Investigator: A. BISHAYEE

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

Experiment # 1 (Acute #1)
Source of Irradiation: ^{137}Cs HDR (Mark-I)
Mice Age, Sex, Strain: 8-9 wks, F, SW
Date In Irradiator: 07/29/98
Type of Irradiation: Acute
Date Out Irradiator: 07/29/98
Animals per group: 3
Date Sacrificed : 07/29/98

Aim: To study GM-CFU survival against acute irradiation.

Results: GM-CFC survival is inversely proportional with acute irradiation (100-250 R)

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(~~<10 µl~~ cell suspension) in mixture of ~~0.5ml~~ 60% HS-DMEM and ~~0.5ml~~ 0.6% bacto agar solution in the presence of ~~100~~ 8.2 U GM-CFS. in 6-well plate
7) Keep the plate~~s~~ ~~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.

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	untreated control
C2							
✓ 1	X-10	#3	2	101.4	0.98	100	Marked on head
✓ 2	X-10	#3	2	101.4	2.46	250	Marked on body
3							
4							
5							
6							

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Preparing Media and Agar:

Culture Medium (Double Strength): 13.37g (1 pack) of D-MEM powder (Gibco, Cat # 12100-046) + 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 just before expt

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

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: Horse serum should be added on the previous day of the experiment.

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.

Multiplication Factor to get # of cells/µl = 2x Coulter Count (# of Cells/ml = 2000 x Coulter Count)

Group #	Coulter Count without ZG	Avg	# cells per µl	Coulter Count with 2 drops ZG	Avg	# cells per µl	Cells/female
C1	Not performed			2264, 2118, 2205	2195	4,391,333	1,463,777
C2	↓						
1				533, 551, 535*	539	1,079,333	359,777
2				3376, 3344, 3256	3325	6,650,666	2,216,888
3							
4							
5							
6							

Average # of cells per µl =

DILUTIONS

* This low count may be due to problem associated with density gradient procedure.

For dilution — See the attached sheets

Exp. # _____

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 (~~one~~ 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 ~~petri dishes (3 petri dishes per group~~ 6-well plate, 3 wells for each dilution).
4) Mix 1.7ml agar + x ml of 2x DMEM with 60% HS + y ml cell suspension + ~~0.408~~ ml GMCSF (x + y = 12 ml) in a dilution tube. containing 20 µl of GM-CSF in each well
5) Add 1 ml of mixture 4 to each ~~petri dish~~ well, mix properly and let it gel for about 15 minutes.
6) Repeat steps 4 and 5 for each ~~group~~ dilution
7) Repeat steps 1 to 6 for each ~~dilution~~ 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: Agar was solidified at 37°C, no stock bottle was used following occasional heating at microwave oven

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

5/5/98

Group #	Dose (rads) #	# of cells plated	# CFU-GM counted	Avg	SF
C1	0	$1X10^6$	187, 192, 179	186	
~~C2~~ C	0	$3X10^5$	82, 74, 91	82.33	—
~~3~~	0	$1X10^5$	10, 12, 15	12.33	
2	100	$3X10^5$	23, 21, 27	23.6	0.28
3	100	$1X10^5$	7, 8, 5	6.66	
4	250	$1X10^6$	31, 43, 50	41.33	
5	250	$3X10^5$	10, 8, 7	8.33	0.10
6	250	$1X10^5$	4, 3, 2	3	

Cell Count

①

	Coulter count for 2and E zap-o-tube	Avg. Count	Cells/ml	Total # of cells	Cells/flask
①	2264, 2118, 2205	2195.6	4,391,333	8782666	1,463,777
②	533, 551, 535	539.6	1,079,333	2158666	359777
③	3376, 3344, 3256	3325.3	6,650,666	13301332	2,216,888

Dilution

1. 9 x 13 mm glass tube (for dilution) (4)
2. 3 x 13mm ē 5.5 ml Agar in each
3. 3 x 13 mm ē 5.5 2X DMEM
4. 5 x 6-well plate ē GM-CSF (20 µl)

Group 1 :

(control)

Coulter Count for 20 µl (MS = 500 µl) 2264, 2118, 2205	Cells/ml 4, 391, 333

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

Vol. required = $\frac{3400000}{4,391,333}$ = 0.774 ml

For dilution, 1.7 ml agar + 774 µl cells + 925 µl 2X DMEM

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

Vol. required = $\frac{1020,000}{4,391,333} \doteq 0.232$ ml

For dilution, 1.7 ml agar + 232 µl cells + 1.46 ml 2X DMEM

Dilution C : 3.4 ml of 100,000 cells/ml = 340,000 cells

Vol. required = $\frac{340,000}{4,391,333}$ = 0.077 = 77 µl

For dilution, 1.7 ml agar + 77 µl cells + 1.62 ml 2X DMEM

Gr 2 (100R) 3.4 ml of 1,000,000 cells/ml = 3,400,000 Cells

Dil. A : vol reqd = $\frac{3400000}{1079333}$ = 3.15 ml

This dilution was not possible because ~~total~~ the volume of final cell suspension was ~ 2 ml.

Dil. B : vol required = $\frac{1020,000}{1079333}$ = 0.945 ml

1.7 ml agar + 0.945 ml cells + 0.755 ml 2X DMEM

Dil. C : vol required = $\frac{340000}{1079333}$ = 0.315 ml

1.7 agar + 0.315 ml Cells + 1.38 ml 2X DMEM

Gr 3 (250R)

Dil. A Vol. = $\frac{34,00000}{6,650,666}$ = 0.511 ml

1.7 ml agar + 0.511 ml cells + 1.188 ml 2X DMEM

Dil B vol = 0.153 ml ($\frac{1020000}{6650666}$ = 0.153)

1.7 ml agar + 0.153 ml cells + 1.546 ml 2X DMEM

Dil C vol = 0.05 ml = 51 ml ($\frac{340000}{6650666}$ = 0.051)

1.7 ml agar + 51 µl cell + 1.649 ml 2X DMEM

10% → 20X | 1% → 200X

90%

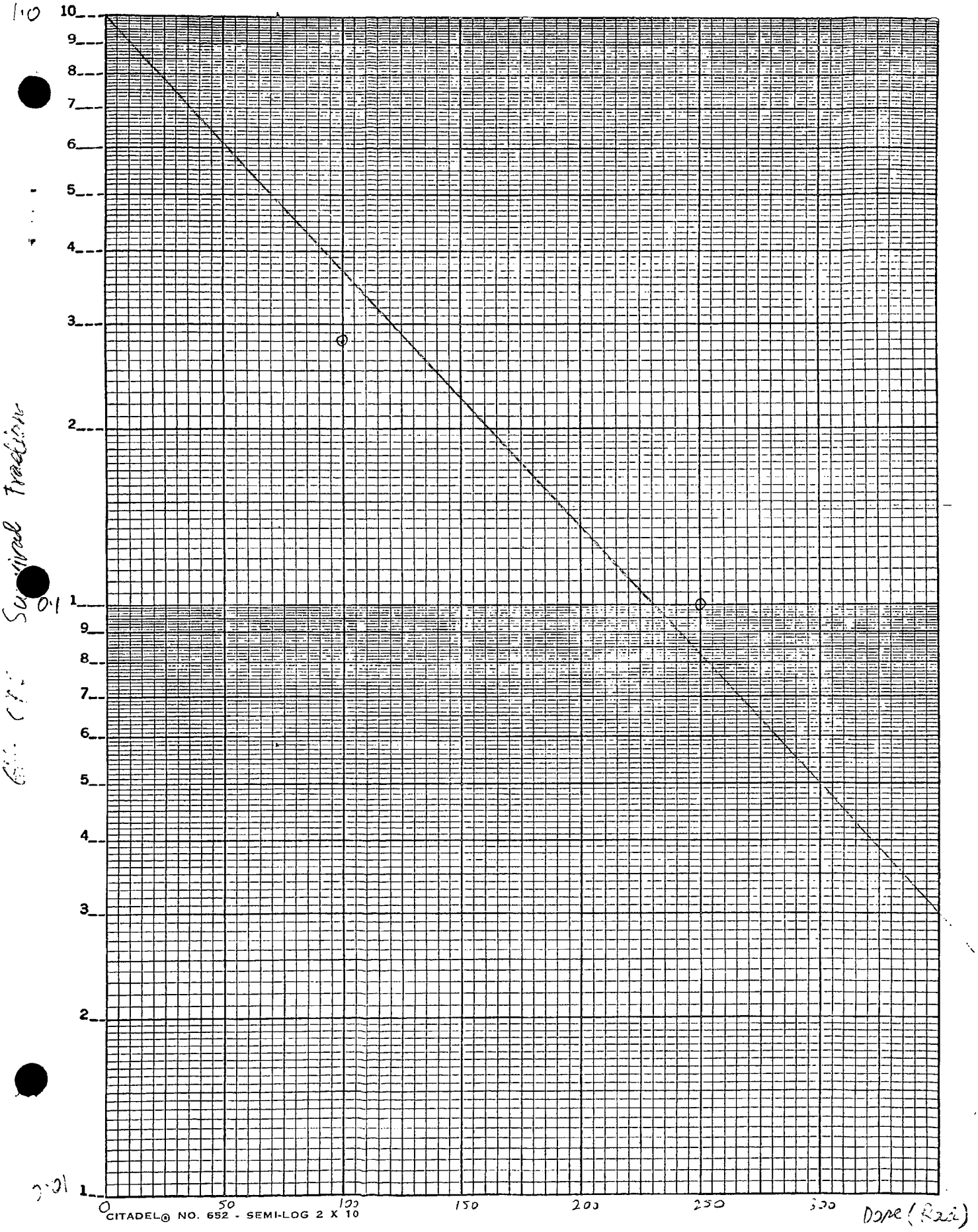
99%

Control

100

200

400


1.0
10
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Dose (Rad)
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