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Granulocyte Macrophage-Colony Forming Unit (GM-CFU) Assay

Experiment # 3 (Survival #1) Source of Irradiation: Radionuclide injection

Mice Sex, Strain, Age: SW, F, 5-6 wk

Type of Irradiation: Chronic

Animals per group: 3

Aims:

i) To determine the bone marrow response with dose after Sn-117m administration.

Summary of Results:

Brief Procedure:

1) Inject animals in groups of 3 with desired activity of Sn-117m intravenously through lateral tail vain.
2) Sacrifice each mouse on optimal day by cervical dislocation and sterilize using 70% EtOH and immediately move it into laminar flow hood.
3) Remove both femurs carefully using sterile instruments and clean the attached tissue thoroughly.
4) Flush the bone marrow with 2% Horse Serum in Dulbecco's Modified Eagles Medium (2% HS-DMEM) using 21G needle and syringe.
5) Separate the mononudear cells by density gradient procedures using Histopaque.
6) Plate the desired number of cells (cell suspension) in mixture of 60% HS-DMEM and 1.7 ml 0.6% bacto agar solution in the presence of 9.2 U (New Sigma Unit) of GM-CFS.
7) Keep the plated petri dishes for 20 min. in laminar flow hood and move them into incubator with 5% CO_2 and 95% air, at 37°C.
8) Count the granulocyte macrophage colonies on 7th day.

Sn-117m injections:

Group # Probe #	Date of injection	Activity injected (μCi/gm)	Date Sacrificed	# of days	Remarks if any
C1	8/28/98	0	9/4/98	7	0.2 ml 0.9% NaCl injected, control
~~2~~					
1	8/28/98	3.95 ~~5.8~~	9/4/98	7	
2	"	8.0 ~~11.7~~	"	"	
3	"	22.3 ~~25.2~~	"	"	
4	"	31.5 ~~46.8~~	"	"	
5	"				
6					
7					
8					

Preparing Media and Agar:

Culture Medium (Double Strength, 2X): 13.37g (1 pack) of DMEM powder (Gibco, Cat # 12100-038) + 490 ml deionized water + 16 µl of L-asparagine (Gibco Cat # 12416-012) at a concentration of 5 µg/µl + 150.4 µl of DEAE dextran (mol. wt. = $2x10^6$, intrinsic viscosity = 0.7) at a concentration of 1 µg/µl (Sigma Cat # D-9885) + 10 ml of penstrep (Gibco Cat # 600-5070, 5,000 units/ml pen, 5,000 µg/ml streptomycin) + 3.7 g of $NaHCO_3$ (Gibco Cat # 11810-025).

Culture Medium (2X) with 60% Horse Serum : Add 60 % Horse Serum to 2X DMEM

Wash Medium: i) Mix equal amounts of culture medium and sterile deionized water.
ii) Add 2% HS

Agar: Prepare 0.6% agar by adding 0.6 g Difco Bacto agar (Difco Cat # 0140-15-4) to 100 ml boiling deionized water. Autoclave on liquid cycle for 20 min.

Comments If any:

Flushing Bone marrow:

1) Remove both femurs from each mouse and place them in a test tube containing wash medium kept in ice, if the femur can not be flushed immediately.
2) Flush the marrow from each femur by aspirating 3 ml of Wash Medium through the femur 5 times with a 21G needle/3 ml syringe in a 50 ml conical centrifuge tube. Follow with two flushes with 1 ml of fresh Wash Medium.
3) Spin the cells at 1200 rpm for 5 minutes at 4°C, decant, break up the cell pellet, resuspend the cells in 5 ml of cold Wash Medium, and vortex the cell suspension.

Comments If any:

One of the bones of Group 4 was broken, so not used in this study.

Counting the Cells:

Add 10µl of cell suspension to 20 ml of Isotone II in a coulter cup and count the cells using coulter counter. Calculate total # of cells in each group.

Coulter Counter Parameters:

Current(I)=500 µA
Full Scale = 1
T_L = 2.7
T_u = 99.9
Manometer Select = 500 µl

Attenuation= 4
Alarm Threshold = off
Preset Gain = 1
Stirrer control = off

Multiplication Factor to get total # of cells in 5 ml = 20,000 x Coulter count

Group #	Coulter Count without ZG	Avg	Total # of cells	Coulter Count with 5 drops ZG	Avg	Total # of cells
C1	NOT PERFORMED					
C2						
1						
2						
4						
5						
6						
7						
8						

Comments if any:

Separating Mononuclear cells and washing the cells:

1) Transfer 3.5 ml of Histopaque (Sigma Cat #H8889) into fresh 15 ml tubes (1 tube per group).
2) Layer the cell suspension carefully on top of Histopaque and centrifuge at 1500 rpm, 4°C, for 30 minutes.
3) Using a Pasteur pipette transfer the mononuclear cells into fresh tubes.
4) Dilute the cell suspension to 15 ml by adding cold Wash Medium into each tube and spin them at 1200 rpm, 4°C, for 5 min.
5) Decant the supernatant, break the pellet, and add 15 ml cold Wash Medium, and spin them again at 1200 rpm, 4°C, for 5 min. Repeat this procedure 2 more times.
6) After 3rd wash break the pellet and resuspend in 2 ml Culture medium (2x DMEM) with 60% HS and keep the tubes in dry bath at 37°C.
7) Add 20 µl of cell suspension to 20 ml of Isotone II in a coulter cup and determine total # of cells in each group using coulter counter

Coulter Counter Parameters: *Same as above*

Multiplication Factor to get # of cells/ml = 2000 x Coulter Count

Group #	Coulter Count without ZG	Avg	# cells per ml	Coulter Count with 5 drops ZG	Avg	# cells per ml
C1				5110, 5125, 5213	5149	10,298,666
C2				~~5007, 5107, 5123~~		
1				5007, 5107, 5123		
2				4813, 4918, 4975		
3				4439, 4556, 4579		
4				4322, 4429, 4372		
5						
6						
7						
8						

Average # of cells per µl ± nl for control = 10,298,666

DILUTIONS

~~GROUP-1~~

Dilution A: (1.0×10^6 cells /ml, Total volume 3.4 ml)

Vol of final cell suspension required = 3400000/cells per ml = $\frac{3400000}{10298666} = 0.330$ ml

1.7 ml Agar + 1.370ml Medium + 0.330ml Cell Suspension

Dilution B: (3.0×10^5 cells /ml, Total volume 3.4 ml)

Vol of final cell suspension required = ~~3400000~~ 1020000/cells per ml = $\frac{1020000}{10298666} = 0.099$ ml

1.7 ml Agar + 1.601ml Medium + 0.099ml Cell Suspension

GROUP-2

Dilution A: (1.0×10^6 cells /ml, Total volume 3.4 ml)

Vol of final cell suspension required = 3400000/cells per ml =

1.7 ml Agar + ml Medium + ml Cell Suspension

Dilution B: (3.0×10^5 cells /ml, Total volume 3.4 ml)

Vol of final cell suspension required = 3400000/cells per ml =

1.7 ml Agar + ml Medium + ml Cell Suspension

GROUP-3

Dilution A: (1.0×10^6 cells /ml, Total volume 3.4 ml)

Vol of final cell suspension required = 3400000/cells per ml =

1.7 ml Agar + ml Medium + ml Cell Suspension

Dilution B: (3.0×10^5 cells /ml, Total volume 3.4 ml)

Vol of final cell suspension required = 3400000/cells per ml =

1.7 ml Agar + ml Medium + ml Cell Suspension

Plating the Cells:

Culture Medium: Maintain four 13mm tubes each containing 4.5 ml of Culture medium in dry bath at 37°C.

Horse Serum: Maintain five 13mm tubes each containing 4.5 ml of Horse Serum in dry bath at 37°C.

Agar: Maintain five 16mm tubes each containing 6.5 ml of Agar in dry bath at 37°C.

1) Warm up dilution tubes (2 or 3 per group) to 37°C in dry bath.
2) Warm up Agar (30 ml) and 60% HS in 2x DMEM (30 ml) in separate tubes to 37°C.
3) Mark the Six-well plates (3 wells for each dilution for each group) containing 20 u l of stock GM-CSF (9.2 U) in each well.
4) Mix 1.7ml agar + x ml of 2x DMEM with 60% HS + y ml cell suspension + 0.02 ml GMCSF (x +y = 1.7 ml) in a dilution tube.
5) Add 1 ml of mixture 4 to each well, mix properly and let it gel for about 30 minutes.
6) Repeat steps 4 and 5 for each dilution.
7) Repeat steps 1 to 6 for each group.
8) Incubate the cells in an incubator at 37°C and 5% CO_2, 95% air for 7 days.
9) On 8th day of incubation count colonies and determine the survival fractions.

Counting the Colonies: (Inverted at 40X or dissecting at 35X)

Group #	Dose / Activity # injected (uCi)	# of cells plated	# CFU-GM counted	Avg	SF
C1	0	$3X10^5$	144, 131, 137	137.33	
C2					
1	3.85	$3X10^5$	122, 131, 136	129.66	0.9441
2	8	$3X10^5$	123, 115, 109	115.66	0.8421
3	22.8	$3X10^5$	111, 105, 117	111	0.8082
4	31.5	$3X10^5$	98, 93, 96	95.66	0.6966
5					
6					
7					
8					