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SEMI-ANNUAL PROGRESS REPORT

Report Prepared by: J. W. Berry, M.D.

Date: July 28, 1952

For Period: January 31, 1952
August 1, 1952

NR 135-861/6-21-50 (Biological Sciences Division)

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CONTRACTOR: The Regents of the University of Colorado

PRINCIPAL INVESTIGATOR: J. W. Berry, M.D.

Hope Lowry, M.D. has withdrawn from this project as explained in her letter to the Office of Naval Research. Although she will be missed there will be others who will carry on in her place. These are J. Carroll Bell, M.D. who has recently joined this group and as mentioned in our more recent application is well qualified and will help to substitute for Dr. Lowry. Also, Howard D. Olson, Ph.D. in Bacteriology will join us September 1, 1952 to carry on and to help with the problems that we are doing under contract with the Office of Naval Research. In addition, we have a research associate Mrs. Marjorie Stitts Knorr who will also be available for part time assistance with the program of the Office of Naval Research.

TITLE OF PROJECT: Research in growth patterns and nutritional requirements of the tubercle bacillus and other acid-fast organisms.

Objectives:

1. To use a slide culture technique by which microscopic growth of the organisms can be observed in order to study the cultural characteristics of the Mycobacteria and to attempt to determine whether there are cultural characteristics which are constantly related to virulence.
2. To use the same technique of culture to study the nutritional requirements of the tubercle bacillus by determining the effects of alterations in medium on the rate of growth and growth pattern.
3. To determine the effects of antibiotics on the rate of growth and cultural characteristics of the tubercle bacillus again using the slide culture technique.

In accordance with our telephone conversation and confirming letters we have during the past few months been investigating the effects of isonicotinic acid hydrazide on the tubercle bacillus and particularly the ability of the tubercle bacillus to become resistant to isonicotinic acid hydrazide.

4. To attempt to solve certain basic problems in the cultivation and isolation of the tubercle bacillus. We are especially interested in attempting to concentrate specimens containing small numbers of organisms by means of a high vacuum system.

Abstract (or Summary) of Results

- a. Since start of project and during current report period (3rd report):
 1. Since report of January 28, 1952 we have continued to work on the saprophytes and are using the methods as described in the previous report and while this work has been slowed down considerably because of our interest in the effect of isonicotinic acid hydrazide it has gone on and our conclusion remains the same except that we are now fairly well convinced that the presence of serpentine growth may or may not mean that the organism is virulent but the absence of serpentine growth is almost certain evidence that the organism is avirulent.
 2. We have completed the serial dilution experiments and while our results have not been analyzed carefully it is fairly sure that solid medium (Loewenstein medium) will produce growth when the organism is diluted about 10 times more than the dilution that will produce growth on the slides; in other words if the slides produce growth at 10^{-6} mgm. per ml. of sputum, Loewenstein's will usually produce growth at 10^{-7} . This is a tentative conclusion that will have to await the detailed analysis of our results which we expect to have done within the next month or so. These results will be contained in our next report in detail.
 3. We have done considerable work on the determination of the effect of isonicotinic acid hydrazide on the growth of the tubercle bacillus, however, this has not been done using the slide culture technique but it has been done as follows. Strains of M. tuberculosis H37Rv, H37Ra, and Ravenel were obtained originally from the Trudeau Laboratory, Saranac, New York; eight to 14 day old cultures of these organisms were used for inoculation into drug containing medium; all cultures are made in Dubos liquid Tween albumin medium contained in 50 ml. Erlenmeyer flasks. Crystalline isonicotinic acid hydrazide was added to these flasks as will be described later.

Procedure: In the original inoculations and in all subsequent transfers .1 ml. inocula were used; a control flask containing no drug and the flask containing serial concentrations of isonicotinic acid hydrazide were inoculated and incubated at 37 degrees C.; all concentrations were run in triplicate; estimates of growth were made periodically usually at intervals of 4 to 7 days; this was accomplished by estimating the amount of sediment present in the flasks and grading this according to the following classification. Trace (plus or minus) means a sediment which appeared definitely to be bacterial growth but which are just perceptible when the contents of the flasks were agitated. A one plus growth equals definite sediment of bacterial growth visible without agitation of the flasks but only slight in amount; four

plus equals maximum growth comparable to that seen in the control flasks at 14 days incubation; 2 plus and 3 plus growth represent serial gradations between 1 and 4 plus growth; it is fully recognized that this method is an arbitrary one and that the possibility of variation of interpretation exists, however, readings were made by the same person each time and estimates of growth were made on each occasion without benefit of knowing what previous estimates had been. In addition, slides were made periodically in order to confirm the gross estimates of growth; the concentration of drug was different with first and second step exposures. Original inoculations were made to flasks containing none (control) .001, .01, .1, 1, 5, 5, and 10 micrograms per ml. of isonicotinic acid hydrazide; when it became apparent that resistance developed rapidly to these concentrations appropriate alterations were devised accordingly first step resistant variants were transferred to flasks containing .01, .1, 1, 10, 100 and 1000 micrograms per ml. of drug. Flasks containing .001, and 5 micrograms per ml. were omitted; this method was followed consistently with each strain, however, in addition to these second step concentrations first step variants of H₃₇Rv resistant to 10 micrograms per ml. were exposed also to concentrations of 25 and 250 micrograms per ml. of isonicotinic acid hydrazide.

Because of the prolonged period required for the growth of variants to become apparent a variation in the manner of transfer of all three strains to the second step flasks was followed. Rather than using as a source of inocula flasks containing the highest concentration of drug in which growth was present an inoculum from each first step flask was transferred to a flask which contained the same concentration of drug in the second step series; in this way the delay period in growth of second step variants was compared with that of bacilli on initial exposure. In as much as the additional concentration of drug were added to the second series of flask another modification was necessitated; first step organisms in 10 micrograms per ml. of drug were transferred to media containing 10, 100 and 1000 micrograms per ml. of isonicotinic acid hydrazide in the second step series of flasks; transfer third step series were accomplished in the same manner with H₃₇Rv, that is, an inoculum from each second step flask was transferred to a flask in the third step series which contained the same amount of drug; with Ravenel and H₃₇Ra, however, the transfers to third step flasks were accomplished by a more commonly used procedure; the second step flasks with the highest drug concentration in which there was maximum bacterial growth was used to inoculate the control flasks and all drug containing flasks in the third step series; therefore, H₃₇Ra organisms in .1 microgram per ml. of isonicotinic acid hydrazide were used to inoculate while with the Ravenel strain, bacilli growing in 10 micrograms per ml. of drug were used as inocula.

Results: We have some preliminary results of these

experiments; with H37Rv growth was apparent at 1 microgram after 4 days and this did not change until 21 days; after 21 days of incubation growth was apparent in the medium containing 10 micrograms of isonicotinic acid hydrazide.

With the Ravenel strain growth appeared first in 1 microgram after 4 days incubation; after 17 days incubation growth was apparent in the medium containing 10 micrograms of isonicotinic acid hydrazide.

With H37Ra growth appeared after 10 days in .01 micrograms; in 14 days in .1 micrograms; 17 days in 1. micrograms, 24 days in medium containing 5 micrograms and only after 38 days did growth appear in medium containing 10 micrograms per ml.

After transfer H37Rv showed growth in 250 micrograms after 7 days, in 1000 micrograms per ml. after 17 days.

With the Ravenel strain growth appeared after 4 days in 1 microgram, after 17 days in 10 microgram; on transfer growth appeared in 7 days in concentration of isonicotinic acid hydrazide of 1000 microgram.

These results are quite different than most of the results in the few studies already reported. We cannot explain this difference but we are fairly confident that our results are accurate since we are using standard organisms, standard procedure, and we know the concentration of isonicotinic acid hydrazide in our flasks is correct since we have determined the concentration chemically.

4. There has been no essential change in this part of the project since the previous report. We are able to concentrate the organisms well by means of high vacuum but as yet we cannot consistently nor even frequently produce good growth with the resultant concentrate. This work is being continued but we are not making very rapid headway. It might also be stated that we have had considerable difficulty in maintaining a high vacuum but recently by having the joints re-ground we think we have solved this problem. For the last two months we have been able to consistently maintain a high vacuum in the system.

NR:

Plans for Future:

Immediate:

1. We propose to continue the work on the saprophytic organisms as described in the previous report and we are hopeful that the work can be brought to a conclusion during the next two months.
2. This work as mentioned previously has been completed in so far as establishment of base line is concerned but our results have not been completely analyzed but will be by the time of our next report.
3. We have started but very recently to study the effect of isonicotinic acid hydrazide on various acid fast organisms in addition to the ones mentioned previously; this work has not progressed far enough to form any conclusion as yet. We plan to study the effect of isonicotinic acid hydrazide on various known laboratory strains plus "wild" pathogenic organisms secured from patients, both streptomycin sensitive and streptomycin resistant organisms.
4. We shall continue to try to determine why we do not secure good growth from the sputum concentrated by means of high vacuum. We know that keeping the temperature less than 20 degrees C. will not allow us good growth. We think that perhaps by adding some protective agent we may be able to protect the tubercle bacilli and we are now working on this phase of the problem in addition to that already mentioned.

Long Range:

1. As mentioned in previous report our long range plans have not changed except we are now in possession of a new antibiotic. This substance has been obtained from the filtrate of media in which *Fusaria bostrycoides* has been grown; the substance is the purple red pigment which has been named bostrycoidin. This substance appears to be quite bacteriostatic and perhaps bactericidal and also in animals it is quite non-toxic. We hope to evaluate this material in our laboratory and in conjunction with the Department of Biochemistry and Dr. Florian Cajori who is going to study the substance chemically and particularly its metabolic effects on the tubercle bacillus.