SECOND QUARTERLY PROGRESS REPORT OF RESEARCH

carried out by

LONG ISLAND BIOLOGICAL ASSOCIATION

for

THE BIOLOGICAL DEPARTMENT, CHEMICAL CORPS, CAMP DETRICK

On Contract DA-18-064-CML-2360

January 1 - March 31, 1953

RESEARCH STUDIES OF FACTORS INDUCING RESISTANCE TO TRANSMISSIBLE MOUSE LEUKEMIA

by

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In the period between January 1st and March 30th 1953, seven experiments have been performed in the Camp Detrick series, numbered CD7 - CD13. The nature of the problems, the biological materials and the basic procedures have been described in the previous report.

CD7.

The attempt to obtain the protective material in the supernatant after a saline suspension of line I_b leukemic cells had been gently agitated (see CD5) was repeated using 3% Knox's special gelatin solution in the suspending medium. This necessitated the use of a non-refrigerated centrifuge of lower speed and shaking the material at room temperature instead of in an ice bath. To obtain a more nearly cell-free supernatant, the first supernatant was recentrifuged. But even so, this supernatant II was probably not cell-free. The sediment from the second run contained enough cells to kill the mice so treated about four days ahead of the controls in the challenging dose given after two days. Of the 15 mice treated with this supernatant II, 14 died with the controls and one survived. In experiment CD5 three out of 25 survived, although the deaths of the others, mostly before the controls, proved that potent leukemic cells had been present in the supernatant. In this experiment (CD7) the coincidence with the control deaths seems to imply that there were no potent cells in the supernatant II, but it is entirely possible that enough cells were present to balance out the effect of some resistance to the challenging dose. Since all of the 45 controls in the challenging dose of 4^{-8} x standard in these two experiments have died, the survival of four in 40 strongly suggests an effect of the treatment.

CD8.

This experiment returned to the question of whether repeated treatments with heated (46° C/14 min.) line I_b cells will prolong the duration of the resistance (see CD6). In the earlier experiment, the challenging dose was

given on the ninth day after the third treatment. Since this proved too soon, the time was extended to 14 days in the present case. Four groups of five mice were given three daily treatments with heated line I_b cells, and five litter mates of each of these groups were given a single treatment. In three of these sets of litter mates, all receiving a single treatment died with the controls but two of the five mice receiving three treatments in each of these sets either survived or lived twice as long as the controls. In the fourth set, however, two of the single treatment mice survived as well as two of those receiving three treatments -- none of the controls in the challenging dose survived. Three treatments compared with one evidently do prolong the resistant period, but they do not yield as strong or as enduring resistance as that following a single treatment to which a very few unheated cells have been added. CD9.

Will the protective action of supernatants be increased by disrupting the leukemic cells? Leukemic cells may be disrupted by suspension in distilled water and they may be mechanically torn by forcibly squirting a suspension from a syringe (without needle) held firmly against a flat surface. Both of these means were employed in this experiment. The original suspension of line I_b leukemic cells was made with saline; this was replaced by sterile distilled water, and every 3 ml separately was "squirted" for 100 shots. The supernatant from this after 2000 x G, 30 min. was recentrifuged twice, each time as before, and the final supernatant injected into 14 mice and challenged in two days. Part of the squirted material was not centrifuged, but heated (46° C/14 min.) to prevent any remaining leukemic cells from producing leukemia, and so to reveal whether the resistance inducing substance was still operative in the whole material. The sediment from the first centrifuging after "squirting" was resuspended in H_2O and injected into 15 mice as a check on the presence of potent leukemic cells and a similar check was made with the saline supernatant

from the preliminary run (2000 x G/40 min.) after four hours at room temperature.

In spite of the distilled water and squirting, the final supernatant gave the same kind of result as previously (out of 14, 2 survived - 3 lived longer than any control); but the heated material, which in saline and not "squirted" would have protected all, actually did protect only 4 out of 11. Sediment from the first centrifuging after squirting failed to produce leukemia, and only 1 in 15 survived challenged in 14 days, whereas had a very few potent leukemic cells been present and been resisted, complete resistance would have been expected. Thus, rather than increasing the protective effect of the supernatant, the distilled water plus the squirting seemed to be responsible for a reduction in the protective action of the whole material. The failure of the unheated sediment to produce any leukemia and to protect only 1 in 15 from the challenge dose in 14 days indicates that the rough treatment of the cells had effectively destroyed them. A side light from this experiment was the discovery that centrifuging 2000 x G/40 min. left in the supernatant so many leukemic cells, that even after 4 hours at room temperature the preliminary saline supernatant killed the 4 mice inoculated as though inoculated with a dose of 4^{-6} or 4^{-7}, that is, 4 to 16 times the dose used for challenging (4^{-8} x standard). Hence the importance of recentrifuging, and in the future, the use of higher speed and longer time, as well.

CD10 and CD11.

Will heating line I_b leukemic cells (46° C/14 min.) liberate protective material into the medium?

The effectiveness of line I_b leukemia in producing resistance after this mild heating is firmly established. One possible action of this gentle heat might be to change cell-wall permeability. Accordingly, supernatants after the standard heating procedure were tested for resistance in these two experiments. The centrifugation was carried out in a zero-degree room using 3250 x G/60 min.

The first supernatants were centrifuged before injection into the mice. The sediment from the first run was resuspended and injected.

In these experiments the leukemic cells were suspended in standard concentration, whereas in previous experiments the supernatants were obtained from suspensions diluted to 1/4 standard (4^{-1}). Thus, although the mice have been given about 1 ml of the supernatants throughout, the concentration in these two experiments was about four times greater.

The results give 4 in 10, and 5 in 10 surviving the challenging dose of 4^{-8} in 48 hours. In relation to the concentration of the dose of supernatant these results are in agreement with previous ones, and give little, if any, indication that the heating increased the effectiveness of the supernatants. However, the more intense and repeated centrifuging give these results greater significance than any previous ones, in that the supernatants were more nearly cell-free.

It may be noted that the challenge dose for CD11 was less than for CD10, because the controls lived somewhat longer and one survived. The delay of deaths after supernatant treatment gives similar evidence. The greater survival after treatment with "white layer" in CD11 may be interpreted as partly the result of a larger dose of this material and partly the result of the smaller challenge dose.

CD12.

With this experiment attention is turned to the possibility that the protective material provided by line I_b leukemic cells is not stored in or on these cells, but is a cell product. In this case, instead of attempting to extract by various methods, the procedure would be to maintain the life of the cells for as long as possible *in vitro* before testing supernatants.

In vivo, leukemic cells, after 46° C/14 min., in all probability being of the same genetic constitution as the hosts, continued to metabolize. The 48

hours during which resistance has been found to increase might well be an index of the accumulation of some metabolites. With this, as well as practical considerations in mind, a period of 12 hours was chosen for the incubation (37° C) of the leukemic cell suspension, before the recentrifuged supernatant was tested in vivo (B). If cells with leukemic potency survived, the sediment might kill the hosts or, in case their numbers were very small, might yield firm resistance. So the sediment after incubation was in part inoculated into 10 mice as a test of surviving leukemic potency (F), in part heated 46° C/14 min. to inactivate the possibly surviving leukemic cells and permit a test of the survival of the capacity to induce resistance (C). This experiment also included a group of 10 mice treated with the recentrifuged supernatant removed from the original saline suspension of cells after refrigerated shaking for 30 min. and before incubation (A). The challenging dose for this group was controlled by 10 mice (D), and another 10 mice controlled the challenging dose used for the mice given the incubated supernatant and the incubated and heated sediment (E). Under the given conditions, 12 hours incubation at 37° C proved to destroy both the capacity to transmit leukemia and the capacity to induce resistance. The only resistant mice in the experiment were two of the ten given preliminary supernatant before incubation (A) and one of these after 23 days came down with a modified distribution of leukemic invasions centering in the hypodermis, the muscles of the abdominal wall and the mesentaries leaving the spleen small. This is a condition characteristic of cases in which struggle between induced resistance and leukemic growth is not quite completely settled and leukemic cells finally, in some cases after a delay of months, get the upper hand, especially in the hypodermis. The only group in this experiment that survived was that of the 10 unchallenged mice given the unheated sediment after incubation (F).

CD13.

With 12 hours incubation at 37° C proving too long, a very simple experiment was set up to test how long potent leukemic cells would survive. After 2 hours incubation, 10/10 died but the interval before death corresponded to a dilution of 4^{-4} x Standard. After 4 hours incubation 9/10 died at intervals expected from a dilution of 4^{-6} - 4^{-7} x Standard. After 6 hours incubation 4/10 died at intervals corresponding to dilution of 4^{-11} x Standard. With nutritive media and rotation during incubation, the survival of leukemic cells may be extended, but even four hours should give enough time for experiments to indicate the probable success or failure of this approach. No detailed protocol for this purely technical experiment is included.

Exp. CD7 - CSH 2170 I_b 2081

Treatment with supernatant II from shaken line I_b suspended in 3% gelatin-saline.
Challenged in 2 days with I_b 4^{-8}

1/7/53 10:50 A.M. - 2:20 P.M. From first killing of spleen donors to last treatment. Room temperature for all materials.

11:50 - 12:20 28 cc I_b 4^{-1} - Std. = spleen wt. x 2 - suspended in 3% gelatin in saline shaken on mechanical shaker set at 90.

7 cc to each of 4 test tubes balanced in pairs, in plastic jackets.

12:30 - 1:00 To International Clinical centrifuge - maximum speed 600 x G.

6 cc removed from each tube to new tubes.

1:15 - 1:45 Recentrifuged

5 cc from each of 3 tubes. 1.0 cc to each of 15 mice.

4th tube failed to upright at end of run so most of contents lost. Used remainder "sediment" from all tubes to treat 5 mice.

1/9/53 Challenge Dilutions made with chilled saline; all vials kept in crushed ice; 4^{-8} made in two lots from 4^{-6}. First lot used to inoculate first 10 treated mice and their 10 litter mate controls, before second lot was mixed and used for the second 10 treated mice and their litter mate controls.

CD7 - Results

Interval before death - Days after I_b 4^{-8}

Box	6	6+	7-	7	7+	8-	8	8+	9-	9	9+	9 1/2	10-	10	10 1/2...	11
A_1 Supernat. II													5			
A_2 Controls											1		3	1		
B_1 Supernat. II											2	1	1		1	
B_2 Controls													2	1	1	1
C_1 Supernat. II											1		2	1	.....1 neg	
C_2 Controls												1	3		1	
Totals Supernat.											3	1	8	1	1 .....1 neg	
Controls											1	1	8	2	2	1
D_1"Sediment II" 4						1										
D_2 Controls													4		1	
Total Controls											1	1	12	2	3	1
4^{-8} controls CD5									9	5	6	2	1	2		

CD8 CSH 2175 I_b 2084 - 4^{-6} Heat series 129

Spleens from CSH 2168 I_b 2081 - standard saline suspension - ml saline = spleen weight in g x 2.

1/13/53 - 6 ml standard, in 3 ml lots - 5 min in 24° C - 14 min in 46° C. treat 20 mice

1/14/53 - 6 ml standard " " "
treat same 20 mice

1/15/53 - 12 ml standard " " "
treat same 20 mice and 20 littermates

1/29/53 - Challenged all 40 mice and 10 untreated controls--dose= 4^{-6} x Std.

Results given by sets of littermates.

Set of litter mates	Intervals before death in 1/4 days: 8th	9th	10th	... 19th	Survived
A 3 treatments	1	1 1			2/5
1 treatment	1	1 1			2/5
B 3 treatments	1	2			2/5
1 treatment	2	1 1	1		0/5
C 3 treatments		2	1		2/5
1 treatment	3	1 1			0/5
D 3 treatments	1	1 1		1	1/5
1 treatment	1	3 1			0/5
Totals					
3 treatments	3	6 1 - 1	- 1	1	7/20
1 treatment	7	5 2 1 3	- - - 1		2/20
Controls same ages, not same litters as above	1 1	5 3			-

CD9. CSH 2179 I_b 2085

Will suspension in distilled water and disruption of cells by "squirting" yield more resistance from supernatant?

2/3/53 5 spleens from CSH 2175 I_b 2084 - standard suspension - saline ml = 2x spleen wt. in g.
9 ml Std. + 27 cc saline = 4^{-1} filtered through cotton.
Centrifuged (15° C) - 3600 RPM/40 min (2000 x G) Supernatant inoc. 4 ♂♂(E)

Sediment resuspended in H_2O
"Squirted" 100 shots for each 3 cc separately
Half of material heated 46° C/15 min - treated 1 ml 11 ♂♂ (C)
Half of material centrifuged - 2000 x G/30 min.
Supernatant recentrifuged 2000 x G/30 min.
Supernatant 2nd recentrifuged 2000 x G/30 min, inoc. 14 ♂♂ .9 cc (A)
Sediment I resuspended in H_2O - inoc. 1 ml 15 ♂♂ (B)

2/5/53 Challenged A and C (D = controls) I_b 2085 - 4^{-8}

2/17/53 Challenged B (F = control - unstaggered litters) I_b 2087 - 4^{-8}

Intervals before death in 1/4 days

Treatment	9th	10th	11th	12th	13th	19th	Survived
A. Supernatant	1	3 1 3 1		1	1	1 recovering accident?	2/14
C. Heated sediment	2	3 2					4/11
D. Controls on A & C	7	8 4 1					0/20
B. Unheated sediment No leuk. in 14 d. then challenged	3	4 6 2					1/16
F. Controls for B challenged	4	3 1 1	1 small mouse				0/10
E. Preliminary 3 saline supernatant No challenge	1						0/4

CD10.

Does heating increase the protective effect of supernatants?

15 ml, line I_b. Standard suspension, heated 46° C/14 min (3 ml per heating vial)
7.4 ml into each of two lusteroid tubes - 3250 x G/60 min.
Supernatant recentrifuged - 3250 x G/60 min.
Supernatant II -.9 ml to 9 ♂♂; 1.0 cc to 1 ♂ (A)
Sediment I from one tube resuspended - .25 ml to 10 ♂♂ (B)
White layer of sediment I (upper) from other tube, in highly concentrated dose to 10 ♂♂ (C)
All mice challenged in 48 hours with line I_b. 4^{-8} x Std.; controls (D)

CD11.

Procedure as for CD10. The only differences were in the greater amount of standard suspension and the slightly larger dose of the supernatant - 1.1 ml, and larger dose of the "white layer" material, which was taken from a centrifuge tube containing 10 ml instead of 7.4 ml as in Exp. CD10.

Time of death in 1/4 days after challenge with 4^{-8}

	9th	10th	11th	12th	Delayed	Survived
A. Supernatant						
CD10		6				4/10
CD11		1	4			5/10
B. Sediment (entire)						
CD10						10/10
CD11				1		9/10
C. "White layer" of sed.					20 d	
CD10		1 1	2	1	1	4/10
CD11		1				9/10
D. Controls on 4^{-8}						
CD10	3	3 2 1	1			0/10
CD11		1 6	1 1			1/10

CD12. CSH 2193 I_b 2091
CSH 2194 I_b 2091

Will leukemic potency and the capacity to induce resistance survive after line I_b leukemic cells are incubated - 37° C/12 hours?

24 ml Standard suspension I_b 2090, shaken in ice bath, setting "100" - 30 min. Centrifuged in refrigerated room 3250 x G - 60 min.
Supernatant recentrifuged (as before) - 1.00 ml to 10♂♂ (A), challenged in 48 hours. Challenge control (D).
Sediment resuspended - to 37° C incubator, 12 hours.
Centrifuged - 3250 x G/60 min.
Supernatant recentrifuged (as before) 1.0 cc to 10 ♂♂ (B)
Sediment resuspended - part - heated - 46° C/14 min - .25 cc to 10 ♂♂ (C)
(B) and (C) challenged in 48 hours - control on challenge = (E)
Another part of resuspended sediment - .25 cc to 10 ♂♂ - no challenge (F)

Time of death in 1/4 days

Treatment	9th	10th	11th	Delayed	Survived
A. Supernatant before incubation	2	4 1 1		23 d / 1	1/10
D. Controls on A challenge		8 1			0/9
B. Supernatant after incubation	1	3 3 3			0/10
C. Sediment after incubation and 46° C/14 min.		4 4 1	1		0/10
E. Controls on B and C challenge		3 4 3			0/10
F. Sediment after incubation - unchallenged					10/10