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# RESEARCH STUDIES OF FACTORS INDUCING RESISTANCE TO TRANSMISSIBLE MOUSE LEUKEMIA

by

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The fourth quarterly report required by the contract under which this work has been carried out, covers the months of July, August and September 1953, and includes ten formal experiments (CD 20-29) cataloged below.

List of experiments herein reported.

CD 20 - Supernatant III from untreated standard suspensions of leukemia $I_b$, compared with supernatant III after rotation at 37°/30 min. challenged in 1 hour and 20 days.

CD 21 - Supernatant III from untreated $I_b$ challenged in 6 days; rotated at 37°/30 min., challenged in 6 days and in 25 days.

CD 22 - Repeated CD 20.

CD 23 - Supernatant III from untreated $I_b$: 1) mixed with challenging dose ($4^{-8}$) and injected at once and after roation at 37°/60 min.
2) challenged after 1 hour, and after 22 days.

CD 24 - Repeated CD 23.

CD 25 - Supernatant V (8600 G) challenged ($4^{-6}$) in 1/2 hour and after 20 days ($4^{-8}$).

CD 26 - Repeated CD 25, using challenge dose of $4^{-8}$.

CD 27 - Supernatant V (8600 G) as compared with supernatant III (5500 G) challenged ($4^{-8}$) "at once".

CD 28 - Repeated CD 27 except each supernatant from a different standard suspension but the same challenging suspension.

CD 29 - Repeated CD 28 with an added group - supernatant III (11,000 G).

EXPERIMENTS

CD 20.

The rotation (at 37°) of a standard suspension of line $I_b$ leukemia before centrifuging was indicated, by CD 19, to be unnecessary for the protective effect of supernatant III. This is confirmed by experiments CD 20, 21 and 22. In CD 20, section I, a more rigid test was made by centrifuging one part of a single standard suspension of line $I_b$ cells without any treatment and the other part after rotation in 37°/30 min. The supernatant III (5500 G) from each of these sources was injected into a group of mice and both groups were challenged in an hour with the same dilution ($4^{-8}$) of leukemic cells. Section II of this experiment, started independently with another lot of leukemic spleens used only untreated standard suspension from which supernatant III was injected into 2 groups and challenged respectively in an hour and in 20 days.

The resistance shown in the 20 day group was comparable to that at any other times tested (4/10 survived). This was highly surprising because in the work with line $I_b$ leukemic cells subjected to 46° C for 14 min., protection was given in 2 days, but by a week it had virtually gone, unless a very few untreated leukemic cells were added to the heated ones, or the length of heating reduced to 7 min., in which case a very few leukemic cells remained intact. Both of these cases give good resistance at 20 days.

Could it be that supernatant III (3 x 5500 G/10+5 min.) might still include a very few leukemic cells? In this case there were not enough cells (if any) in the supernatant III to produce leukemia in 20 days but in CD 18 the supernatant III, challenged in 6 days (group D), gave 2 deaths 3 days ahead of the first death of the controls.

In CD 20, Section II, the repetition of the immediate challenge after supernatant III from untreated standard suspension (E), was apparently without result, since 9/10 of the controls in the challenging dose survived, and the

one death was delayed. In spite of all efforts to interpret and avoid them, such failure of the controls has seriously interfered with several of the experiments in this last quarter, even involving the last experiment, CD 29. After so many experiments in which the controls regularly died, and the correlation between dosage and the time of the appearance of big spleens has been so high that these have been produced unfailingly on the date desired, it might be supposed that an intrinsic change in the properties of the leukemic cells might account for this unexpected survival of controls.

But this cannot be the correct interpretation, because along side of the cases of failure, other controls have given the expected 100% deaths. Differences in the number of cells in dilution $4^{-8}$ arising from variations in accuracy of weighing spleens, or measuring saline, or from differences in the length of time between killing the spleen donor and inoculating the diluted dose of cells with the hosts can account for differences of a day or even two in the average intervals before death, but not for the survival of controls. A possible interpretation has been suggested, covering all the observations, by the results of the final experiment (CD 29) when an experimental test of its validity was no longer possible.

The site of inoculation has always been swabbed with alcohol before the needle is inserted. If instead of being moistened the hair is dripping wet with alcohol, this may wet the needle upon withdrawal. If the needle is returned quickly in this condition to the vial containing the $4^{-8}$ dilution of cells, an appreciable amount of alcohol may be introduced - especially when the volume of the cell suspension is small. One of the precautions devised against survival of controls has been to mix only small volumes in the final step of dilution $4^{-8}$, for there has been considerable evidence that the last syringefuls from volumes, say, of 10 ml, have lost some potency. Certain it is that in some cases the hair has been dripping wet with alcohol and in other

cases only moistened. Just when the use of such excess alcohol began is uncertain, but it seems probable that it was a result of the growing pressure that developed this early summer and eliminated every seemingly unnecessary movement - such as squeezing out the cotton before applying the alcohol. This could account for a reduction in the lathality of the challenging suspension in any syringeful after the first, according to how much alcohol was applied to the hair of the mouse given the last dose of that syringeful (5 mice per box, 5 doses per syringeful). The controls have always been inoculated after the test group. This could account for a gradual weakening of the 4-8 dilution in a big series of inoculations; it could also account for the abrupt loss of lethality that appeared in both sections of CD 29.

Returning to CD 20 - in Section II the first syringeful of the challenging dilution given to group E, resulted in 2 deaths on the 11th day and 3 survivors; the second syringeful given to the first control box F1 gave 1 death at the limit of 13 days and 4 survivors; the third syringeful given to the second box of tests, E2, as well as the fourth syringeful given to the second box of controls gave no deaths at all. It is entirely possible that the results of the first syringeful are valid - that 3/5 survived on account of the prior injection of supernatant III. In the following three boxes, the survivors may have received challenging doses rendered sub-lethal by alcohol introduced when the syringe was returned for the second filling and the usual ten squirts given to insure uniform distribution of the cells.

CD 21.

In comparing supernatant III from rotated and untreated standard suspension of $I_b$ as challenged in 6 days, this experiment was divided into two independent sections for preparation and injection of the supernatants but on the 6th day, one challenging dilution of 4-8 was used for all the mice.

Besides this, one box of 5 mice in each section was injected with supernatant III from rotated standard suspension and not challenged until the 25th day.

In the first section, after supernatant III from rotated suspension (A1) 1/5 survived; against 3/5 after supernatant III from untreated suspension (C1); and 5/0 - controls (D1). In the second section, 2/5 survived in the "rotated" group (A2), 5/5 survived in the "untreated" group (C2) and 5/5 controls (D2) survived. Since all these mice were inoculated with dilution $4^{-8}$ from the same vial, one syringeful per box of five in the above order, it is evident that the last two syringefuls had lost their lethality. This sudden change between the fourth and fifth syringefuls was preceded by a progressive lengthening of the intervals before death of the mice that died. At the time this seemed to indicate a deterioration with time (although the dilution vial was kept in crushed ice), so in subsequent experiments, when more than four syringefuls were required of the same challenging dilution, the last step of diluting was duplicated in two vials. It should be noted that settling out of the cells could not account for the failure of the last syringefuls, for the suspension was thoroughly agitated before each syringeful was withdrawn, by 60 syringe squirts of 1 ml in blending the dilution at first, and 10 squirts before each successive syringeful.

Although incomplete, this experiment gives evidence that supernatant III from both rotated and untreated standard suspension gives resistance at 6 days, comparable to that appearing earlier. Further, groups B1 and B2, injected with supernatant III from the rotated standard suspension showed comparable resistance when challenged in 25 days; 3/10 survived, against 0/10 controls on challenging dose ($4^{-8}$).

CD 22.

The effect of rotating standard suspension at 37° was further checked by comparing supernatant III from rotated and untreated cell-suspension challenged

in an hour. One group (C1 and C2) with "untreated" supernatant III was not challenged at once but held as controls on the possible lethality of the supernatant. The two sections of this experiment were performed entirely independently with different challenging dilutions, on successive days. Again the rotation seemed to have no effect - all the controls died and the unchallenged group in the first section (C1) developed no signs of leukemia until, after 22 days, it was challenged ($4^{-8}$) and 1/5 survived. But the unchallenged group in the second section (C2) developed two cases of leukemia with death on the 13th day. This is the first direct evidence of leukemic cells in supernatant III, although the delayed challenging in this and earlier experiments was planned primarily to check this possibility which had been suggested by the death of two mice with supernatant III two days ahead of the earliest death known for that challenging dose.

CD 23.

Since some resistance can be established when the challenging dose (.2 ml dilution $4^{-8}$) is given an hour after supernatant III (1.0 ml) from untreated standard suspensions, the question is raised whether the material responsible for this resistance acts upon the leukemic cells directly or upon the host. To approach this question, the challenging dilution was mixed with supernatant III before injection in the proportion of 0.2 : 1.0 with 1.2 ml per dose. One group of mice was inoculated at once, another group inoculated after the mixture of supernatant III and challenging dilution had been incubated for 1 hour by rotation in 37°. Controls (D1) were inoculated after the unmixed portion of the challenging suspension had been held in the cold room (2°) for one hour. All of the above was performed twice, independently from the beginning, with the addition, in the second section, of a group (2 boxes C1 and C2) injected with supernatant III alone, and challenged with the controls (D2) in one hour.

This entire experiment, including (for the first time) the preparation of the challenging dilution, was performed in the cold room (2°), anticipating excellent results. But of the 40 mice included in the two sections only 3 died, and these were in the first section with the mixture given before rotation (A1). Alcohol carried over on the needle could explain the survival of controls in the first section but not the unexpected survivors in the first box of the second section. At the time it seemed suggestive that the challenging dilution in this and in no other experiment was prepared in the cold room. In the following repeat experiment, in which all other procedures were carried out in the same way except that the challenging dilution was made in the laboratory instead of in the cold room, the results are significant.

CD 24.

The repetition of CD 23 included the same groups with the exception of the group given supernatant III alone; replacing this, a group (C1 and 2) was given the challenging dose after it (alone) had been rotated 37°/60 min. The two independent sections of this experiment gave identical results and can be combined. All controls died, and 4/10 survived the mixture of supernatant III and challenging dilution $4^{-8}$ - (1.2 ml dose) inoculated at once. However, all those given the incubated mixture survived 10/10. This would have seemed an emphatic answer to the question of direct action of supernatant III upon the cells, had the rotation of the challenging dose alone been omitted. But all of this group (C1 and 2) also survived - so that the survival of the group with the rotated mixture depended upon the effect of the rotation in 37° upon the leukemic cells, rather than the effect of supernatant III upon them.

CD 25.

If leukemic cells in supernatant III are responsible for the resistance found at 20 days, supernatant after more intense centrifugation should fail

to give any protection at this time. Will the complete removal of leukemic cells wipe out the resistance to immediate challenge? The rest of the experiments were directed toward these questions. Supernatant III (3 x 5500 G) is slightly clouded, and in the last run some sediment is thrown down. By increasing the speed of the centrifuge to give 8600 G, and increasing the number of runs to five, a brilliantly clear supernatant V was obtained, which gave no visible sediment after the fifth run. Beginning with this experiment the use of the cold room was given up entirely. In CD 25, two groups were given supernatant V; one group challenged at once, one group in 20 days. In view of the recent survival of controls and at that time suspecting an intrinsic change in the potency of the leukemic cells, the immediate challenge was increased to $4^{-6}$. In each section of the experiment all the supernatant V mice died.

Before the time for the 20 day challenge, dilution $4^{-8}$ in other experiments was killing all the controls, so that any intrinsic reduction in the virulence of the leukemic cells seemed ruled out. Accordingly dilution $4^{-8}$ was used for the 20 day challenge utilizing the $4^{-8}$ dilution prepared for the second section of CD 27. When this second section of CD 27 was being carried out, only one possible donor for the immediate challenge remained alive, and this one died naturally a few minutes before its spleen would have been taken. Since the mouse was hardly cold when the spleen was removed, no trouble was anticipated, but all the fifteen controls inoculated with the $4^{-8}$ dilution from this spleen survived, as well as 17 of the 20 mice in the test groups. As had become the practice, the $4^{-8}$ dilution was made in two vials, a different syringe for each vial. It is interesting that the first syringeful from each vial was responsible for the three deaths that did follow. In this case, and in all other experiments, the deaths have invariably resulted from leukemia, with typical spleens.

CD 26.

Again supernatant V was used for challenge at once and in 20 days. Although the challenge dose was $4^{-8}$, all treated and control mice died promptly after the immediate challenge. From the 20 day challenge 1/10 survived, but the litter-mate of this one in the controls also survived.

The question raised under CD 25 is unanswered and the termination of the contract prevented further challenging at 20 days. There remained opportunity to compare supernatant V and supernatant III when challenged at once.

CD 27.

In order to start with the same material to produce supernatant III and supernatant V and control the time and the temperature of larger and faster centrifuging, all the required standard suspension was placed in one centrifuge tube and the supernatant III (3 x 5500 G/10+5 min.) prepared. Then this was divided between two tubes, one of which was given 3 x 8600 G, and the supernatant removed each time to a clean tube to produce what will be called supernatant "V"; the other tube was given the same centrifuging but each time the sediment was resuspended without removal of the supernatant, leaving it supernatant III. In the first section of the experiment, 1/5 given supernatant III survived and 0/5 survived given supernatant "V" - all controls died. In the second section, the donor of the challenging dilution had died naturally as already mentioned and the only death was from the first syringeful, one mouse given supernatant "V" - which died on the 14th day. On the chance that the challenge had not included enough cells even to immunize, these three groups were reinoculated 12 days later in connection with the final experiment, CD 29. All of the reinoculated controls died, as well as all of the previously uninoculated controls; of the group given supernatant III 3/5 survived and of the group given supernatant "V" 3/4 survived. The prompt death of the controls makes this result seem to have some significance for the comparison of superna-

tants III and "V". Compared with the first section of this experiment these proportions of survivors are high and a suspicion is raised that in the original challenge there may have been enough cells in the first two syringefuls to partially immunize (witness one death), but the last syringeful given to the controls had been reduced below the immunizing level, perhaps by alcohol carry-over.

CD 28.

Instead of starting with the same standard suspension of $I_b$ for both supernatants III and V, in this experiment the two supernatants (III = 3 x 5500 G; V = 5 x 8600 G) were centrifuged independently from different standard suspensions but within each of the two sections, were challenged by the same $4^{-8}$ dilution. Thus, group A was challenged an hour after treatment with supernatant V, and group B challenged a few minutes after treatment with supernatant III. In the first section, after supernatant V, 1/5 survived; after supernatant III, 2/5 survived and all controls died; in the second section, 1/5 survived after each supernatant and 1/5 of the controls. On face value, the second section is completely negative. In comparison with the first section and considering the uncontrolled phenomenon of deterioration of dilution $4^{-8}$ in successive syringefuls that has been so obviously present in some cases, it seems altogether probable that the survivors in the first two groups and in the third (controls) survived for different reasons, and for the purposes of the comparison of the two supernatants, the two sections may reasonably be combined, indicating 2/10 survivors for supernatant V and 3/10 for supernatant III.

CD 29.

In the first experiment, mice for a fourth staggered group were available, so CD 28 was repeated with the addition of a group given supernatant III run

at 11,000 G, in this way producing a crystal clear supernatant without increasing the total time in the centrifuge as required for supernatant V. Within each section all three groups were challenged with the same $4^{-8}$ dilution at the same time: in group A (supernatant V) this was within 2 hours; in group B (supernatant III, 5500 G) this was in 1 hour; and for group C (supernatant III, 11,000 G) this was in a few minutes.

The results were disconcerting but dramatic. All the controls on dilution $4^{-8}$ in each of the two sections survived although half of the supernatant-treated mice died. In the first section the deaths occurred in the first two syringefuls of dilution $4^{-8}$; the 3rd syringeful (C1, supernatant III, 11,000 G) as well as the 4th syringeful (controls) gave 5/5 survivors. The total time elapsed in inoculating was less than 20 minutes. In the second section, the first three syringefuls gave deaths (3, 4, and 4 respectively) and the fourth none. It was the abrupt breaks between the 2nd and 3rd syringefuls in the first section and between the 3rd and 4th syringefuls in the second section that at long last, in defying every other interpretation that had been devised for the failure of controls, pointed to alcohol carried over by the needle as the explanation of the weakening of the virulence of the challenging dose in successive syringefuls. For this could account for gradual change quite as easily as for the abrupt changes here exhibited.

In section I, 4/5 survivors after supernatant V (A1) is unusually high; one of these, however, was a borderline case, with a large spleen, which nevertheless recovered. With all the supernatant III (11,000 G) mice (C1) surviving, the first impression was that the $4^{-8}$ dilution must have been weak to start with, but this is not supported by the groups from CD 27 reinoculated with a second vial of dilution $4^{-8}$ made at the same time from the same $4^{-6}$ dilution. As described above, the 3rd and 4th syringefuls in these reinoculations were fully potent and killed all the reinoculated controls as well as all

the controls (4th syringeful) being inoculated for the first time. By the time these mice were inoculated the watch glass of alcohol for swabbing was probably running dry!

As it stands the tally of results for this experiment seems to say that treatment with these supernatants increases the susceptibility of the mice rather than induce resistance. In view of all that has gone before, this is untenable. On the other hand, if the evidence of erratic reduction in the virulence of successive syringefuls of the same $4^{-8}$ dilution is accepted, whether or not alcohol carry-over is the correct interpretation, one seems justified to consider the results for A1 and 2, B1 and 2, and C2 as reasonably good for the comparison of resistance induced by the three supernatants. However fragmentary the evidence in all of these experiments none of it suggests that the resistance induced by supernatant III (5500 G) is eliminated by more intense centrifugation.

CD 20. CSH 2232 $I_b$ 2106
CSH 2233 $I_b$ 2106
CSH 2245 $I_b$ 2109

VI.30.1953

Supernatant III from untreated, compared with rotated (37°), standard suspension of $I_b$, challenged at once and in 20 days. This checks CD 19, using a single original cell suspension.

Groups

Section I A. Supernatant III from untreated standard suspension $I_b$.

B. Supernatant III from rotated (37°/30 min.) standard suspension $I_b$.

C. Controls on challenge ($4-8$) given in 1 hour.

Section II D. Supernatant III from untreated standard $I_b$ - unchallenged for 20 days, then given $4-8$.
Controls for D challenge.

E. Supernatant III from untreated standard $I_b$.

F. Controls on challenge ($4-8$) for E, given in 1 hour.

Section I.

7:00 - 9:00 A.M. Prepared standard suspension (43.0 ml) in two lots of 9 spleens each; while collecting each lot of spleens held in staining jar packed in ice and salt. First lot, after suspension, held in flask packed in ice and salt. Second lot, after suspension, combined with first lot and equal volumes, in alternate 2 ml portions, to centrifuge tube (a1) and another flask.

9:00 - 9:35 Flask rotated (50 p.m.) - to tube b1.

9:45 - 10:00 Tube b1 - 5500 G/10+5 min. - supernatant I (1.4 ml) to tube b2.

10:07 - 10:22 Tube b2 - 5500 G/10+5 min. - supernatant II (13 ml) to tube b3.

10:30 - 10:45 Tube b3 - 5500 G/10+5 min. - supernatant III (10.5 ml) to vial.

10:50 - 10:54 Injected 10 ♂♂ group A - 1.0 ml per mouse.

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9:05 - 9:20 A.M. Tube a1 - 5500 G/10+5 min. - supernatant I to tube a2.

9:25 - 9:40 Tube a2 - 5500 G/10+5 min. - supernatant II to tube a3.

9:45 - 10:00 Tube a3 - 5500 G/10+5 min. - supernatant III to vial - held in 2° room till supernatant from tube b3 ready to inject.

10:40 - 10:44 Inject 10 ♂♂ group B - 1.0 ml per mouse.

11:47 - 12:03 Challenged groups A and B (C = controls) .2 ml $4^{-8}$ CSH 2232 $I_b$ 2106.

Section II.

1:30 - 3:30 P.M. Prepared 40.0 ml standard $I_b$ in two lots as in Section I, all in tube d1.

3:32 - 3:47 Tube d1 - 5500 G/10+5 min. - supernatant I to tube d2.

3:55 - 4:10 Tube d2 - 5500 G/10+5 min. - supernatant II to tube d3.

4:17 - 4:32 Tube d3 - 5500 G/10+5 min. - supernatant III to vial.

4:45 - 4:56 Injected 1.0 ml per mouse, groups D and E.

5:59 - 6:08 Challenged ($4^{-8}$) groups E and F (C = controls) CSH 2232 $I_b$ 2106.

VII.20.1953 - CSH 2245 $I_b$ 2109

1:00 Challenged ($4^{-8}$) group D (at that time all negative) and 10 ♂ (correlated) controls.

CD 20 Results

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

| | 9th | 10th | | | 11th | | 12th | | 13th | Survived |
|---|---|---|---|---|---|---|---|---|---|---|
| Supernatant III from standard suspension $I_b$: | | | | | | | | | | |
| Section I. | | | | | | | | | | |
| A. Untreated | 1 | 1 | | 4 | | 1 | | | | 3/10 |
| B. Rotated - 37°/30 | | 1 | 1 | 2 | 2 | 1 | | 1 | | 2/10 |
| C. Controls on challenge in 1 hour CSH 2232 $I_b$ 2106 | | | 1 | 1 3 | | 4 | | | | 0/9 |
| Section II. | | | | | | | | | | |
| D. Untreated - challenged in 20 d. | | | | 1 | 1 | 3 | | 1 | | 4/10 |
| Controls on challenge (4-8 of D CSH 2245 $I_b$ 2109 | | | | 2 | | 3 | 3 | 1 1 | | 0/10 |
| E. Untreated - challenged in 1 hour | | | | | | *2 | | | | 8/10 |
| F. Controls on challenge (4-8) of E CSH 2233 $I_b$ 2106 | | | | | | | | | **1 | 9/10 ! |

* Inoculated with 1st syringeful.

** Inoculated with 2nd syringeful.

CD 21. CSH 2240 $I_b$ 2108

Supernatant III from rotated 37°/30 and untreated standard suspension of $I_b$, challenged in 6 days and in 25 days.

Groups

Section I
- A1. Supernatant III rotated 37°/30 - challenged in 6 days.
- B1. Supernatant III rotated 37°/30 - challenged in 25 days.
- C1. Supernatant III untreated - challenged in 6 days.
- D1. Controls on challenge $4^{-8}$ in 6 days.

Section II
- A2.
- B2.
- C2.
- D2.

} as above

Section I.

VII.8.1953

2:05 - 4:14 P.M. Prepared 32.6 ml standard suspension in two lots of 7 spleens each; combined and divided 2 : 1 between flask and centrifuge tube C, in alternating 4 and 2 ml portions. Tube held in cold room till flask rotated.

4:15 - 4:50 Flask rotated 37°/30+5 min. - to tube a1.

4:55 - 5:10 Tubes a1 and c1 - 5500 G/10+5 min. - supernatants I to tubes a2 and c2 respectively.

5:20 - 5:35 Tubes a2 and c2 - 5500G/10+5 min. - supernatants II to tubes a3 and c3 respectively.

5:45 - 6:00 Tubes a3 and c3 - 5500 G/10+5 min. - supernatants III 10+ ml and 5 ml respectively to vials a and b.

6:12 - 6:22 Inject 1 ml vial c to 5 ♂♂ group C.
Inject 1 ml vial a to 10 ♂♂ groups A and B.

Section II.

6:30 - 8:45 P.M. Prepared 35.75 ml standard suspension $I_b$ in 2 lots of 9 spleens each; combined and divided in alternate 4 ml and 2 ml portions between flask and centrifuge tube c1, which was held in cold room while flasks rotated.

8:45 - 9:20 Flask rotated 37°/30+5 min. - to flask a1.

9:25 - 9:40 Tubes a1 and c1 - 5500 G/10+5 min. - supernatants I respectively to tubes a2 and c2.

9:47 - 10:02 Tubes a2 and c2 - 5500 G/10+5 min. - supernatants II respectively to tubes a3 and c3.

10:15 - 10:30 Tubes a3 and c3 - 5500 G/10+5 min. - supernatants III, 10 ml from tube a3 to vial a.

10:45 - 10:55 Injected from vial c 1.0 ml per mouse to group C2. Injected from vial a 1.0 ml per mouse to groups A2 and B2.

VII.14.1953 - CSH 2240 $I_b$ 2108

8:40 - 9:05 P.M. Challenged ($4^{-8}$) groups A1, A2, C1, C2, and controls D, D2.

VIII.2.1953 - CSH 2255 $I_b$ 2111

5:45 Challenged ($4^{-8}$) groups B1 and B2, and 10 ♂ controls.

CD 21 Results

| | Time of death in 1/4 days after challenge (4-8) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 9th | 10th | 11th | 12th | 13th | 14th | Survived |
| VII.8.1953 Supernatant III from std. suspension $I_b$ | | | | | | | |
| In 6 d. challenged 4-8 CSH 2240 $I_b$ 2108 | | | | | | | |
| Section I. | | | | | | | |
| 8:40 P.M. A1. Rotated 37°/30 | | 1 2 | 1 | | | | 1/5 |
| 8:45 P.M. C1. Untreated | | | 1 1 | | | | 3/5 |
| 8:50 P.M. D1. Controls on 4-8 | | | | 1 1 | 1 2 | | 0/5 |
| Section II. | | | | | | | |
| 8:55 P.M. A2. Rotated 37°/30 | | | | | 1 | 1 1 | 2/5 |
| 9:00 P.M. C2. Untreated | | | | | | | 5/5 |
| 9:05 P.M. D2. Controls on 4-8 | | | | | | | 5/5 |
| In 25 d. challenged 4-8 CSH 2255 $I_b$ 2111 | | | | | | | |
| Section I. | | | | | | | |
| 5:45 P.M. E. Controls on 4-8 | 2 | 2 1 | | | | | 0/5 |
| 5:49 P.M. B1. Rotated 37°/30 | 2 | 1 | | | | | 2/5 |
| Section II. | | | | | | | |
| 5:52 P.M. B2. Rotated 37°/30 | | 3 1 | | | | | 1/5 |
| 5:56 P.M. E. Controls on 4-8 | | 3 | 1 | 1 | | | 0/5 |

CD 22. CSH 2241 $I_b$ 2108
CSH 2242 $I_b$ 2108
CSH 2257 $I_b$ 2111

VII.15-16.1953

Further comparison of supernatants from untreated and rotated 37°/30 suspensions of $I_b$.

Groups

Section I
A1. Supernatant III from rotated 37°/30 standard $I_b$ challenged in 1 hour.

B1. Supernatant III from untreated standard $I_b$ challenged in 1 hour.

C1. Supernatant III from untreated standard $I_b$ challenged in 22 days.

D1. Controls on challenge $4^{-8}$ of A1 and B1.

Section II
A2.
B2.
C2.
D2.
} as above

Section I.

9:10 - 11:25 A.M. Prepared 35.5 ml standard suspension $I_b$ (in cold room) in 2 lots of 7 spleens each: combined and divided, in alternate 2 ml and 4 ml portions between flask and centrifuge tube b1: tube held in cold room until flask rotated.

11:30 - 12:05 Flask rotated 37°/30+5 min. - to tube a1.

12:13 - 12:28 Tubes a1 and b1 - 5500 G/10+5 min. - supernatants I to tubes a2 and b2 respectively.

12:42 - 12:57 Tubes a2 and b2 - 5500 G/10+5 min. - supernatants II to tubes a3 and b3 respectively.

1:07 - 1:22 Tubes a3 and b3 - 5500 G/10+5 min. - supernatants III - 5+ ml to vial a; 10+ ml to vial b.

1:30 P.M. Inject 1 ml per mouse vial a to group A1.

1:35 Inject 1 ml per mouse vial b to groups B1 and C1.

2:35 - 2:40 Challenge ($4^{-8}$) groups A1 and B1 - D1 controls.

Section II.

VII.16.1953

10:05 A.M. - 12:15 P.M. Prepared 33.9 ml standard suspension $I_b$ in 2 lots of 8 and 6 spleens; combined and divided in alternating 2 ml and 4 ml portions between flask and tube b1; tube held in cold room until flask rotated.

12:20 - 12:55 Flask rotated 37° - to tube a1.

1:00 - 1:15 Tubes a1 and b1 - 5500 G/10+5 min. - supernatants I to tubes a2 and b2 respectively.

1:22 - 1:37 Tubes a2 and b2 - 5500 G/10+5 min. - supernatants II to tubes a3 and b3 respectively.

1:43 - 1:58 Tubes a 3 and b3 - 5500 G/10+5 min. - supernatants III - 5+ ml to vial a, 10+ ml to vial b.

2:10 Inject vial a - group A2 - 1 ml per mouse.

2:00 Inject vial b - groups B2 and C2 - 1 ml per mouse.

3:20 Challenge ($4^{-8}$) - groups A2, B2 and D2 controls.

VIII.6.1953 - 2257 $I_b$ 2111

5:05 P.M. Challenged C1 (22 d.) ($4^{-8}$)

CD 22 Results

| | Time of death in 1/4 days after challenge ($4^{-8}$) | | | | | | | | | | | | | Survived |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 9th | | 10th | | 11th | | | 12th | | 13th | | 14th | | |
| Supernatant III from standard $I_b$ | | | | | | | | | | | | | | |
| A1. Rotated 37° challenged in 1 hour | 1 | | 3 | 2 | 1 | | | 1 | | | | | | 2/10 |
| B. Untreated - challenged in 1 hour | | | 1 | 2 | 1 | 2 | | 1 | | | | | | 3/10 |
| D. Controls on $4^{-8}$ in 1 hour<br>CSH 2241 $I_b$ 2108<br>CSH 2242 $I_b$ 2108 | | | 2 | 1 | 1 | | 2 | 3 | | | | 1 | | 0/10 |
| C1. Untreated - challenged in 22 days | | | 4 | | | | | | | | | | | 1/5 |
| Controls for C1 $4^{-8}$ in 22 days<br>CSH 2257 $I_b$ 2111 | | | 1 | | 3 | | | 1 | | | | | | 0/5 |
| C2. Untreated - unchallenged | | | | | | | | | | 1 | 1 | | | 3/5 |

CD 23. CSH 2243
CSH 2244
VII.17.1953

Is there direct interaction between supernatant III and the challenging line $I_b$ cells?

Will incubation of the mixture of supernatant leukemic cells increase the number of survivors?

Groups

Section I A1. 1.2 ml mixture supernatant III and dil. $4^{-8}$ at once.

B1. 1.2 ml mixture supernatant III after rotation 37°/60 min.

D1. Controls on dil. $4^{-8}$ after 1 hr. in 2° room.

Section II A2. 1.2 ml mixture supernatant III and dil. $4^{-8}$ at once.

B2. 1.2 ml mixture supernatant III after rotation 37°/60 min.

C1 and C2. 1 ml supernatant III (above)

Section I.

CSH 2243 $I_b$ 2108

8:55 - 10:45 A.M. Prepared 37.75 ml standard suspension $I_b$, in two lots of 8 spleens each, in 2° room, combined in tube 1; mice injected just outside cold room.

10:50 - 11:10 Tube 1 - 5500 G/15+5 min.; supernatant I (19 ml) to tube 2.

11:15 - 11:30 Tube 2 - 5500 G/10+5 min.; supernatant II (17 ml) to tube 3. Prepare dilution $4^{-8}$ - 2.2 ml to flask in cold room and held there 1 hour.

11:45 - 12:00 Tube 3 - 5500 G/10+5 min.; supernatant III (11 ml) to flask.

12:10 Inoculate A1 - 1.2 ml mixture from flask per mouse.

12:10 - 1:10 Flask rotated 37°/60 min.

1:10 Inoculate B1 - 1.2 ml mixture from flask after rotation.

12:20 Inoculate D1 - .2 ml dilution $4^{-8}$ - control.

Section II.

1:35 - 3:50 P.M. Prepared 39.80 ml standard suspension line $I_b$ in two lots of 10 and 8 spleens - combined in tube 1.

3:55 - 4:15 Tube 1 - 5500 G/15+5 min.; supernatant I (25 ml) to tube 2.

4:20 - 4:35 Tube 2 - 5500 G/10+5 min.; supernatant II (23 ml) to tube 3. Prepared dil. $4^{-8}$ - 2.2 ml to flask 1 - rest held in 2° room 1 hour.

4:42 - 4:57 Tube 3 - 5500 G/10+5 min.; supernatant III - 11 ml to flask 1, 10+ ml to vial.

5:14 Mix contents of flask and inoculate A2 - 1.2 ml per mouse.

5:14 - 6:14 Flask to rotate - 37°/60 min.

5:18 Inject C1 and C2 - 1 ml supernatant III from vial.

6:15 Inoculate B2 (1.2 ml per mouse) from flask after rotation.

6:37 Inoculate D2 - .2 ml $4^{-8}$ after 1 hour in 2° room and resuspended by 60 shots.

Results

| | | Survived | |
|---|---|---|---|
| A1 - A2 | - mixture at once | 7/10 | (3 deaths 13-14 d. = delay; 2 "survivors" had "pads" at 24 d.) |
| B1 - B2 | - mixture rotated | 10/10 | |
| C1 - C2 | - supernatant alone | 10/10 | |
| D1 - D2 | - controls | 10/10 | (!) |

CD 24. Repetition of CD 23

Is there direct interaction between supernatant III and line $I_b$ cells?

Section I. CSH 2251 $I_b$ 2111

A1. Supernatant III and dil. $4^{-8}$ mixed - inoculated at once.

B1. Supernatant III and dil. $4^{-8}$ mixed - after rotated 37°/60 min.

C1. Dil. $4^{-8}$ after rotation 37°/60 min.

D1. Controls on dil. $4^{-8}$ before rotating.

7:30 - 8:55 A.M. Prepared 30.3 ml standard suspension $I_b$ in 2 lots of 6 spleens each - combined in tube 1.

9:00 - 9:20 Tube 1 - 5500 G/15+5 min.; supernatant I (20 ml) to tube 2.

9:25 - 9:45 Tube 2 - 5500 G/15+5 min.; supernatant II (15 ml) to tube 3.

9:52 - 10:25 Tube 3 - 7000 G/28+5 min.; supernatant III - 11 ml to flask II (red).
Prepared dil. $4^{-8}$ -outside cold room in ice - 6.4 ml in flask I (blue).
Removed 2.2 ml $4^{-8}$ to flask II (red) and mixed.

10:23 Inoculate D1 - .2 ml $4^{-8}$ from flask I.

10:35 Inoculate A1 - 1.2 ml mixture from flask II.

10:37 - 11:37 Flasks I and II rotated 37°/60 min.

11:40 Flask I - inoculate .2 ml - C1.

11:45 Flask II - inoculate 1.2 ml - B1.

Section II. CSH 2252 $I_b$ 2111

A2. Supernatant III and dil. $4^{-8}$ mixed - inoculated at once.

B2. Supernatant III and dil. $4^{-8}$ mixed - after rotated 37°/60 min.

C2. Dil. $4^{-8}$ (above) after rotated 37°/60 min.

D2. Dil. $4^{-8}$ at once - controls.

10:45 - 12:25 P.M. Prepared 31.0 ml standard suspension $I_b$ in two lots of 6 spleens each - combined in tube 1.

12:30 - 12:50 Tube 1 - 5500 G/15+5 min.; supernatant I (19 ml) to tube 2.

12:55 - 1:15 Tube 2 - 7000 G/15+5 min.; supernatant II to tube 3.

1:18 - 1:49 Tube 3 - 7000 G/26+5 min.; supernatant III - 11.0 ml to flask (red) containing 2.2 ml dil. $4^{-8}$.
Prepared dil. $4^{-8}$ - 6.4 ml in flask (blue) - 2.2 ml $4^{-8}$ to flask (red).

1:45 Inoculated D2 - .2 ml $4^{-8}$ from flask (blue), then flask to rotator.

2:00 Mix flask (red) inoculated A2 - 1.2 ml per mouse - then

2:02 - 3:02 Flasks (red and blue) rotated 37°/60 min.

3:07 Inoculate C2 - .2 ml from flask (blue) $4^{-8}$.

3:11 Inoculate B2 - 1.2 ml from flask (red) mixture).

CD 24 Results

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

| | 10th | 11th | 12th | Survived |
|---|---|---|---|---|
| **Section I.** CSH 2251 $I_b$ 2111 | | | | |
| A1. Mixture supernatant III and $4^{-8}$ - inoc. at once | | | 1 1 1 | 2/5 |
| B1. Mixture - after rotation 37°/60 min. | | | | 5/5 |
| C1. Dilution $4^{-8}$ after rotation 37°/60 min. | | | | 5/5 |
| D1. Dilution $4^{-8}$ at once | 2 | 1 1 | | 0/4 * |
| **Section II.** CSH 2252 $I_b$ 2111 | | | | |
| A2. Mixture - at once. | | 1 | 1 1 | 2/5 |
| B2. Mixture - after rotation | | | | 5/5 |
| C2. Dilution $4^{-8}$ after rotation | | | | 5/5 |
| D2. Dilution $4^{-8}$ at once | 1 3 | 1 | | 0/5 |

* 5th ♂ killed by accident

CD 25. CSH 2256 $I_b$ 2111
CSH 2257 $I_b$ 2111
CSH 2264 $I_b$ 2115

VIII.6.1953
VIII.26.1953

Will crystal clear supernatant give any protection at once? at 20 days? Survival of controls on dilution $4^{-8}$ prompted the use of dilution $4^{-6}$ for the immediate challenge - $4^{-8}$ was used at 20 days.

This experiment was performed entirely in laboratory, including centrifuging for the first time not in 2° room.

Groups

Sections I and II A1 and A2. 1 ml supernatant V - challenged ($4^{-6}$) at once.

B1 and B2. Controls on $4^{-6}$

C1 and C2. 1 ml supernatant V - challenged ($4^{-8}$) 20 days.

D1 and D2. Controls on $4^{-8}$

Section I.

8:55 - 10:00 A.M. Prepared 29 ml standard suspension $I_b$ in 2 lots of 6 spleens each - combined in tube 1.

10:03 - 10:18 Tube 1 - 8600 G/10+5 min.; supernatant I (17 ml) to tube 2.

10:23 - 10:38 Tube 2 - 8600 G/10+5 min.; supernatant II (16.5 ml) to tube 3.

10:42 - 10:57 Tube 3 - 8600 G/10+5 min.; supernatant III (15 ml) to tube 4.

11:02 - 11:17 Tube 4 - 8600 G/10+5 min.; supernatant IV (13 ml) to tube 5.

11:20 - 11:35 Tube 5 - 8600 G/10+5 min.; supernatant V no sediment visible.

11:40 - 11:45 Supernatant V - 1 ml per mouse to A1 and C1.

12:07 - 12:10 P.M. Challenged A1 and B1 - .2 ml $4^{-6}$. CSH 2256 $I_b$ 2111.

Section II.

1:40 - 2:40 P.M. Prepared 28.75 ml standard suspension in 2 lots of 6 spleens each - combined in tube 1.

2:45 - 3:00 Tube 1 - 8600 G/10+5 min.; supernatant I (20 ml) to tube 2.

3:05 - 3:20 Tube 2 - 8600 G/10+5 min.; supernatant II (18 ml) to tube 3.

3:25 - 3:40 Tube 3 - 8600 G/10+5 min.; supernatant III (16 ml) to tube 4.

3:45 - 4:00 Tube 4 - 8600 G/10+5 min.; supernatant IV (14 ml) to tube 5.

4:05 - 4:20 Tube 5 - 8600 G/10+5 min.; supernatant V no visible sediment.

4:25 - 4:30 Inject A2 and C2 - 1 ml supernatant V.

4:55 - 5:00 Inoculate A2 and B2 - .2 ml $4^{-6}$. CSH 2257 $I_b$ 2115.

VIII.26.1953 (20 d. test)

5:28 - 5:38 P.M. Challenged C1 and C2, D1 and D2 - .2 ml $4^{-8}$.
CSH 2264 $I_b$ 2115.
(Donor of the best spleen available for this challenge died naturally a few minutes before spleen removed.)

CD 25 Results

Time of death in 1/4 days after challenge ($4^{-6}$ or $4^{-8}$)

| | 8th | 9th | 10th | 11th | 12th | 13th | Survived |
|---|---|---|---|---|---|---|---|
| **Section I.** | | | | | | | |
| A1. Supernatant V - $4^{-6}$ in 1/2 hr. CSH 2256 $I_b$ 2111 | 1 4 | | | | | | 0/5 |
| B1. Controls on $4^{-6}$ | 4 | 1 | | | | | 0/5 |
| **Section II.** | | | | | | | |
| A2. Supernatant V - $4^{-6}$ in 1/2 hr. CSH 2257 $I_b$ 2111 | 5 | | | | | | 0/5 |
| B2. Controls on $4^{-6}$ | 2 | 1 | 1 | | | | 1/5 ! |
| **Sections I and II.** | | | | | | | |
| C1 and C2. Supernatant V - $4^{-8}$ in 20 days CSH 2264 $I_b$ 2115 (Donor had just died naturally.) | | | | | | 2 * | 8/10 |
| D1 and D2. Controls on $4^{-8}$ | | | | | | | 10/10 ! |

* inoc. 1st syringeful - 2nd vial of $4^{-8}$

CD 26. CSH 2259 $I_b$ 2112
CSH 2260 $I_b$ 2112
CSH 2265 $I_b$ 2116
VIII.12.1953
IX.1.1953

Supernatant V - challenged at once ($4-8$) and in 20 days. Repetition of CD 25.

Groups

Sections I and II A1 and A2. 1 ml supernatant V (8600 G) - from standard $I_b$ - challenged in 1/2 hr.

B1 and B2. Controls on challenge ($4-8$) of A.

C1 and C2. 1 ml supernatant V (as above) - challenged in 20 days.

D1 and D2. Controls on challenge ($4-8$) of D.

Section I.
VIII.12.1953

8:40 - 9:45 A.M. Prepared 28 ml standard suspension $I_b$ in two lots of 6 and 5 spleens - combined in tube 1.

9:50 - 10:05 Tube 1 - 8600 G/10+5 min.; supernatant I (18 ml) to tube 2.

10:10 - 10:25 Tube 2 - 8600 G/10+5 min.; supernatant II (16 ml) to tube 3.

10:30 - 10:45 Tube 3 - 8600 G/10+5 min.; supernatant III (15 ml) to tube 4.

10:48 - 11:03 Tube 4 - 8600 G/10+5 min.; supernatant IV (13 ml) to tube 5.

11:08 - 11:23 Tube 5 - 8600 G/10+5 min.; supernatant V (no visible sediment).

11:27 - 11:30 Inject A1 and C1 - 1 ml supernatant V.

11:57 - 12:00 Challenged A1 and B1 - .2 ml $4-8$. CSH 2259 $I_b$ 2112.

Section II.

12:45 - 1:45 P.M. Prepared 23.40 ml standard suspension $I_b$ in 2 lots of 5 spleens each - combined in tube 1.

1:50 - 2:05 Tube 1 - 8600 G/10+5 min.; supernatant I (17 ml) to tube 2.

2:10 - 2:25 Tube 2 - 8600 G/10+5 min.; supernatant II (15 ml) to tube 3.

2:30 - 2:45 Tube 3 - 8600 G/10+5 min.; supernatant III (13 ml) to tube 4.

2:50 - 3:05 Tube 4 - 8600 G/10+5 min.; supernatant IV (12 ml) to tube 5.

3:10 - 3:25 Tube 5 - 8600 G/10+5 min.; supernatant V 10+ ml to vial - (very slight sediment).

3:29 - 3:32 Inject A2 and C2 - 1 ml supernatant V. CSH 2260 $I_b$ 2112.

Section I and II. (20 d. test)
IX.1.1953

7:57 - 8:06 A.M. Challenged C1, D1, C2, D2 - .2 ml $4^{-8}$. CSH 2265 $I_b$ 2115.

CD 26 Results

| | Time of death in 1/4 days after challenge (4-8) | | | | | |
|---|---|---|---|---|---|---|
| | 10th | 11th | 12th | 13th | 14th | Survived |
| Section I. CSH 2259 $I_b$ 2112 | | | | | | |
| A1. Supernatant V - 4-8 at once | 4 | | 1 | | | 0/5 |
| B1. Controls on 4-8 | 5 | | | | | 0/5 |
| Section II. CSH 2260 $I_b$ 2112 | | | | | | |
| A2. Supernatant V - 4-8 at once | 4 | 1 | | | | 0/5 |
| B2. Controls on 4-8 | 4 | 1 | | | | 0/5 |
| Sections I and II. CSH 2265 $I_b$ 2116 IX.1.1953 - 20 d. test | | | | | | |
| 7:57 A.M. C1. Supernatant V - 4-8 in 20 d. | 1 | 2 1 1 | | | | 0/5 |
| 8:00 A.M. D1. Controls on 4-8 | 1 | 1 2 | 1 | | | 0/5 |
| 8:03 A.M. C1. Supernatant V - 4-8 in 20 d. | | 1 1 1 | 1 | | | 1/5 * |
| 8:06 A.M. D2. Controls on 4-8 | | 1 | | 1 | 2 | 2/5 * |

* litter mates

CD 27. CSH 2263 $I_b$ 2115
CSH 2264 $I_b$ 2115
VIII.26.1953

Comparison of supernatant III and supernatant V - challenged at once. Supernatant III was prepared as usual, then divided in two parts - one part was continued with removal of successive supernatants, the other part centrifuged simultaneously, but each time sediment resuspended.

Groups

Sections 1 and II A1 and A2. Supernatant III challenged ($4^{-8}$) at once.

B1 and B2. Supernatant V challenged ($4^{-8}$) at once.

C1 and C2. Controls on $4^{-8}$

Section I.
VIII.26.1953

8:20 - 10:10 A.M. Prepared 34.40 ml standard suspension $I_b$ in 2 lots of 7 spleens each - combined in tube 1.

10:10 - 10:25 Tube 1 - 5500 G/10+5 min.; supernatant I (21 ml) to tube 2.

10:29 - 10:44 Tube 2 - 5500 G/10+5 min.; supernatant II (19 ml) to tube 3.

10:50 - 11:05 Tube 3 - 5500 G/10+5 min.; supernatant III 6.5 ml to tube 4, 9.0 ml to tube 5.

11:10 - 11:30 Tubes 4 and 5 - 8600 G/15+5 min.; tube 4 sediment resuspended - from tube 5 supernatant IV 8 ml to tube 7.

11:35 - 11:55 Tubes 4 and 6 - 8600 G/15+5 min.; tube 4 sediment resuspended - from tube 6 supernatant V to tube 7.

12:00 - 12:15 Tubes 4 and 7 - 8600 G/15+5 min,; tube 4 sediment resuspended - tube 7 supernatant VI removed and injected.

12:20 Inject supernatant IV (tube 4), A1 - 1 ml per mouse.

12:15 Inject supernatant VI = "V" (tube 7), B1 - 1 ml per mouse.

Prepared dil. $4^{-8}$ during last run.

12:37 - 12:40 Challenged A1, B1 and C1 (controls) - .2 ml $4^{-8}$ - CSH 2263 $I_b$ 2115.

Section II.

1:35 - 2:45 P.M. Prepared 31 ml standard suspension $I_b$ in 2 lots of 7 and 6 spleens - combined in tube 1.

2:50 - 3:05 Tube 1 - 5500 G/10+5 min.; supernatant I (20 ml) to tube 2.

3:10 - 3:25 Tube 2 - 5500 G/10+5 min.; supernatant II (18 ml) to tube 3.

3:30 - 3:45 Tube 3 - 5500 G/10+5 min.; supernatant III 6.5 ml to tube 4, 9.0 ml to tube 5.

3:50 - 4:10 Tubes 4 and 5 - 8600 G/15+5 min.; tube 4 sediment resuspended - from tube 5 supernatant IV to tube 6.

4:15 - 4:35 Tubes 4 and 6 - 8600 G/15+5 min.; tube 4 sediment resuspended - from tube 6 supernatant V to tube 7.

4:45 - 5:00 Tubes 4 and 7 - 8600 G/15+5 min.; tube 4 sediment resuspended and injected, tube 7 supernatant VI removed and injected

5:04 Inject supernatant III (tube 4), B2 - 1 ml per mouse.

5:10 Inject supernatant VI = "V", A2 - 1 ml per mouse.

5:15 - 5:21 Challenge B2, A2 and C2 - .2 ml $4^{-8}$ - CSH 2264 $I_b$ 2115 dilution $4^{-8}$ prepared during centrifugation). Donor for this challenging dilution (last spleen available) had died naturally a little before last run, so removed spleen within a few minutes and diluted to $4^{-3}$ - held in ice until A2 and B2 treated before making final dilutions.

CD 27 Results

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

| | 9th | 10th | 11th | | Survived |
|---|---|---|---|---|---|
| Section I. CSH 2263 $I_b$ 2115 - $4^{-8}$ VIII.26.1953 | | | | | |
| A1. Supernatant III | 1 | 1 1 | 1 | | 1/5 * |
| B1. Supernatant "V" | 1 | 1 1 | 2 | | 0/5 |
| C1. Controls on $4^{-8}$ - challenge at once | 2 | 3 | | | 0/5 |
| Section II. CSH 2264 $I_b$ 2115 Donor died naturally before spleen removed $4^{-8}$ - vial 1 | | | | | |
| 5:17 PM A2. Supernatant III | | | | | 5/5 |
| 5:15 PM B2. Supernatant "V" | | | | 3/4 - 14 / 1 | 4/5 |
| 5:21 PM C2. Controls on $4^{-8}$ challenge at once | | | | | 5/5 |

* at 23 d. = "pad"

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IX.7.1953
CSH 2267 $I_b$ 2117

Reinoculated after 12 days - A2, B2 and C2 on chance first challenge had included no living cells. But death of one B2 mouse 2 days later (also 2♂♂ given same challenge dose in CD 25 - 20 d. test)proved $4^{-8}$ contained some live cells on 8/26.

| | 9th | 10th | 11th | 12th | 13th | Survived |
|---|---|---|---|---|---|---|
| 10:03 AM A2. Supernatant III | | | | 1 | 1 | 3/5 |
| 10:08 AM B2. Supernatant "V" | 1 | | | | | 3/4 |
| 10:11 AM C2. Controls on $4^{-8}$ (CSH 2264) | 1 | 4 | | | | 0/5 |
| 10:14 AM 5 ♂♂ not previously inoculated | | 5 | | | | 0/5 |

CD 28. CSH 2265 $I_b$ 2116
CSH 2266 $I_b$ 2116
IX.1.1953

Supernatant III compared with supernatant V prepared from different lots of standard suspension and centrifuged independently - challenged ($4^{-8}$) at once.

Groups

Sections I and II A1 and A2. 1.0 ml supernatant V (5 x 8600 G/10+5)

B1 and B2. 1.0 ml supernatant III (3 x 5500 G/10+5)

C1 and C2. Controls on challenge (at once) $4^{-8}$

Section I.

4:10 - 5:10 A.M. Prepared 14.75 ml standard suspension in two lots of 3 and 4 spleens each. As removed, spleens placed in 4 ml iced saline until minced - this saline used in suspending. Combined in tube 1.

5:10 - 5:25 Tube 1 - 8600 G/10+5 min.; supernatant I to tube 2.

5:28 - 5:43 Tube 2 - 8600 G/10+5 min.; supernatant II to tube 3.

5:46 - 6:01 Tube 3 - 8600 G/10+5 min.; supernatant III to tube 4.

6:09 - 6:24 Tube 4 - 8600 G/10+5 min.; supernatant IV to tube 5.

6:26 - 6:41 Tube 5 - 8600 G/10+5 min.; supernatant V (5+ ml)

6:47 Inject supernatant V - 1.0 ml per mouse - into A1.

During above centrifuging, prepared 14 ml standard suspension $I_b$ (in tube 6) in two lots of 3 and 4 spleens each - spleens being chilled immediately upon removal and all saline chilled and suspension kept in ice bath.

6:43 - 6:58 Tube 6 - 5500 G/10+5 min.; supernatant I to tube 7.

7:01 - 7:16 Tube 7 - 5500 G/10+5 min.; supernatant II to tube 8.

7:19 - 7:34 Tube 8 - 5500 G/10+5 min.; supernatant III to vial.

7:40 Inject B1 - 1.0 ml per mouse - supernatant III.

Dilution $4^{-8}$ prepared during last runs.

7:45 - 7:50 Challenge ($4^{-8}$) A1, B1, C1. CSH 2265 $I_b$ 2116.

Section II.

8:35 - 9:10 A.M. Prepared 15 ml standard suspension $I_b$ in two lots of 3 spleens each - spleens into cold 4 ml saline immediately upon removal.

9:14 - 9:29 Tube 1 - 8600 G/10+5 min.; supernatant I (10 ml) to tube 2.

9:31 - 9:46 Tube 2 - 8600 G/10+5 min.; supernatant II (8 ml) to tube 3.

9:53 - 10:08 Tube 3 - 8600 G/10+5 min.; supernatant III (7 ml) to tube 4.

10:11 - 10:26 Tube 4 - 8600 G/10+5 min.; supernatant IV to tube 5.

10:29 - 10:44 Tube 5 - 8600 G/10+5 min.; supernatant V - 5+ ml to vial.

10:50 Inject 1.0 ml supernatant V - A2.

During above centrifuging, prepared 15.5 ml standard suspension $I_b$ in two lots of 3 spleens each, kept cold from moment of removing each spleen - to tube 6.

10:48 - 11:03 Tube 6 - 5500 G/10+5 min.; supernatant I (8 ml) to tube 7.

11:06 - 11:21 Tube 7 - 5500 G/10+5 min.; supernatant II (7 ml) to tube 8.

11:25 - 11:40 Tube 8 - 5500 G/10+5 min.; supernatant III - 5+ ml to vial.

11:45 Inject B2 - 1.0 ml supernatant III.

During above centrifuging prepared dilution $4^{-8}$.

11:51 - 11:59 Challenged A2, B2, C2 - .2 ml dilution $4^{-8}$ - CSH 2266 $I_b$ 2116.

CD 28 Results

| | Time of death in 1/4 days after challenge ($4^{-8}$) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 9th | 10th | 11th | 12th | 13th | 14th | Survived |
| Section I. CSH 2265 $I_b$ 2116 | | | | | | | |
| 7:45 AM A1. 1.0 ml supernatant V--challenged in 1 hr. | | 2 | | 1 1 | | | 1/5 |
| 7:47 AM B1. 1.0 ml supernatant III--challenged in 7 min. | | 2 | | | | 1 | 2/5 |
| 7:50 AM C1. Controls on challenge ($4^{-8}$) | | 1 | 1 2 | | 1 | | 0/5 |
| Section II. CSH 2266 $I_b$ 2116 | | | | | | | |
| 11:51 AM A2. | | 1 | 3 | | | | 1/5 |
| 11:55 AM B2. as above | | | 1 1 | 2 | | | 1/5 |
| 11:59 AM C2. | | | 1 1 | 2 | | | 1/5 |

CD 29. CSH 2267 $I_b$ 2117
CSH 2268 $I_b$ 2117
IX.7.1953

Continuing comparison of supernatant III (5000 G) and supernatant V (8600 G) and adding supernatant III (11,000 G).

Groups

Sections I and II A1 and A2. 1.0 ml supernatant V (5 x 8600 G) challenged in 2 hrs.

B1 and B2. 1.0 ml supernatant III (3 x 5500 G) challenged in 1 hr.

C1 and C2. 1.0 ml supernatant III (3 x 11,000 G) challenged in 8 min.

D1 and D2. Controls on challenge $4^{-8}$ given.

Section I.

5:20 - 6:10 A.M. Prepared 12.65 ml standard suspension $I_b$ in 2 lots of 3 spleens each. Spleens immediately into iced saline upon removal - combined in tube 1.

6:15 - 6:30 Tube 1 - 8600 G/10+5 min.; supernatant I (9 ml) to tube 2.

6:35 - 6:50 Tube 2 - 8600 G/10+5 min.; supernatant II (8 ml) to tube 3.

6:55 - 7:10 Tube 3 - 8600 G/10+5 min.; supernatant III (7 ml) to tube 4.

7:13 - 7:28 Tube 4 - 8600 G/10+5 min.; supernatant IV (6.5 ml) to tube 5.

7:30 - 7:45 Tube 5 - 8600 G/10+5 min.; supernatant V (5+ ml) - inject A1.

7:50 Inject A1 - 1.0 ml supernatant V.

During above centrifuging prepared 12.80 ml standard suspension in 2 lots of 3 spleens each - (as above) - to tube 6.

7:50 - 8:05 Tube 6 - 5500 G/10+5 min.; supernatant I (10 ml) to tube 7.

8:10 - 8:25 Tube 7 - 5500 G/10+5 min.; supernatant II (7 ml) to tube 8.

8:28 - 8:43 Tube 8 - 5500 G/10+5 min.; supernatant III 5+ ml - inject B1.

8:50 Inject B1 - 1.0 ml supernatant III (5500 G)

During above centrifuging prepared 12.50 ml standard suspension Ib in 2 lots of 3 spleens each (as above) - combined in tube 9.

8:46 - 9:01 Tube 9 - 11,000 G/10+5 min.; supernatant I (7.5 ml) to tube 10.

9:05 - 9:20 Tube 10 - 11,000 G/10+5 min.; supernatant II (6.0 ml) to tube 11.

9:25 - 9:40 Tube 11- 11,000 G/10+5 min.; supernatant III - inject C1.

9:45 Inject C1 - 1.0 ml supernatant III (11,000 G)

During above centrifuging prepared dilution $4^{-8}$ and started challenging.

9:40 - 9:57 Challenged - .2 ml $4^{-8}$ - A1, B1, C1, D1 controls on $4^{-8}$. CSH 2267 $I_b$ 2117.

Section II.

2:15 - 2:45 P.M. Prepared 14.75 ml standard suspension $I_b$ in 2 lots of 3 spleens each - kept chilled from moment of removing spleen (as above) - combined in tube 1.

2:50 - 3:05 Tube 1 - 8600 G/10+5 min.; supernatant I (10 ml) to tube 2.

3:06 - 3:23 Tube 2 - 8600 G/10+5 min.; supernatant II (9 ml) to tube 3.

3:28 - 3:43 Tube 3 - 8600 G/10+5 min.; supernatant III (7 ml) to tube 4.

3:47 - 4:02 Tube 4 - 8600 G/10+5 min.; supernatant IV to tube 5.

4:05 - 4:20 Tube 5 - 8600 G/10+5 min.; supernatant V inject A2 after tube 6 in centrifuge.

4:25 Inject A2 - 1.0 ml supernatant V.

During above centrifuging prepared 15.55 ml standard suspension $I_b$ in 2 lots of 3 spleens each (as above) to tube 6.

4:24 - 4:39 Tube 6 - 5500 G/10+5 min.; supernatant I (10 ml) to tube 7.

4:41 - 4:56 Tube 7 - 5500 G/10+5 min.; supernatant II (7 ml) to tube 8.

5:00 - 5:15 Tube 8 - 5500 G/10+5 min.; supernatant III 5+ ml - inject B2.

5:20 Inject B2 - 1.0 ml supernatant III (5500 G).

During above centrifuging prepared 15.20 ml standard suspension $I_b$ in 2 lots of 3 spleens each - (as above) - combined in tube 9.

5:18 - 5:33 Tube 9 - 11,000 G/10+5 min.; supernatant I (10 ml) to tube 10.

5:37 - 5:52 Tube 10 - 11,000 G/10+5 min.; supernatant II (7 ml) to tube 11.

5:55 - 6:10 Tube 11 - 11,000 G/10+5 min.; supernatant III 5+ ml - inject C2.

6:15 Inject C2 - 1.0 ml supernatant III (11,000 G). CSH 2268 $I_b$ 2117.

During above centrifuging prepared dilution $4^{-8}$.

6:10 - 6:25 Challenged .2 ml $4^{-8}$ - A2 after 2 hrs., B2 after 1 hr., C2 after 7 min. D2 - controls on $4^{-8}$.

CD 29 Results

| | Time of death in 1/4 days | | | | | |
|---|---|---|---|---|---|---|
| | 10th | 11th | 12th | 13th | 14th | Survived |
| Section I. CSH 2267 $I_b$ 2117 | | | | | | |
| A1. 1.0 ml supernatant V (8600 G) challenged in 2 hrs. | | | 1 | | | 4/5 |
| B1. 1.0 ml supernatant III (5500 G) challenged in 1 hr. | | | | 1 1 | 1 | 2/5 |
| C1. 1.0 ml supernatant III (11,000 G) challenged in 8 min. | | | | | | 5/5 ! |
| D1. Controls on challenge 4-8 | | | | | | 5/5 ! |
| Section II. CSH 2268 $I_b$ 2117 | | | | | | |
| A2. 1.0 ml supernatant V (8600 G) challenged in 2 hrs. | | 2 | 1 | | | 2/5 |
| B2. 1.0 ml supernatant III (5500 G) challenged in 1 hr. | | 2 1 | | 1 | | 1/5 |
| C2. 1.0 ml supernatant III (11,000 G) challenged in 7 min. | | 1 | 1 | 2 | | 1/5 |
| D2. Controls on challenge 4-8 | | | | | | 5/5 ! |

FINAL REPORT OF RESEARCH

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# RESEARCH STUDIES OF FACTORS INDUCING RESISTANCE TO TRANSMISSIBLE MOUSE LEUKEMIA

by

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In initiating, directing and supervising the work under this contract, Col. Joseph Victor, M.D. has been closely associated with every step. He made frequent trips to Cold Spring Harbor for personal conferences, and by mail and telephone, has been in continuous contact as the individual experiments have been formulated and as the results have been recorded.

The underlying objective coordinating the diverse experiments has been to determine whether similarities in the phenomena of resistance inducible against certain bacteria and certain transplanted leukemias, indicate a common basic mechanism, so that further analysis in one case (leukemia) will illuminate the other. The general conclusion may be drawn that a common basic mechanism is not indicated; that diverse courses of events may lead to survival after a challenging dose (of different or the same pathogens) that is lethal to the controls; hence, that survival under one set of conditions may not a priori be equatable to survival under another set of conditions.

The concept is supported that the induction of resistance does not establish a static state, but rather depends upon the relative rates of the hosts' reactions and of the proliferation of the challenging organisms. Any influence modifying the rate of either process may decide between death and survival. Once an animal survives, the active resistance so acquired largely determines the outcome of further challenging. The chief interest appears to be in the initial steps that lead to survival after the first challenge. What are the determining factors, in a given case, that are responsible for the success of the hosts' reactions in out-running the growth of the lethal challenging dose of the cells?

Within the case of line $I_b$ leukemia, in hosts of the strain of origin, more than one such factor appears to have been found. Previously it had been learned that virtually 100% survival could be ensured by treatment with normal tissue from an unrelated strain of mice, as well as by treatment with

thoroughly washed leukemic (line $I_b$) cells after they had been subjected to 46° for 4 minutes. The work under this contract adds evidence of what appears to be another factor influencing survival. This factor is found in the supernatant from saline suspensions of cells from line $I_b$ leukemic spleens.

Although the progress of the work has been seriously handicapped by the failure to concentrate this factor enough to yield a high proportion of survivors, it seems highly probable that this factor is not a direct product of the leukemic cells, but was elaborated by the donor of the spleen in reacting to the invading leukemic cells with which it had been inoculated.

Since the challenging dose may be inoculated immediately after the supernatant or the two may even be mixed and injected together with as much protection as at 2, 6, or 20 days, it appears that the factor responsible for the survivals is independent of the leukemic cells, in spite of the fact that the production of supernatants absolutely cell-free has been found to be difficult and uncertain. If a few cells remained in the supernatant these would merely add to the lethality of the challenging dose when the two are combined. The experimental procedure in this case has merely reduced the number of leukemic cells to a minimal lethal dose without diluting the protective factor in the original "standard" cell suspension.

The resistance found when the challenging dose was delayed for 20 days raises a question as to the possible action of cells that might remain in the supernatant. According to previous experiments with heated (46°) line $I_b$ cells, a few living leukemic cells were necessary to obtain full resistance persisting for 20 days after injection of the heated cells. In the last quarter of this contract year, an attempt has been made to determine whether living cells were also responsible for the resistance found 20 days after treatment with a supernatant. But the year has terminated before an answer to this question could be obtained.

The detailed protocols have been recorded in the four quarterly progress reports. Without commenting upon experiments concerned with testing techniques that were not adopted, the results contribute to the following questions:

Will dog blood, frozen or fresh, induce resistance to a challenging dose of line $I_b$ leukemia given in 1, 2 or 3 days? (CD 3 and 4) The data indicate emphatically that this is not the case.

Will repeated treatments with line $I_b$ leukemic cells after being heated 46°/14 min. produce more persistent resistance than a single treatment? (CD 6 and 8) When challenged at 14 days, somewhat more of the mice receiving three treatments survived than of those receiving one treatment.

Can resistance be induced by supernatants from line $I_b$ cell suspensions? The majority of the 29 experiments deal with this question. The early ones were based on the idea that a substance responsible for resistance was in some way produced by, or was a part of, the leukemic cells, and might be separated from the cells by appropriate means. Thus, before testing supernatants for protective properties, the cell suspensions were rotated in an ice bath and at 37°; they were heated (46°/14 min.) and repeatedly centrifuged and resuspended in fresh saline to see if a protective substance was removed or was continuously produced; the cells were disrupted by distilled water and by the shearing action of forceable "squirting"; the cell suspensions were incubated at 37°. Supposing that the protective material might be unstable, the time of preparation of the cell suspension was cut down by dividing each experiment into two independent sections: the material was kept cold by ice baths and finally by carrying out all operations, short of injecting the mice, in a 2°-room. But under no condition was enough resistance obtained to protect more than 50% of the mice, and in most cases only between 10% and 30%. No evidence was found to support the assumption that the material in the supernatant responsible for this resistance originated in or was produced by the

leukemic cells. None of the various treatments of the leukemic cell suspensions increased the resistance induced by the supernatants from entirely untreated cell suspensions.

Since the resistance induced by dog blood against certain bacteria and by heated leukemic cells was at its height in 2 days, the challenge in many experiments was given in 2 days. But varying this time from 0 to 25 days gave virtually the same degree of resistance at all times tested.

Table 1

Variation in Time of Challenge (dilution $4^{-8}$) after Treatment with Supernatant III

Experiments are included in which the cell suspensions were rotated as well as centrifuged, but all cases with erratic controls are omitted. Survivors/number in group.

| Day of challenge | | | | | | |
|---|---|---|---|---|---|---|
| 0 | 1 | 2 | 6 | 20 | 22 | 25 |
| 19/60 | 4/10 | 17/50 | 10/20 | 4/10 | 1/5 | 3/10 |

This result stands in striking contrast to the results for heated cells, which showed a rapid strengthening of resistance for two days followed by a rapid weakening after 3 days. This evidence of different mechanisms in the resistance from supernatants and from heated cells is supported by the fact that after three successive washings, the heated cells were as effective in inducing resistance as at first, while the supernatants from resuspended cells, whether heated or not, were ineffective.