2/17/89

STUDY OF OSTEOPOROTIC FRACTURES (V2)

Protocol for Obtaining Biological Samples

I. SERUM AND WHITE BLOOD CELLS

1. Introduction:

The objectives of this part of the examination are:

a) Obtain blood for white blood cells and serum from each participant in two different vacutainer tubes.

b) Distribute white cells and serum into cryotubes.

c) Freeze white cells and serum at -20C until one storage box is filled.

d) Ship to our friends at BRI.

2. Equipment:

GLOVES, disposable, non-sterile.
Horizontal centrifuge (same specifications as for baseline protocol).
Non-self defrosting freezer, -20C; dry ice.
8.5 ml (BD 4606) or 10 ml yellow-top tubes, containing acid citrate dextrose (ACD), two per subject.
15 ml red-top tubes, coated (BD 6432 or BD 6532), one per subject
10 ml red-top tubes, non-coated (BD 6441), two per subject (bone loss cohort only).
Vacutainer set-ups; 20 or 21 g needles.
4 ml cryotubes, with yellow and blue inserts.
Plastic disposable transfer pipets (with built in bulbs).
Borosilicate disposable transfer pipets; rubber bulbs for pipets
Wooden applicator sticks.
Cryotube storage boxes (100 cell); 100 cell cryotube inserts
Sterile normal saline (A liter should last a long time. Keep in 'fridge.)
Insulated shipping boxes for sending cryotubes to BRI

NOTE: Time of blood collection is no longer critical, but should be recorded. The only dietary guideline is NO CAFFEINE since midnight.

3. Procedures:

A. VENIPUNCTURE

PUT ON GLOVES

a) The participant must have been seated for 10 minutes before venipuncture. This standardizes the degree of orthostatic hemoconcentration.

b) Before drawing the blood, a preprinted label showing the participant's ID code should be placed on each vacutainer tube. It is essential to then check the ID code on each tube to ensure that the specimen being collected belongs to the participant. This can best be done be holding the tube next to the ID number on the participant's chart and calling out the number. Then ask the participant to say her name aloud and verify it against the name on the chart.

c) Draw blood from an antecubital vein whenever possible. Use a tourniquet to produce venous distention so that a needle can be inserted. A blood pressure cuff inflated midway between systolic and diastolic blood pressure is most effective and is highly recommended. Do not leave the tourniquet in place for more than 2 minutes. This avoids excessive hemoconcentration. If the 2 minute interval is exceeded, abandon the arm temporarily and attempt to obtain the specimen from the other arm.

d) Draw blood using the vacutainer system (one red-top 15 ml Becton-Dickinson #6432 or #6532; and two yellow-top 8.5 ml, Becton- Dickinson #4606). For detailed instructions, use those supplied with the vacutainer tubes. A syringe may be used for participants with veins that are too small or fragile for the vacutainer system. For the bone loss cohort, draw two additional tubes (two red-top 10 ml uncoated, Becton-Dickinson # 6441).

e) Draw yellow-top first, as close to two full tubes as possible.

f) Gently turn the yellow-top tubes a few times to allow the anticoagulant to mix with the blood.

B. BLOOD PROCESSING - FOR RED-TOP TUBES ONLY

PUT ON GLOVES

a) Allow the filled red-top tubes to stand at room temperature for at least 60 minutes but for no more than 120 minutes. This procedure is necessary to allow an adequate clot to form.

b) After clot formation and before centrifugation, remove the red-top stoppers and gently free the clot from the sides of the tube with a clean plain wooden applicator stick. Replace the stoppers. Balance the tubes of blood for centrifugation. Use a horizontal centrifuge; angle heads are not satisfactory.

c) Centrifuge the blood for 10 minutes at room temperature at a setting known to yield a relative centrifugal force (RCF) of at least 1000 x g at the bottom of the tubes. The table below gives those combinations of centrifuge speed in revolutions per minute (rpm) and rotating radius (r) that will yield an RCF value of 1000 x g. RPM should be read from a tachometer or rev counter when the centrifuge is normally loaded. Radius (r) is measured in centimeters from the center of the rotor shaft to the bottom of the vacutainer tube when the tube is in a horizontal position.

r (cm)	12	14	16	18	20	22.5	26
rpm	2800	2600	2400	2250	2100	2000	1900

Do not use a brake to slow down the centrifuge.

d) Remove serum from the clot by aspiration with a clean borosilicate or plastic transfer pipet (the clot may sometimes stick to plastic). Use a new pipet for each subject. Transfer the serum to prelabeled (see e., below) cryotubes with blue stopper insert. Fill the cryotube up to the line that is already marked on the tube (this yields 3.6 to 4.0 ml). Do not go above the line because expansion space is needed when the serum freezes. If there is not enough serum to fill the tube, fill it as much as possible.

e) Each cryotube should be individually labeled with the ppt ID and filled with serum. Use a pen with permanent ink. Dr. Leef has recommended that "Sharpies" be used to print the ID codes. Keep the labeled cryotubes away from solvents such as alcohol or acetone as these will erase the ID code. Before transferring the serum, the vacutainer tube and cryotube should be held side by side and the numbers read aloud to check that the ID code numbers match. Do not set up production lines of labeled empty cryotubes. The chance of error is increased by the latter procedure.

f) If a serum sample is reddish in color, determine if it is hemolyzed or simply contaminated with red blood cells. One can tell the difference by recentrifuging the vacutainer tube. This will pellet any contaminating red cells and the serum will clear. If the sample is hemolyzed the red color will remain in the serum. If the patient is still in the clinic another red-top tube should be obtained. Otherwise, the hemolyzed sample should be processed.

g) Insert the blue insert into the serum cryotube cap. Some caution should be used in capping the cryotubes. Screw the caps on firmly to secure them tightly against the rubber gasket, but do not apply an extreme amount of pressure. To promote rapid freezing, place the cryotubes upright in a footless metal rack that is in contact with a shelf in a -20 C freezer.

C. PROCESSING FOR YELLOW-TOP TUBES

PUT ON GLOVES.

a) Blood may sit in yellow-top tubes until end of day, or may be spun immediately. Keep at room temperature. (Blood will not clot, but may begin to settle after a few hours. This is not a problem.)

b) Centrifuge tubes at approximately 2000 rpm for 20 minutes. Do not brake.

c) Result should be three-layers in tube: bottom = red blood cells; middle = buffy coat (white blood cells); top = plasma. The buffy coat will be MUCH smaller than the other two layers.

d) Using a plastic pipet, remove and dispose of plasma (or you may save/use for your own purposes). Begin near the top, and work your way down. Stop just above the buffy coat, leaving a little plasma behind.

e) Gently swirl the tube to loosen the buffy coat. Pipet out the buffy coat, using a plastic pipet, with the remaining plasma. Squirt into a 4 ml cryotube. Follow same directions about labelling and capping cryotubes as for serum. Insert a yellow insert into the cryotube cap.

f) Place cryotubes upright in footless rack in contact with shelf in -20C freezer.

4. Summary of Important Rules

a) The participant must be seated for 10 minutes prior to venipuncture.

b) The tourniquet must not be in place for more than 2 minutes.

c) The tourniquet must be released before blood is drawn.

d) Vacutainer tubes and cryotubes must be pre-labeled with the participant's ID code number. Vacutainer tube ID numbers must be checked with the participant's chart immediately before venipuncture. Cryotube ID numbers must be checked with each respective vacutainer tube before transferring the samples.

e) The red-top vacutainer tubes must be kept at room temperature for at least 60 minutes but no longer than 120 minutes before centrifugation.

f) Samples are frozen by placing cryotubes upright on a -20 C freezer shelf.

5. Potential problems with obtaining white blood cells and serum

a) Yellow-top tube isn't full: Work with what you've got.

b) There are some red cells in the buffy coat: Not a big problem.

c) There are lots of red cells in the buffy coat, or buffy coat "sticks" to the red cells: 1) Recentrifuge; 2) It its still a problem, add about 5 ml sterile normal saline to the yellow-top tube. Spin again for 10 minutes. Then to step 4 (except top layer is now saline, not plasma).

d) There isn't any buffy coat: Spin for another 5-10 minutes.

i) There still isn't any buffy coat (and other samples in the same batch did layer). Save lowest portion of plasma layer. Record.

ii) There still isn't any buffy coat (and other samples in the same batch did NOT layer). Probable centrifuge failure. Save specimens at room temperature for up to 72 hours while fixing/replacing.

e) Yellow-top is full, but couldn't get red-top: Treat (i.e., SAVE) plasma from yellow-top as if it is serum from red-top. Record on grid as participant ID - B*. Also, keep a list of all such participants.

II. WHOLE BLOOD ON BLOTTERS FOR PCR ANALYSIS

a) Supplies:

Filter paper: #903 Newborn screening blotter, roll of 3 inch by 600 feet cut into 3 in by 3 in pieces
Drierite: 5 lbs Indicator Mesh #8
40 ZipLoc™ plastic bags (food storage size)
Airtight plastic container for storage of ZipLoc™ bags

b) Label the corner of a 3 in. by 3 in. piece of filter paper with the subjects ID and namecode, using a #2 pencil.

c) Before spinning, draw about 1/2 cc of whole blood from a yellow-top tube with a plastic pipette. If blood has settled, mix thoroughly before pipetting.

d) Decant the 1/2 cc of whole blood onto the filter paper. Hold up a corner of the filter paper while decanting so that the blood doesn't soak through to the paper towel.

e) Place the whole blood/filter paper on a paper towel and allow to air dry.

f) When the blood is completely dry, place the numbered filter paper in a ZipLoc™ plastic bag. The plastic bag should contain 1 tspn Drierite. Store about 50 pieces of filter paper in each plastic bag, clipped (plastic clip) together in ID order. Record the ID numbers contained in each ZipLoc™ bag on a piece of paper taped to the inside of the bag and visible from without.

g) Store the plastic bags in the airtight plastic container along with 1 tblspn Drierite for each bag. If available, store the plastic container in a refrigerator.

h) Monitor the Drierite in the baggies and the plastic container each week. If the Drierite changes from blue to pink-red in color, replace it with a fresh sample. The Drierite may be recycled by warming it in an oven until it turns blue again.

III. TEMPORARY CRYOTUBE STORAGE AND SHIPMENT

a) After samples have been frozen by placing cryotubes upright on a -20 C shelf (overnight), place cryotubes in ID numerical order into a storage box using the inserts.

b) Use the cryotube storage box grid for recording the position of cryotubes, by ID number, within the shipping boxes sent to BRI. (This is a back-up identification system in case the ID numbers on the tube are obliterated after prolonged storage at - 180 degrees.) As the filled tubes are placed into the slots formed by inserts, write the ID number which is on the tube into the corresponding box on the paper grid. Write a dash and the letter B or Y after the ID to record whether the tube cap contained a blue (serum) or yellow (WBC) insert

c) Since the box does not have a definite up or down, right or left, you will have to mark the upper right corner of the cardboard box and the insert. (The paper grid is already marked "upper right" and "upper left".) Orient the box so that the oval holes along the bottom of the box are facing toward you on one side and away from you on the other side. In a clearly visible spot in the upper right corner of the box and the insert (to the right and away from you), punch a whole in the cardboard with a single hole paper punch.

d) Store samples at -20 C in the storage box until 100 cryotubes have been filled and frozen. This should take about two weeks.

e) When the box is ready for shipping, record on the grid form the dates over which the samples were collected, your clinic, the date the box was shipped to BRI and the number of tubes being shipped.

f) Send one copy of the form to BRI with the box and keep one copy yourself.

g) When the box arrives at BRI it will be assigned a unique identifier and placed into storage. A copy of the grid with the identifier will be sent to the coordinating center.

h) A box of samples should then be shipped in an insulated shipping box on dry ice by a 24 hour-air carrier (such as Federal Express). To ensure that that the samples can be received at BRI the next day, ship Monday - Wednesday only. The insulated shipping box and carrier can be chosen by each Center. However, the shipping box must provide insulation and have inner dimensions sufficiently large to handle 1 storage box (o.d. 5.25 x 5.25 x 4.75 inches) and 5-10lbs. of chipped dry ice to keep the samples cold even if shipment is interrupted for a day or two. (If in doubt, err on the side of too much dry ice.)

i) Serum should be shipped to:

Don Dover
Biomedical Research Institute
12264 Wilkins Ave. Bay E
Rockville, Maryland 20852