MEMORANDUM

BRISTOL LABORATORIES

UNIT OF BRISTOL-MYERS COMPANY

FROM J. Lederberg

TO J. Lein

C.C.

DATE August 17, 1955

SUBJECT New Antibiotics Screening Program-Consultantship Arrangement

Dear Joe:

Let me take up some of the issues in your last communication in order.

I am not terribly surprised that agar diffusion techniques prove to be probably unsuitable for routine use. I think they could be made to work but only in the hands of a professionally skilled worker. In the long run you would have to repeat most of your experiments with liquid culture mixtures in any event so there may be little enough point in using the agar diffusion methods. I imagine if the need required it would not take much ingenuity to design an automatic filling machine which would automatically deliver different ratios to input fluids upon setting a dial or some such thing. In a setup like yours I suppose the human hand is the more appropriate instrument.

As far as the general screening program is concerned, I can see very few things that we have not already discussed and for which there already has been a decision on one basis or another. I fully sympathize with your problem in having to make decisions often on an expedient basis or even less than that without having very many of the pertinent facts at all but I can't see that you have actually gone very far off any possible path.

The only points I would bring up again would be the queries (1) Should one adhere exclusively or primarily to Actinomycetes as the sources of new antibiotics? (2) Whether a mutation program still could not be applied more routinely, because while I think your argument that mutations leading to increased production are likely to be on the whole infrequent, it is probably valid that only experience would really tell. My suspicion would be that when you start with relatively low yields it should not be extremely difficult to obtain mutants which give moderate performance. I would agree that going from moderate to superior strains represents the most difficult step. If I am not mistaken there was an entirely similar experience in the development of penicillin-producing strains. I think it would be worthwhile with any culture which seems to be producing a possibly promising or new antibiotic but in relatively small quantity to do platings along the lines of Kelner's technique to try and look for improved production, even simply as the basis for further preliminary analysis. Of course, a much more exhaustive job would be done whenever you approach the question of routine production. The matter of replating the cultures from the wild in order to separate out the possible heterokaryons would seem to me to be much too important to be by-passed at almost any cost and some of the occasional inconsistencies in production from one run to another might very well be due to this cause. I would think that as soon as a culture seems to be producing any antibiotic in which you would have any interest it would be worthwhile to replate it and do further studies on two or three reisolated single colonies.

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I was extremely happy to hear of the successful outcome of your discussions with Biochemistry on further emphasis on less potent broths since this is perhaps the most obvious vulnerable point for the search for new materials. These are certainly the items which will require the most skill in order to handle and from that point of view are the most likely to have been overlooked by your competitors. In addition one would imagine that there will be relatively few antibiotics of very high potency on first isolation and more of less and less potency and therefore the cream having apparently been more or less skimmed it is necessary to go into thinner and thinner veins.

I would again emphasize the possibility of discovering adjuvant or accessory antibiotics, i.e., compounds which by themselves might have little antibiotic activity and not enough to justify marking them alone but which could be extremely important in maintaining the usefulness of other more striking activites. This may not be expressed in the normal course of events in terms of simple synergism and I am not sure what the best methods would be of picking up useful accessory agents. It might be worthwhile, however, to consider what might be done with more or less reconstruction experiments involving infections with challenges consisting of primarily an organism sensitive to a primary antibiotic containing a few organisms resistant to it to see whether an accessory antibiotic would have the desired effect of eliminating the last residual resistant organisms. With tuberculosis especially this would be an extremely useful and pertinent result either with streptomycin resistance or to a lesser degree with isoniasid resistance.

From your previously expressed interest in combination effects I would judge that you have already given considerable thought in the matter of synergisms. Now the first proposal that I had made before with regard to accessory antibiotics would not necessarily be reflected in any obvious synergistic effect since the primary antibiotic would probably be the one which is most readily manifest in <u>in vitro</u> experiments. Synergisms represent the second approach and here again, of course, there should be many opportunities for the interaction of two antibiotics which separately are too weak to be very much good by themselves. I would imagine that the point at which the most emphasis should be given to such accessory effects would be where an antibiotic has proven to be more or less active in <u>in vitro</u> tests, say even in the presence of serum, but which fail when used by themselves in <u>in vivo</u> challenges. In such cases it might be necessary or desirable to go right ahead with mixed therapy without the benefit of very much knowledge of the <u>in vitro</u> interactions, the latter not necessarily being of very great relevence to the therapeutic effects.

I was startled and interested by your interest in the search for virus-inducing agents on the basis of the azaserine effect. It hadn't occurred to me that this correlation would be of direct chemotherapeutic interest but of course it is. It is doubtless true that most of the agents that have been successfully used in anti-tumor therapy would have this inducing effect on lysogenic bacteria, X-rays, nitrogen mustard and azaserine. I have been acquainted with this effect of azaserine for some time but had forgotten to think of azaserine as an antibiotic produced by an Actinomycete.

I think one should look a little bit at what is behind this correlation and I think the root of it is some fundamental common basis of mutagenic or more accurately so-called radiomynetic activity, that is to say, all of the agents that I have enumerated very closely resemble X-radiation - their biological effects. To the compounds indicated

one can add such common agents as formaldehyde, hydrogen peroxide, and in fact I think there are reasonably good grounds for believing that hydrogen peroxide is a key media in many of the other radiomynetic effects of chemicals and of radiation in particular.

I have some notions of my own about what the next step in this correlation is. I have the feeling that the one thing that is common to most of the radiomynetic agents is that they are potentially powerful alkalating agents, that they are capable of substituting on free amino or carboxyl groups under physiological conditions. I think even the peroxide can fit into the picture if one recalls that in the first place hydrogen peroxide forms complexes with many organic compounds which have reactive hydrogen and that these organic peroxides in turn can function as alkalating agents. In this connection you might be interested to know that such familiar alkalating agents as acetic anhydride, acetyl chloride, dimethyl sulfate and ethylene oxide also have radiomynetic effects in other test systems, e.g., chromosome breakage and in the induced breakdown of diploids in Escherichia coli. You will see something about this in table 10 in our paper in the 1951 Cold Spring Harbor Symposium and rather more recently in a paper that I think you will very much want to see - I believe this is a review by Loveless in either volume 1 or volume 2, probably volume 2, of ADVANCES IN CANCER RESEARCH. Loveless there has reviewed what is common in the chemical reactivity of a variety of carcinogenic, mutagenic and anticarcinogenic agents. Now not all of the agents indicated have been tested on the lysogenic system but there are already so many parallels that I would be willing to bet that practically any compound or agent which is mutagenic in this way and which breaks chromosomes and breaks down diploids of Escherichia coli is going to be found to be an inducing agent provided its toxic effects on the bacteria do not already override any possibility of this lysogenic induction. Therefore on this basis the search for compounds with inducing activity would be equivalent to a search for powerful, natural, mutagenic agents and I believe it has been shown that azaserine already falls into this category (Demerec). What may be unique about these antibiotics that distinguishes them from such destructive chemicals as acetic acid anhydride and acetyl chloride is that they are relatively stable in aqueous solution so that they have some possibility of entering the target cell that you are aiming at.

Precisely why so many radiomynetic agents should in fact prove to have anti-tumor activity is not at all clear any more than what is certain why X-rays do have some differential effect on tumor tissue, presumably this is all bound up with the question of rates of growth and the like which distinguish tumor tissue from other cells. There may be also problems of differential penetration which will be one of the few points of access to differential tumor chemotherapy. I think the question of screening for anti-tumor agents on this basis then perhaps reduces to what is the most effective method of looking for agents with radiomynetic activity. If I were working in a research laboratory primarily, I would think that the breakdown of diploids would be one of the best systems for examining antibiotics from this point of view and you would get results along the lines of table 10 that I referred to in the Cold Spring Harbor Symposium. I regret to say that these diploids are so hard to handle that there is really no possibility of adapting them to routine use in this way. However, instead of diploids of Escherichia coli it might be possible to work up techniques based on the breakdown of diploid heterocycles in other organisms, especially yeast. Here again, there may be many technical problems in working out the nature of the effects of the killing agent, but the handling of the yeast culture itself would be very much less of a problem. I think for the moment that this approach, this use of a test for genetic effects, is something

that ought to be thought about in the long run but is a long ways from being anywhere near ready for routine application. Any test of this kind is bound to mean much more attention to the antibiotic effects of your reagent than you are likely to want to give in a routine preliminary screening program. These are things that you might do on an intermediate level before going on to test your material, let's say on an Ascites tumor situation.

That leaves now the question of what other systems would be most appropriate for looking for this kind of activity. Another system that would be possible would be one which involves testing for active mutagenic potency and some of Demerec's fundamentally rather sloppy but still workable assay systems, for example, the induced reversion from SD to SR again might be applicable in a research laboratory atmosphere but I would shudder to think of having to set up such a program on a routine basis. I think that the possibility of lysogenic induction as a screening technique does deserve closer consideration. I think it is possibly the one which is most likely to be readily useable which is the least likely to be misinterpreted in the details of its results but again it is going to require a lot of attention in trying to set it up on a routine basis. Now you are quite right that almost the only way of looking for effects of this kind is going to be to look for the production of phage rather than for the gross destruction of the lysogenic bacterium. One might hope that one could get a differential on that basis and that is the reason that I gave you the strains but you would be bound to miss many reagents as you already know that you would if you relied on an observable difference in the survival of LP+ as compared to LPS bacteria. You are quite right too in supposing that the normal untreated lysogenic strain is going to produce phage without the intervention of some inducing agent. However, there ought to be and there is as a rule a very sharp distinction between the amount of phage which is produced spontaneously and the amount which is produced under the influence of a suitable inducing agent. Now one will have to pay some attention to detail on this because in order to get effective induction it is necessary that the cells being treated, until the time to which the material is added, be in optimal physiological condition and you are also going to have to work out some more or less semi-quantitative way of measuring the phage that is produced in the presence of an antibiotic. That means of course that you are going to have to do these tests for this particular purpose to begin with in a liquid medium.

I think the quantitative assay does not necessarily present too many problems because one can fairly easily set up a loopful dilution type of technique which will get around that question, that is to say, you might use a routine along the following lines, grow your bacteria in broth, use them at log phase, that is to say, add the antibiotic to them and make sure they are being adequately aerated during the time that the antibiotic is working, say some 2, 3 or 4 hours later take a loopful of the LP+ bacteria and antibiotic mixture and dilute that in a cc of water and then take a loopful of that and spread it on a streptomycin-resistant indicator.

We have found that EMB agar without sugar but with peptone is one of the better types of media to use for the detection of plaques of lambda. They show up rather more sharply I think than they do on straight nutrient agar - that is a straight surface plate technique that you can use. Now in order to use this method you will have to have a streptomycin-resistant mutant of a lambda-sensitive strain. I see no reason at all that the W1485 strain could not be directly adapted - I'm not sure off hand whether or not we have a W1485 streptomycin-resistant mutant of our own. If we do, I will gladly send it to you. Another stock that you could use would be strain C or some stocks

related to it. There may be some advantages to this. I will arrange to send you a few different lambda-sensitive strains that you can look at yourself and see which one of them would be the best indicator for your purposes. I think you may have to prepare your own streptomycin-resistant mutants of each of these but that is routine and shouldn't hold you up for very long. I just don't think that we have very many of them. At any rate the ones that I will send you next will include strain C and another one that we may already have that is streptomycin-resistant which is sensitive to lambda.

Does this kind of a protocol seem too difficult to be worthwhile for your purposes? I do not see many ways in which it can be simplified and you are going to have to work out a few of the details with known agents like azaserine or nitrogen mustard or ultraviolet light in order to see exactly how it is going to go.

One alternatively could use differential quantitative measurements of killing of lysogenic as against sensitive bacteria but on the whole I think that would be less reliable than would be the production of phage. The reason would be that if even only 15% of the bacteria are lysed that represents a very large output of phage as compared to the normal situation whereas that reduction in viable titer would not mean a great deal at all.

Do you think I have given you enough detail on the probable method? This is something that I am sure you have pretty well thought out for yourself. I think rather than try to spell the whole thing out for you I'll give it to you as a form and then if you want to try it you can come up with this or that question and we'll do our best to help you out. I think you could get started right now with your W1485 strain and I will see about digging up some of these others. In the meantime I will give the matter some more thought myself and see if I can imagine the way of getting at the question. On the whole though, I think the use of a streptomycin-resistant indicative strain on a streptomycin medium would be the best approach to looking for the production of lambda. I think too that there is little question that the lambda system is perhaps the best for doing this type of work. There are many other inducible bacteria you could probably pick up any of them. I think there is enough generality in the effect of mutagenic agents on different organisms that there would not be a great deal of point in spreading your effort over too wide a variety of responding organisms. This is rather a different situation, I think, from the usual modes of antibiotic activity.

This itself may be something of a hint because I think it would be reasonable then to suppose that most any antibiotic with a narrow spectrum of activity among the bacteria is not going to be a likely candidate for this sort of work whereas those antibiotics that have some effect on a variety of organisms are those which are most likely to work.

That just now led me to another suggestion. Another technique of looking for such differential effects may in fact be at least as good as any of these, and that is taking advantage of the rather remarkable differential insensitivity to radiation of strain B and strain B/r. Now as you probably know this differential is not unique to UV light and to X-rays but to some unknown mechanism appears to apply also to other kinds of radiomynetic agents so I think that one might give some attention to the very simple possibility of comparing strain B and B/r, and any antibiotic which differentiates seriously between them would be a likely candidate as a radiomynetic agent. I don't know off hand whether azaserine has been tested directly -- I would

be willing to predict that there would be a significant differential between the two. No one knows what the basic mechanism is for this difference in sensitivity. This in turn has led to another thought that might be quite apropo to this kind of analysis, and that is a comparison of the survivorship of haploid as against diploid yeast. Since there are quite remarkable differences in the susceptibility of strains of yeast of different ploidy to radiomynetic agents, particularly UV and X-rays, I would assume without knowing it to be a fact that the same is likely to be true with other agents.

Do you happen to know whether azaserine is active on yeast? If you tell me that it is at all I will be glad to dig up some haploid and diploid yeast strains for you and you may want to see for yourself if there is a differential in killing activity. One point about almost all of these tests is that they are going necessarily to involve some sort of semi-quantitative procedure in liquid medium because none of the effects are going to be all or no inhibition or bactericidal effect such as you have been accustomed to seeing with ordinary antibiotic activity.

Let me know if any one of these approaches seems interesting enough to you that you want to pursue it further and I will do my best to dig up the material. I can, I think, send you stocks of B and B/r or very closely related derivatives of them. I haven't retested these myself for their differential response to irradiation. You can get, of course, the basic data on them from Dr. Evelyn Witkin at Cold Spring Harbor if you want. I am not sure that they haven't been working with this particular problem for quite some time and I am really not at all sure whether they could help you a great deal further on their specific properties. One of these methods certainly should be suitable for what you are ultimately aiming at here.

To turn to another question, I certainly did make a bad guess as to what you intended to use these coli strains for. I am a little surprised that you presented the possibility of biological protection to cover antibiotic suppression of enteric formula simply as a promotional stunt and I really wonder if this is what you think of it. It seems to me that you are touching here on some of the most subtle and most important problems of host-parasite relationships, and I would certainly encourage you to go on in this direction and would lend every effort I could to help, provided that it was clearly understood that there are some very serious problems involved here that are going to take a good deal of experimental work before they can have any hope of use. Against that should be measured, I think, the great importance that this approach would have. I don't know why you should think that this is so absolutely silly. In the first place, behind this notion is a considerable tradition of bacteriological therapy which had as one of its poorer expressions, for example, the fad of drinking Lactobacillus vulgaris milk some while ago. In the German literature of the 20's and 30's there is a good deal to be said about so-called "coli therapy", the fundamental reasoning of which was quite similar to what you have in mind as a cover for antibiotic suppression of other organisms although of course the practical situation was a little different. In fact, a couple of years ago I talked to Dr. Finland at Massachusetts General Hospital about just this possibility -- it's one that had occurred to him too. This was just at the time when he was first describing these very serious Staphylococcal enteritides that followed upon Aureomycin administration and of course the first point that he would bring up was just how dangerous an experiment it would be to introduce any new organism into an unknown situation. Despite those reservations, they're ones which you have expressed yourself of course, it seems to me that there is some very important worthwhile work that needs to be done here and that an important adjuvant to antibiotic therapy of the gut is going to be replacement with suitable healthy so-called flora when they question however whether there is such

a thing as a healthy flora but it may not turn out that each individual will have his own range of microfloral adaptations. This is something that will require a great deal of experiment. Frankly I don't think that your approach is going to work; I think that using a purineless mutant is not going to be the answer. If I am not mistaken it has been pretty well shown by the wofk of Burrows and Bacon that the reason that purineless mutants of the Salmonellas are relatively non-pathogenic is precisely because they failed to grow sufficiently in the habitat into which they are introduced unless artificial growth factor supplements are administered to the animals as well. Now if your're going to give purine along with purineless bacteria I think you might as well give up that particular block in the first place. I think too that not many physicians are going to be convinced by a purely apriori argument that purineless organisms under any circumstances are really likely to be less virulent than the normal. There are some very serious questions as to what one should consider as the normal flora and perhaps as a first approach in experimental animals I would use for administration and prevention of overriding infections that flora which does recurr in organisms which have been chronically treated with drugs like Aureomycin, but which animals have remained healthy. I am not proposing giving Staphylococci, Candida, Proteuses etc. The organisms I think should be repurified and studied to get some sort of expectation of their later behavior when they are reinoculated. So I don't think that this is a silly idea, Joe, not at all, but I do think it's not something that one can afford to go into lightly. I have every hope that you have the facilities to be able to do a proper study of it since I don't think that anyone else is doing very much along these lines. Of course one place one would hope to have gotten information on this question would be from the germ-free animal studies of Renier's group at Notre Dame. I have had occasion to look over that work again lately; the performance so far has been very little beyond the technical accomplishment of maintaining animals in germ-free condition. They give hints of experiments that they are going to do on pure culture inoculating germ-free animals but in fact very little is done about it. A person who might be able to give you some information on these effects would be Mortimer Star (at the Department of Bacteriology at the University of California) Davis - he was interested although he was primarily a plant pathogenic bacteriologist. He was interested for awhile in the growth stimulating effects of antibiotics and felt sure that their modification of intestinal flora was an important part of it. At any rate he did notice that after the administration of streptomycin to turkeys there was an eventual recurrence of streptomycin-resistant coliforms and that when this finally happened his growth-stimulating effects ceased. I think he may have had a note or two about this in the Bacteriological Proceedings but I'm not sure.

You may be interested in a case that happened here in our department. The baby girl of one of our assistant professors came home from the hospital with what eventually proved to be a pure culture of Serratia marcescens in her gut. No other organism could be isolated at first for a period of about two weeks. After she began to go on a diet of non-sterile light foods she eventually picked up some coliform as well. She is still excreting this Serratia. I'll be glad to send you a culture of this organism if it would have some interest for you as an organism that could readily be followed in artificial infection experiments along these lines. There is at least the justification that in one instance it had no pathogenic effects whatsoever. I think you may find some resistance among pediatricians to the administration of a chromogenic organism. I know that this particular family was alarmed because of the red diapers for a period of some time. This question is one that I'm really quite interested in myself, Joe, and wish there were an opportunity to do more research on.

To: J. Lein August 17, 1955 New Antibiotics Screening Program - Consultantship Arrangement

There ought to be better facilities for germ-free research and maybe something eventually is going to be done about it at institutions other than Notre Dame.

That about ties up these main points. The only thing that I want to add now, Joe, is a note that you may or may not have seen in an issue of NATURE, July 16, 1955, p. 121. There is a paper by Simonti and Simonti on genetic recombination in Streptomyces. They worked with a strain of Streptomyces coelicolor, and they have about the same category of evidence as was represented in the first paper in NATURE by Tatum and myself on E. coli. Obviously as a result of this Gallen, Bradley and I are going to have to reevaluate our own position in this work and we're going to have to discuss this somewhat with you. The main thing that I would have been concerned about in freely discussing our work was that I did not want to get any information from you which had to be held confidential and I did not want to have a purely one-sided discussion on work of this kind. I will just leave it at that, Joe, and if you can see any reasonable common grounds that we can meet on to talk about the genetics of Actinomycetes I'll be glad to reopen the question. However, as long as we were directly occupied with this work ourselves it would do us, I think, a good deal of harm to be influenced by information which was not publicly available. Esther and I are taking about 10 days off on a trip to Colorado and for that reason there may be some delay in your receiving some of the cultures that I promised although I left instructions behind for them. I will be back just before the end of the month.

I've just learned that we are going to have our laboratory remodeled sometime during the fall semester and if that is the case there may be a very suitable occasion for me to take off and pay you another visit in Syracuse some time in October or November or early December if that would suit your plans as well. Let's leave that open for the time being but I thought I would just mention it to you. Probably by the time this has been assimilated we will have gotten back from Madison and I will wait to hear from you again.

Yours sincerely,

Joshua Lederberg

/jlg