Transport Container

B: Back Table Equipment

- Straight Osteotome
- Mallet
- Scissors
- Forceps
- Sterile Jars [Nalgene]
- Large Basin
- Sterile Plastic Bags

C: Equipment For Bone Marrow Isolation

- Sterile Wraps
- Large Stainless Steel Basin
- Medium Stainless Steel Basin
- Sterile Scalpel Blades
- One Mallet
- Straight Osteotome 1 1/4"
- Scissors
- Forceps
- Sterile Gauze
- Processing Solution [1L]
- 10cc Disposable Syringes
- Needles [16 G]
- 40 ml Aerobic Culture Bottles [Organon Teknika, Durham, NC]
- 40ml Anaerobic Culture Bottles [Organon Teknika, Durham, NC]
- Protective Face Shield

D: Equipment For Laminar Flow Hood

- Sterile Wraps
- Basins [medium]
- Rongeurs (2-Large/2-Medium/2-Small)
- Sterile Gauze
- Sterile Jars [Nalgene]
- Stainless Steel Container
- Stainless Steel Jars
- Stainless Steel Sieves [Pore Size 180 & 425 μm]
- Stainless Steel Racks For 250ml Conical Tubes
- Sieve Pans
- Conical Tubes [250ml]

APPENDIX II

SOLUTION 1: PROCUREMENT SOLUTION

• Dulbecco's Modified Eagle's Medium	500ml
• Human Serum Albumin [25%]	50ml
• Bacitracin [50,000 U]	5ml
• Polymixin B [500,000 U]	5ml
• Heparin [1000 U/ml]	10ml
• Hepes Buffer [1M]	12.5ml

SOLUTION 2: PROCESSING MEDIUM

• X-Vivo 10* [BioWhittaker, Walkersville, Md.]	1000ml
• Human Serum Albumin [25%]	100ml
• Bacitracin [50,000 U]	10ml
• Polymixin B [500,000 U]	10ml
• Heparin [1000 U/ml]	10ml

SOLUTION 3: RESUSPENSION MEDIUM

• RPMI 1640	1000ml
• Human Serum Albumin [25%]	100ml
• Gentamicin (50 mg/ml)	10ml
• Heparin (1000 U/ml)	10ml
• Hepes Buffer [1M]	25ml

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* modified