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RESEARCH STUDIES OF FACTORS INDUCING RESISTANCE TO TRANSMISSIBLE MOUSE LEUKEMIA

by

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The third quarterly report required by the contract under which this work is being carried out covers the months of April, May and June 1953 and includes the six formal experiments (CD 14-19) cataloged below as well as studies on the technique of elimination of white cells from supernatants.

It was demonstrated that 3% gelatine in saline, compared with saline alone, dramatically hastened the throwing down of red cells and other materials, so the supernatant was crystal clear while that of the saline suspension of the same suspension of leukemic cells handled in the same way was cloudy. However, there were many more white cells in the gelatine supernatant. The use of gelatine was not continued.

In the first experiment, using 5500 G, two runs of 60 minutes were used. But this proved unnecessarily long so the runs were cut down to 30 minutes, then, after still further checking, to 10 minutes; but a third run was added, so the "supernatant III" means two recentrifugations of the original supernatant. After the second and third runs the visible sediment consisted of a small semitransparent disc tightly adherent to the tube.

List of experiments herein reported.

CD 14 - Successive supernatants from heated cells (46°/14 min.).

CD 15 - Supernatants after rotation in 37° bath for 1/2, 2, 4 hours. Leukemic potency of cells after rotation for 6 hours in 37°.

CD 16 - Supernatant III after 37°/30 min. in 24 and 48 hrs. challenged by dilution 4^{-8}; in 48 hrs. by dilution 4^{-9}. Supernatant IV (from resuspended sediment II rotated 37°/60 min.) rotated 37°/4 hrs. and held 2°/4 hrs.

CD 17 - Primary supernatant III after rotation in 37°/30 min. injected at once in comparison with same supernatant rotated 37°/4 hrs. and held at 2°/4 hrs.

CD 18 - Supernatant III after rotation in 37°/30 challenged in 1 hr., 2 days and 6 days.

CD 19 - Supernatant III from untreated standard suspension of I_b, compared with supernatant IV after rotation in 37°/30. Resuspended sediment I from each of above supernatants after third washing, rotated in 37°/80 min. and each supernatant IV tested.

EXPERIMENTS

CD 14.

Can protective effect be obtained from supernatants from line I_b cells heated (46°/14 min.) after removal of primary supernatants? The protection that appeared in the supernatant of heated cells in CD 10 and 11 (9/20 survived) left a question whether the protective action depended upon something contributed by the heated cells, or was originally present. So in this experiment, after the standard suspension of I_b was rotated 30 min. in ice bath, the recentrifuged supernatant was tested. The resuspended cells were heated (46°/14 min.) and after rotating 60 min. in ice, the supernatant II removed and tested; again the heated cells were resuspended, rotated, and the supernatant II tested; shaking and centrifuging was again repeated, but this supernatant was discarded. For the fourth time the heated cells were resuspended, shaken, centrifuged and the supernatant II this time tested. None of the mice given any of the supernatants survived; not even after the primary supernatant. However, part of the heated cells after their first centrifugation were injected into mice, and heated cells after their final centrifugation were also injected into mice. All of the mice receiving heated cells survived the challenging dose (4^{-8}) given 2 days later. This experiment runs counter to the idea that the heated cells yield some protective substances when shaken. That repeated washing did not lessen the capacity of heated cells themselves to induce 100% resistance confirms earlier experiments performed in this laboratory.

CD 15.

Will supernatants from I_b become more protective if rotated in a 37° bath, instead of in ice? The time in 37° was varied from 1/2, 2 and 4 hrs. Supernatants II after these three times gave respective survivors 4/10, 2/10, 1/10. In each case one of the mice called a survivor, when killed in 21 days showed signs of subcutaneous infiltration known to be associated with resistance that is not quite complete. There is no evidence that prolonged rotation in 37° increases the protective effect of the supernatants--in fact the reverse is suggested. However, this is not because the leukemic cells are breaking down, because after rotation for 6 hrs. in 37° the cells were virtually as potent in producing leukemia as the original standard suspension. This indicated a much greater viability of leukemic cells than has been observed previously; but this is the first time that viability in vitro has been studied with shaking. It seems that previous studies merely measured the rate of oxygen starvation. This long survival when shaken opens the way to a variety of studies on the metabolites and other physiological activity of leukemic cells in vitro.

CD 16.

Could the proportion of survivors be increased by challenging at a different time or by using a smaller challenging dose? Will protective action survive rotation of supernatant in 37°/4 hrs? Previous experiments with heated cells showing that when subjected to 46° for 7 min. strong resistance at 20 days is induced; when subjected to 46° for 14 min. virtually no resistance at 20 days but 100% resistance at 2 days--raised the question of varying the time of challenging. Preliminary tests indicating that a dose of 4^{-9} or even 4^{-10} killed all mice, encouraged the use of 4^{-9} as a more subtile challenge.

The one day challenge proved as good as that at 2 days--4/10, compared

with 3/10 survivors; but the dose of 4^{-9} gave only 2/10 survivors, and the controls only 3/10.

Still thinking that the protective substance in the supernatant was in some way originated in the leukemic cells, the available resuspended cells seemed possible material for a first effort to answer the question of the stability of this substance as found in supernatants. Accordingly, resuspended cells were rotated--37°/60 min. and the supernatant II divided into two lots--one was rotated in 37°/4 hrs., the other was held at 2° for 4 hrs. Then both injected and the mice challenged in 2 days with 4^{-8}. Since none of the cold controls survived, the result is uninterpretable. The protective effect might have disintegrated in cold as well as in 37° or there may have been almost none of the protective material in the supernatant from the resuspended cells.

CD 17.

Continuing the questions of the preceding experiment, the stability of the protective material was tested using the primary supernatant III after rotation in 37°/30. When injected at once, 3/10 survived (one of these when killed in 25 days showed the superficial infiltrations characteristic of borderline resistance); when rotated in 37°/4 hrs., 1/10 survived (as in CD 16); when held at 2°/4 hrs., 2/10 survived. The resuspended cells from the first centrifugation after rotation in 37°/30 gave a supernatant III which failed to protect any of 10 mice.

To answer conclusively these questions about resistance which at best is so weak that less than half of the mice survive, is almost impossible. The variations in the small number of survivors may or may not have the apparent meaning. However, it will be noted that the evidence of protective material being produced in vitro is very slight and could well depend upon the incomplete removal of pre-existing protective material by a single centrifugation.

If this were indeed the case, several washings of the cells would eliminate the occasional survivors that have raised the question.

CD 18.

Varying time of challenge - 1 hour, 2 days, 6 days - after treatment with supernatant III from standard suspension of I_b after rotation in 37° for 30 minutes.

To reduce possible deterioration during the long preparation of the five lots of standard suspension of I_b (-4 hrs. - 87 ml.), salt was added to the ice bath in which the material was held as accumulated. The time for each run in the centrifuge was reduced to 10 minutes at full speed; the times in protocol include 5 minutes for speeding up. Since CD 16 had shown that challenging in 24 hours seemed to be as effective as in 2 days, to find resistance in this experiment when the challenging dose was given in an hour after treatment was not quite so surprising but this does raise a large doubt as to the identity of the mechanisms responsible for resistance as induced by these supernatants and by heated cells. This doubt is further increased by the resistance appearing when challenge was delayed for six days, whereas cells heated for 14 min. 46° would yield to very little resistance when challenged at this time. Supposing these results are repeatable, they represent the most significant finding that has been obtained under this contract.

CD 19.

Having been started before the results from CD 18 were known, this experiment returned to the question of the production of protective material by leukemic cells in vitro, and included, as a matter of routine, a test of supernatant III from untreated standard suspension as a direct control on the effect of rotation in 37°.

The cells in the sediment from untreated and from rotated suspensions

separately were twice resuspended and these supernatants discarded; again resuspended they were rotated in 37° for 80 minutes. The supernatant III from each of these lots of washed cells were tested by challenging (4^{-8}) in 48 hours, and gave no survivors from either group of 10 mice each. This seems to add considerable weight to the probability that the protective material in the original supernatant was there in the beginning and not put there by the cells during the period of rotation in 37°.

The two parts of this experiment, combined for practical reasons, suddenly found important theoretical relationship in the unexpected result that the supernatant III from untreated standard suspension gave 4/10 survivors when challenged (4^{-8}) in 2 days. Obviously there is some protective material in the leukemic spleens which in saline suspensions is immediately separable by centrifugation from the leukemic cells.

The fact that the supernatant from the rotated material in this experiment gave only 2/10 survivors, cannot be considered significant, for the experiment was performed in two sections and the rotated and untreated material came from cell suspensions from different lots of spleens. However, in samples as large as ten spleens that came from the same litters, the probability of significant difference in the two original cell suspensions is not very high and it is entirely possible that if the rotation at 37° is not necessary for the demonstration of the protective material, it may prove to be a disadvantage.

Thus the final quarter of this year opens with new and unexpected horizons.

CD 14. CSH 2199 I_b 2093
III/31 - IV/2/1953

Can protective effect be obtained from supernatants from line I_b cells heated after removal of primary supernatant? (46°/14 min.)

5:25 - 6:55 A.M. 24 ml. standard suspension from 12 line I_b spleens, shaken for 30 min. in crushed ice in mechanical rotator (90 r.p.m.).

7:40 - 8:40 A.M. 5500 G/60 min., 12 ml. supernatant P, recentrifuged.

9:10 - 10:10 A.M. 5500 G/60 min., Supernatant P_2, in 1.0 ml. doses to 8 ♂♂ (P) 10:25 A.M.

9:30 -- Sediment P - resuspended; 3 ml. to each of 8 heating vials - 24°/5 min.; 46°/14 min. - to crushed ice.

10:35 - 11:35 A.M. Heated material pooled; shaken for 60 min. in crushed ice as above.

11:45 - 12:45 P.M. 5500 G/60 min.

12:55 - 1:55 P.M. Supernatant A, recentrifuged - 5500 G/60 min.

-- 2:10 P.M. Supernatant A_2 in 1.0 ml. doses to 10 ♂♂ (A).

-- 1:30 P.M. Sediment A resuspended (part) in .25 ml. doses to 10 ♂♂ (D).

1:10 - 2:10 P.M. Sediment A resuspended (remainder) shaken /60 min.

2:15 - 3:15 P.M. 5500 G/60 min. supernatant B_1 recentrifuged as before 3:30 - 4:30 P.M.

-- 4:55 P.M. Supernatant B_2 in 1.0 ml. doses to 10 ♂♂ (B)

3:40 - 4:40 P.M. Sediment B, resuspended; shaken/60 min.

4:45 - 5:45 P.M. 5500 G/60 min. - supernatant discarded.

6:00 - 7:00 P.M. Sediment C, resuspended; shaken/60 min.

7:05 - 8:05 P.M. 5500 G/60 min. - Supernatant D, recentrifuged as before 8:20 - 9:20 P.M.

-- 9:30 P.M. Supernatant D_2 in 1.0 ml. doses to 9 ♂♂ (C).

-- 8:35 P.M. Sediment D, resuspended; in .25 ml. doses to 9 ♂♂ (E).

IV/2 10:00 - 10:48 P.M. Group A-F (incl.) inoculated .2 ml. 4^{-8} dilution standard suspension of line I_b transfer 2093.

CD 14. (cont.)

Time of deaths in 1/4 days after challenge with dilution 4^{-8} of line I_b.

Group	9th . . .	10th . . .	11th . . .	12th . . .	13th . . .	Survived
P. Primary supernatant II after shaking/30 min.	4	1 3				0/8
A. Supernatant II after resuspension and 46°/14	4	3 1	1		1	0/10
B. Supernatant II from 1st resuspension of heated cells	7	2 1				0/10
C. Supernatant II from 3rd resuspension of heated cells	8	1 1				0/10
D. Sediment after 46°/14 min.						9/9
E. Sediment after 3rd resuspension of heated cells						9/9
F. Controls in challenging dose 4^{-8}	1	3 2 2	1			1/10

CD 15. CSH 2206 I_b 2096

Will cells produce leukemia after 6 hours rotation in 37° (E & F)?

Supernatants after standard I_b rotated in 37° bath for 1/2 (A), 2 (B), and 4 hours (C).

Rotation by horizontal rotator, 40 per min. Centrifuging 5500 G 60 min. at full speed.

4/21/53

7:20 A.M. Start killing for spleens: 2 ml. saline per gm. spleen.

10:30 A.M. 32.60 ml. standard divided equally in 5 ml. lots between Erlenmeyer flasks. A and C to 37° bath and rotator; 135 ml. volume immersed at an angle.

11:05 A.M. (30 min. plus allowance or warming up) Flask A contents by 5 ml. syringe to tube A1 (55 ml. lusteroid centrifuge tube); to centrifuge; top speed 11:15 - 12:15.

12:20 P.M. Supernatant from A1 - 11 ml. to tube A2 and recentrifuged; 1 more ml. supernatant removed and sediment resuspended in 12 ml. saline.

12:30 P.M. Resuspended sediment A1 to flask B to 37° bath and rotated 2 hours.

1:40 P.M. Tube A2, after 60 min. centrifuging, withdrew 10 ml. supernatant to vial; inject 1.0 ml. to 10 mice - group A - last shot 1:50 P.M.

2:30 P.M. Flasks B and C contents removed with 5 ml. syringes to tubes B1 and C1 (in flask C "white material" stuck around edge of rotation wave) to centrifuge - top speed 2:50 - 3:50 P.M.

3:55 P.M. Supernatant from B1 to B2; supernatant from C1 to C2 - to centrifuge.

4:05 P.M. Sediment from tube C1 resuspended to flask D to 37° bath and rotator. 2h.

5:05 P.M. Tube B2 withdrew 10 ml. supernatant to vial inject 1.0 ml. to 10 mice = B.
Tube C2 withdrew 10 ml. supernatant to vial, 1.0 ml to 10 mice = C.

5:30 P.M. Last injection of above.

6:05 P.M. Flask D from bath; inject .2 ml. to 10 mice - group E.
.05 ml. to 10 mice - group F,
last shot 6:20.

4/23/53

4:10 - 4:25 P.M. Inoc. groups A, B, C and untreated controls D with .20 ml. 4-8. These groups included one mouse each from ten litters of four. Groups E and F. for an independent purpose, are entirely unstaggered.

CD 15. (cont.)

Group	Time of death by 1/4 days after challenge (4^{-8}) 9th . . .	10th . . .	11th . . .	12th . . .	Survived
A. Supernatant II after 1/2 hr. in 37°	1	2 2	1		4/10
B. Supernatant II after 2 hrs. in 37° (resuspension after 37°/30)		2 2 2	1	1	2/10
C. Supernatant II after 4 hrs. in 37°		2 4 1	2		1/10
D. Controls on challenge (4^{-8})		1 6	2 1		0/10

	Days after injection 4th . . .	5th . . .	
E. Sediment - 6 hrs. in 37° - .20 ml. dose		7 3	0/10
F. Sediment - 6 hrs. in 37° - .05 ml. dose		9 1	0/10

CD 16. CSH 2213 I_b 2099
CSH 2214 I_b 2099
V/12-14/53

Reduction in time before challenge and reduction in challenging dose after treatment with supernatant from standard I_b suspension rotated 37°/30 min.

Will protective action survive rotation of supernatant in 37°/4 hrs?

8:20 A.M. Began killing donors, 26 spleens yielded 64.45 ml. standard suspension plus 4.10 ml. from preceding routine transfers - all held in crushed ice as collected, after passing cotton filter.

11:35 - 12:10 P.M. In two flasks rotated (40 per min.) 37°/35 min.

12:30 - 1:00 P.M. To centrifuge tubes a_1 and a_2 - 5500 G/30.

1:15 - 1:45 P.M. Supernatant I to tubes a_3 and a_4 - 5500 G/30.

2:05 - 2:35 P.M. Supernatant II to tubes a_5 and a_6 - 5500 G/30.

3:05 - 3:15 P.M. Supernatant III to vial - in 1.0 ml. doses to 30 ♂♂ (groups A, B, and C).

1:35 - 2:35 P.M. Sediment I from 1st centrifuge run above, resuspended, to 2 flasks, rotated 37°/60 min.

3:00 - 3:30 P.M. To centrifuge tubes g_1 and g_2 - 5500 G/30 min.

3:50 - 4:20 P.M. Supernatant I to tubes g_3 and g_4 - 5500 G/30 min.

4:30 - 8:30 P.M. Supernatant II - 1/2 to flask, rotated 37°/4 hrs.
Supernatant II - 1/2 to tube g_5 to 2° room - 4 hrs.

8:45 - 9:00 P.M. Rotated supernatant II in 1.0 ml. doses to 10 ♂♂ (G)
Supernatant II from cold room in 1.0 ml. doses to 10 ♂♂ (H)

V/13/53

3:10 - 3:20 P.M. Challenged (4^{-8}) group A and controls D.

V/14/53

2:15 - 2:30 P.M. Challenged (4^{-8}) groups B, G. H, and controls E.

2:35 - 2:45 P.M. Challenged (4^{-9}) group C and controls F.

CD 16. (cont.)

	Time before death in 1/4 days after challenge										Survived
	9th		10th			11th		12th		13th	
A. Supernatant III after 37°/30 Challenged in 24 hrs - 4^{-8}			2		1	1	1			1	4/10
D. Controls for A			1	3	5		1				0/10
B. As A, but challenged in 48 hrs. 4^{-8}		1	2		1	1		2			3/10
E. Controls for B		2	7	1							0/10
C. As A, but challenged in 48 hrs. 4^{-9}				1		2	2		1	2	2/10
F. Controls in 4^{-9} for C						4		2		1	3/10
G. Supernatant II from resuspended sediment I, rotated 37°/60, rotated 37°/4 hrs. challenge 4^{-8} - 48 hrs.		3	4		1				1		1/10
H. Supernatant II as for G but held 4 hrs. at 2°			5	3	2						0/10
E. Controls for G and H (see above)											

CD 17. CSH 2219 I_b 2101
V/26-28/53

Does rotation 37°/4 hrs. inactivate effectiveness of primary supernatant after 37°/30 min. standard I_b?

Do cells produce X-substance in 37°/30?

Groups:

A. 1.0 ml. supernatant III after rotation in 37°/30 min. injected at once.
B. 1.0 ml. " " " rotated 37°/4 hrs.
C. 1.0 ml. " " " held 0° 4 hrs.
D. 1.0 ml. supernatant III from resuspended sediment I rotated 37°/30 min.
E. Controls on challenge 4^{-8} in 48 hrs.

9:00 A.M. - 12:40 P.M. Preparation of 71.90 ml. standard suspension from 31 spleens from CSH 2214 I_b 2100 - in ice bath during preparation.

12:40 P.M. 35.8 ml. standard suspension in each of two flasks in 37° bath rotated 35 min.

1:40 - 2:10 P.M. 35 ml. in each of two centrifuge tubes 5500 G/30 min.

2:35 - 3:05 P.M. Supernatant I, 22 ml. per tube 5500 G/30 min.

3:20 - 3:35 P.M. Supernatant II, 18 ml. per tube 5500 G/15 min.

4:00 P.M. Supernatant III inject 10♂♂ (A)

3:55 P.M. Supernatant III 10+ ml. to flask rotated 37°/4 hrs; inject 10 ♂♂(B)
Supernatant III 10+ ml. to 0° room 4 hrs; inject 10 ♂♂ (C)

3:00 - 3:30 P.M. Sediment I, resuspended to original volume rotated 37°/30 min.

3:45 - 4:15 P.M. 5500 G/30 min.

4:30 - 5:00 P.M. Supernatant I 5500 G/30

5:10 - 5:25 P.M. Supernatant II 5500 G/15

5:40 P.M. Supernatant III inject 1.0 ml. 10♂♂ (D)

V/28

4:30 - 4:55 P.M. Challenge A, B, C, D, and E controls 4^{-8}: 50 mice - one suspension of 4^{-8} (12 ml.) mixed with 5 ml. syringe.

CD 17. (cont.)

Time of death in 1/4 days after challenge (4^{-8}) in 2 days

	9th . . .	10th . . .	11th . . .	12th . . .	Delays	Survived 25 d
A. Supernatant III after 37°/30 min.	1	2 1	1	1	18 d / 1	3/10
B. Same supernatant III rotated 37°/4 hrs.		5 2		2		1/10
C. Same supernatant III 0° room 4 hrs.		3 3	1 1			2/10
D. Supernatant III from sediment I after 37°/30 min.	1 1	4 4				0/10
E. Controls in 4^{-8}		4 5	1			0/10

CD 18.

CSH 2222 I_b 2103
CSH 2224 I_b 2103
CSH 2225 I_b 2103
CSH 2226 I_b 2104

Varying time of challenge after treatment with supernatant III from standard suspension I_b rotated 37°/50 min.

Standard suspension collected in ice-salt bath: centrifuge runs (5500 G) cut down to 10 min. plus 5 min. for speeding up.

VI/9/53

6:30 A.M. Began killing donors of spleens: 34 spleens yielded 87 ml. standard suspension.

10:40 - 11:30 A.M. Rotated (50 per min.) in 2 flasks in 37° bath.

11:47 - 12:08 P.M. Centrifuged in 2 lusteroid tubes 5500 G/10 (+5) min.

12:17 - 12:32 P.M. Supernatant I - 5500 G/10 (+5) min.

12:41 - 12:46 P.M. Supernatant II - 5500 G/10 (+5) min.

1:07 - 1:30 P.M. Supernatant III - in 1.0 ml. doses to 40 ♂♂ - groups A, B, C, D.

2:00 - 2:08 P.M. Challenged (0.2 ml. doses 4^{-8}) group A (1 hour).

VI/10/53

1:35 - 1:39 P.M. Challenged (4^{-8}) group B (24 hours).

VI/11/53

1:15 - 1:20 P.M. Challenged (4^{-8}) group C (48 hours).

VI/15/53

4:20 - 4:25 P.M. Challenged (4^{-8}) group D (6 days).

CD 18. (cont.)

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

Time before challenge	9th . . .	10th . . .	11th . . .	12th . . .	Survived
A. 1 hr. CSH 2222		1 2	2		5/10
A - Controls		4	1 2	2 1	0/10
B. 24 hrs. CSH 2224					-
B - Controls - technical error= no leukemia					-
C. 48 hrs. CSH 2225		3 1		1	5/10
C - Controls	2	5 2			1/10 = recovery-- had large spleen
D. 6 days $\frac{7\ d}{2}$ CSH 2226	1	1			6/10
D - Controls		1	1 8		0/10

CD 19. CSH 229 I_b 2104
VI/16-18/53

Can any protective effect be obtained from:

1. Supernatant III, from untreated standard suspension of I_b compared with rotation 37°/30 min?

2. Supernatant III from washed (x3) cells resuspended for 4th time and then rotated 37°/80 min?

In order to reduce the excessive time between killing first donor and first injection of mice, this experiment was performed in 2 sections. In each section 10 spleens were minced together and when suspended in saline at once rotated without chilling. The donors of the two lots of spleens came from the same litters in nearly equal numbers.

Section I.

8:30 A.M. Began killing donors: 10 spleens yielded 18.25 ml. standard suspension.

9:30 - 10:00 A.M. Rotated 37°/30 min. - to centrifuge tube 1.

10:05 - 10:15 A.M. 5500 G/10 +5 min.

10:26 - 10:41 A.M. Supernatant I - to tube 2
Sediment I, resuspended tube 1 } 5500 G/10 +5 min.

10:55 - 11:10 A.M. Supernatant II from tube 2 to tube 3
Sediment II resuspended (2) tube 1 } 5500 G/10 +5 min.

11:30 A.M. Supernatant III from tube 3 - to vial, in 1.0 ml. doses to 10♂♂ (A)

11:20 - 12:40 P.M. Sediment III from tube 1, resuspended, to flask, rotated 37°/80 min.

12:45 - 1:00 P.M. To tube 4 - 5500 G/10 +5 min.

1:15 - 1:30 P.M. Supernatant I to tube 5 - 5500 G/10 +5.

1:45 - 2:00 P.M. Supernatant II to tube 6 - 5500 G/10 +5.

2:25 P.M. Supernatant III to vial, held in 0° room 25 min., then in 1.0 ml. doses to 10 ♂♂ (B)

Section II.

11:45 A.M. Began killing donors: 10 spleens yielded 23 ml. standard suspension.

12:45 - 1:00 P.M. Directly to centrifuge tube a - 5500 G/10 +5 min., with tube 4.

CD 19. (cont.)

1:15 - 1:30 P.M. Supernatant I to tube b
Sediment I, tube a resuspended } 5500 G/10 +5 with tube 5

1:45 - 2:00 P.M. Supernatant II to tube c
Sediment II, tube a resuspended } 5500 G/10 +5 with tube 6

2:10 - 3:30 P.M. Sediment III, tube a, resuspended, to flask, rotated 37°/80 min.

2:33 P.M. Supernatant III, from tube c, to vial, in 1.0 ml. doses to 10 ♂♂(C)

3:35 - 3:50 P.M. Rotated sediment III to tube d - 5500 G/10 +5 min.

3:37 - 4:12 P.M. Supernatant I from tube d to tube e - 5500 G/10 +5 min.

4:20 - 4:35 P.M. Supernatant II from tube e to tube f - 5500 G/10 +5 min.

4:45 P.M. Supernatant III from tube f - in 1.0 ml. doses to 10 ♂♂.

VI/18/53

3:00 - 3:19 P.M. Challenged A, B, C, D and E - 0.2 ml. dose - dilution 4^{-8} of standard suspension of I_b.

Time before death in 1/4 days.

Supernatant III from:-	9th . . .	10th . . .	11th . . .		Survived
Section I					
A. Rotated 37°/30	1 1 2	2 1	1		2/10
B. Washed cells from above rotated 37°/80	1 1 2 2	2 1 1			0/10
Section II					
C. Untreated standard suspension		4 1	1		4/10
D. Washed cells from above rotated 37°/80		1 6	2	1 delayed 18 d.	0/10
E. Controls in challenge dose 4^{-8}	1	2 2 2	3		0/10