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

Experiment # 2 (Acute #2)
Mice Age, Sex, Strain: 10-11 wks, F, SD
Type of Irradiation: Acute
Animals per group: 3

Source of Irradiation: ^{137}Cs HDR1 (Mark I)
Date In Irradiator: 08/11/98
Date Out Irradiator: 08/11/98
Date Sacrificed: 08/11/98

Aim: To study GM-CSC survival against acute irradiation with three dose points

Results:

Brief Procedure:

1) Irradiate the mice acutely with desired doses.
2) Sacrifice each mouse by cervical dislocation and sterilize using 70% EtOH and move it into laminar flow hood.
3) Remove both femurs carefully using sterile instruments and clean the attached flesh 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 mononuclear 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 GM-CFS in six-well plates.
7) Keep the plates 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.

High Dose Rate Irradiation:

Group #	Attenuator Used	Turn Table Position	# of Sources Used	Dose Rate (R/min)	Irradiation Time (min)	Total Dose (R)	Comments if any
C1	—					0	unmarked
C2							
1	X-10	#3	2	101.4	0.98	100	marked on head
2	X-10	#3	2	101.4	1.97	200	" " Body
3	X-10	#3	2	101.4	3.94	400	" " Tail
4							
5							
6							

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Preparing Media and Agar:

Culture Medium (Double Strength, 2X): 13.37g (1 pack) of D-MEM 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 in 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 and tibias 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 10 times with a 21G needle/3 ml syringe in a 50 ml conical centrifuge tube. Follow with two flushes with 0.5 ml of fresh Wash Medium.
3) Spin the cells at 1200 rpm for 5 minutes at 4°C, decant, break up pellet, resuspend the cells in 5 ml of cold Wash Medium, and vortex the cell suspension.

Comments If any:

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 2 drops ZG	Avg	Total # of cells
C1	NOT PERFORMED					
C2						
1						
2						
3						
4						
5						
6						

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.

Exp. # _____

Coulter Counter Parameters: *Same as above*

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

Group #	Coulter Count without ZG	Avg	# cells per µl	Coulter Count with 2 drops ZG	Avg	# cells per µl
C1						
C2	Please See the					
1	separate			Sheet		
2						
3						
4						
5						
6						

Average # of cells per µl =

DILUTIONS

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

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

1) Warm up dilution tubes (three 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 (three wells for each dilution) containing 20 ul of 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 15 minutes.
6) Repeat steps 4 and 5 for each dilusion.
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.

Comments If any:

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

Group #	Dose (rads) #	# of cells plated	# CFU-GM counted	Avg	SF
C1	0	3×10^5	134, 133, 128	131·66	
C2					
1	100	3×10^5	52, 47, 40	46·33	0·3519
2	200	3×10^5	18, 15, 13	15·33	0·1164
3	400	1×10^6	9, 10, 11	10	0·022
4					
5					

Cell count

Group	Coulter count for 20µl Cells (MS = 500ml)	Avg. count	Cells/ml	Total # of cells	Cells/femur
1	3382, 3234, 3202	3272	6,545,333	13090666	2,181,777
2	2717, 2600, 2620	2645	5,291,333	10582666	1,763,777
3	3810, 3745, 3719	3758	7,516,000	15032000	2,505,333
4	4507, 4409 4343	4419	8,839,333	17678666	2,946,444

Dilusion

Group 1

Dilusion A : 3.4 ml of 1,000,000 cells/ml = 3,400,000 cells

$$\text{Vol. required} = \frac{3400000}{6545333} = 0.519 \text{ ml}$$

For dilusion,
1.7 ml Agar + 1.18 ml 2X DMEM + 0.519 ml cells.

Dilusion B : 3.4 ml of 300,000 cells/ml = 1,020,000 cells

$$\text{Vol. required} = \frac{1,020,000}{6545333} = 0.155 \text{ ml}$$

For dilusion,
1.7 ml Agar + 1.54 ml 2X DMEM + 0.155 ml cells

Group 2

Dilution A

Vol. required = $\frac{3400000}{5291333}$ = 0.642 ml

1.7 ml Agar + 1.05 2X DMEM + 0.642 ml cells

Dilution B

Vol. required = $\frac{1,020,000}{5291333}$ = 0.192 ml

1.7 ml Agar + 1.50 ml 2X DMEM + 0.192 ml Cells

Group 3

Dilution A Vol. required = $\frac{3400000}{7516000}$ = 0.452 ml

1.7 ml Agar + 1.247 ml 2X DMEM + 0.452 ml Cells

Dilution B

Vol. required = $\frac{1,020,000}{7516000}$ = 0.135 ml

1.7 ml Agar + 1.564 ml 2X DMEM + 0.135 ml cells

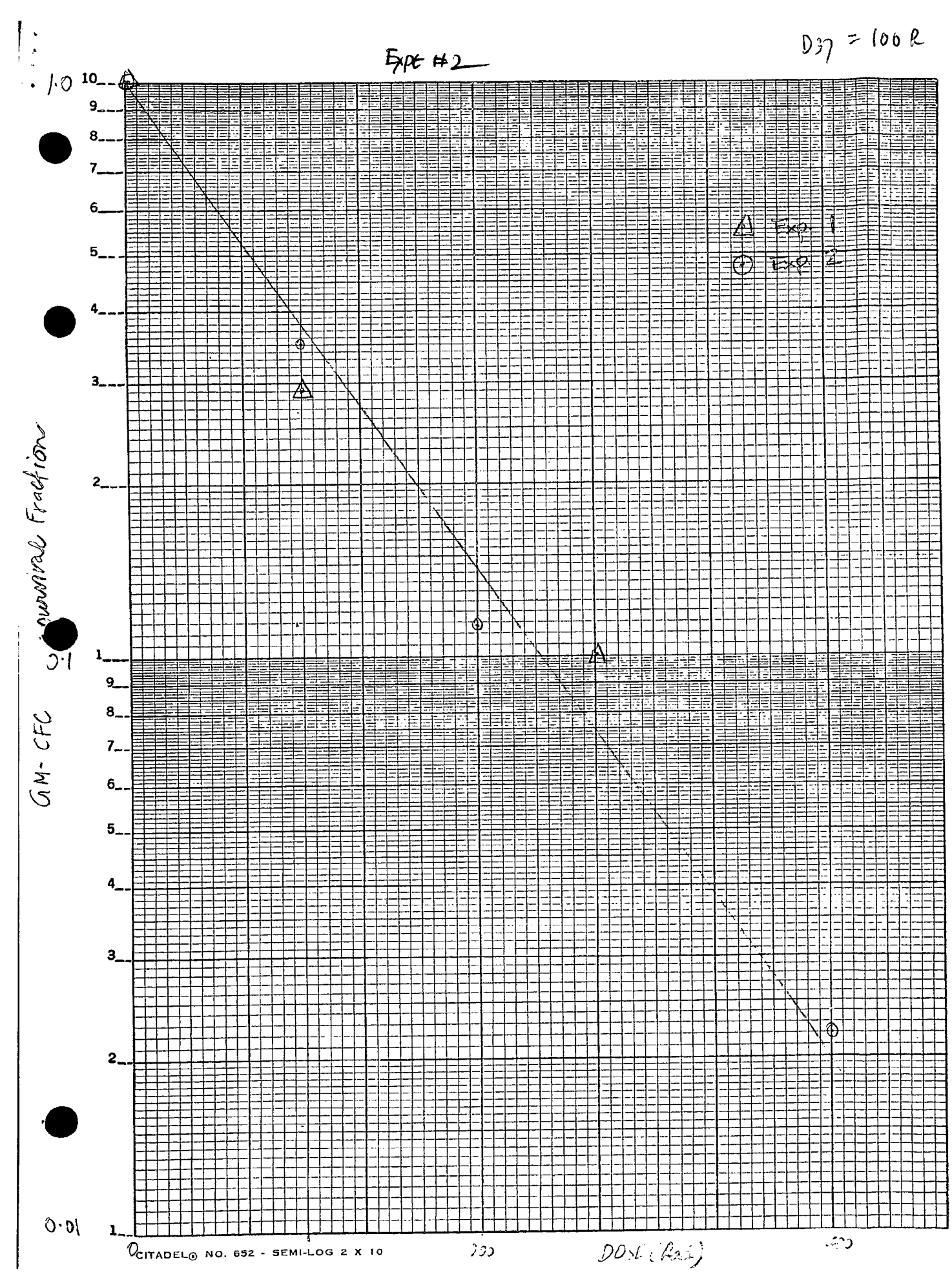
Group 4

Dilution A vol. required = $\frac{3400000}{8839333} = 0.384$ ml

1.7 ml Agar + 1.315ml 2X DMEM + 0.384ml Cells

Dilution B vol. required = $\frac{1,020,000}{8839333} = 0.115$ ml

1.7 ml Agar + 1.58 ml 2X DMEM + 0.115 ml Cells


Expt #2
D37 = 100 R
1.0
10
9
8
7
6
5
4
3
2
1
0.1
0.01
Survival Fraction
GM-CFC
Exp 1
Exp 2
CITADEL® NO. 652 - SEMI-LOG 2 X 10
DOSE (Rad)