MEMORANDUM

April 5, 1961

TO: Members, Enterovirus Committee

FROM: Executive Secretary

SUBJECT: Dr. M. Sigel's Contract Proposal for Rapid Viral Identification.

There is attached a contract proposal from Dr. M. Sigel of the University of Miami School of Medicine, outlining the proposed research project in the general area of rapid viral identification.

Please review this at your earliest convenience. I shall be glad to receive an evaluation from you as to the merits of this proposal. I have written Dr. Sigel acknowledging receipt of this proposal and have suggested that he send in more details as to budget items, i.e., how the monies stated are to be spent, and also more details as to the general methods of attack which he proposes for the problems he desires to work on.

Marvin M. Harris, Ph.D.

Attachment.

Area A - no relation to mission of enterovirus committee.

B. No comment as to importance in research.

C + D) Same as A.
Dr. Marvin Harris  
Viruses and Cancer Program  
National Cancer Institute  
National Institutes of Health  
Bethesda, Maryland

March 28, 1961

Dear Dr. Harris:

As a follow-up to our discussion of two weeks ago, I am sending you this proposal for a contract. You will find the substance of my proposal on page 3 of this letter. The preceding part of the letter contains my thoughts accumulated in the course of many years of association with the problems, practices and developments in diagnosis of virus infections.

I called attention to some of the needs and limitations in publications in 1950-1952. I shall now list only a few of the problems in diagnostic virology, especially as they relate to the quickened tempo of efforts at demonstrating human oncogenic viruses.

1. There is an acute shortage of diagnostic reagents, especially standardized sera for identification of viral agents.

2. There is a severe shortage of personnel with either the experience or the interest for clinical or diagnostic virology. The few virologists who emerge from schools and colleges every year are more often than not interested in the more exciting fundamental and challenging aspects of virology, principally in basic virological problems.

3. Most of the classical techniques require much time for completion.

4. The existing virus diagnostic services are very much hindered by the ignorance on the part of the hospitals and clinicians in matters of utilization of the available services and such things as collection, handling, preservation and shipment of specimens.

5. Most biological systems employed for the isolation of viruses have built-in hazards, that is: latent viruses or other microbial agents which may interfere with the isolation or obversely provide spurious isolates which
may be mistaken by the worker for the actual agent sought. In this connection, it is worth stressing that, especially in animal experiments, inoculation of secretions or tissue extracts may activate and thus stimulate the multiplication of latent viruses.

6. Serologic testing is complicated by group reactions, anamnestic reactions and non-specific reactions. Moreover, serologic tests display variations with regard to sensitivity with different viral antigens.

Several developments of fairly recent origin have provided a few solutions and remedies to the complexities cited above. I am referring especially to methods of viral purification, the, theoretically, most significant and, practically, highly promising achievements with fluorescent microscopy and the availability of wide spectrum antibiotics able to suppress a host of non-viral agents. One could, of course, point to the very important contribution provided by the newer methods of tissue culture. This undisputed blessing of tissue cultures in the form of the recognition of the existence of many previously unknown viruses has, nevertheless, created the acute problem of identifying these agents and associating them causally with diseases.

These problems assume an additional dimension in the area of viruses in relation to cancer. On top of the large multiplicity of different types of cancer, one is faced with the colossal task of sorting out the even larger array of viruses which may be encountered in nature. One doesn't even know what question to ask in the sorting out process. Should we be looking for highly virulent viruses, or latent and moderate viruses? - for viruses not yet branded as causes of known diseases or viruses with definite disease syndromes? Assuming that some human cancers are caused by viruses, will these be viruses causing direct effects by way of the cell genome or will they be causing secondary effects by releasing some carcinogen from damaged cells or will they display their oncogenic properties only under the proper combination of conditions and factors?

Experience with viruses associated with cancer in animals suggest that similar viruses of man may have a rather narrow host range. Thus, after all is said and done, when the virus or a virus is isolated which has many of the earmarks of a human cancer virus, how will one prove that this virus causes cancer in man? The last consideration is the basis of our own project supported by N.I.H. exploring the utility of heterologous transplants for the detection of oncogenic viruses.

I am sure that much of what I have discussed above is known to you and to Dr. Scudder and I may have paraphrased many of your own concepts and thoughts. A thought, however, that as a virologist with many years experience in the virus diagnostic field and, more recently, in the area of viruses and cancer, I should like to reiterate the points which have contributed major difficulties and provided a challenge in my own activities. I think we all agree that large expansions of basic research are
Dr. Marvin Harris

an absolute necessity to increase our understanding so as to permit us to ask the pertinent questions before even attempting to supply the answers. Along with such increasing tempo and scope of research, there is a great need for more qualified and competent investigators and technicians, which means more and better training programs. Both of these vital areas of endeavor depend on new or improved tools. It is heartening that the National Cancer Institute has provided the means and organized a group, under Dr. Scudder's leadership, to take positive steps in this direction. Although I do not possess full knowledge of all the ramifications and objectives of your program, I think that the ones that you and Dr. Scudder mentioned to me, namely production and standardization of certain reagents and the interest in new or improved procedures, are most commendable.

I should now like to come to the part dealing with the interests of this organization in helping to resolve some of the existing problems, difficulties and pitfalls:

The Variety Children's Research Foundation, by virtue of its nature and types of activities combining basic research with clinical and virus diagnostic activities, is in a position to cooperate with your program along several lines of mutual interest. As I mentioned to you in Washington, we would like to be considered for contractual cooperation in the development of certain reagents, especially anti-sera. At the present moment, however, we wish to submit for your consideration a proposal for a contract bearing on methodology.

The work which I consider pertinent can be divided into four areas. If desirable to both parties a single contract could cover all four areas; or the initial contract could cover one or two areas and then extend into the remaining two. In three areas experimental models will be employed. In the fourth, specimens from naturally occurring human infections would constitute the source and basis for testing. For convenience, I shall outline the broad technical aspects and give the rationale at the end of each area.

AREA A: Development of methods and approaches for the purpose of enhancing the efficiency of the detection or isolation of viruses.

1. The use of tissue culture cells as "protectors" of virus at the time of collection, handling and shipment of specimens containing one or more viruses.

2. As a corollary, the use of tissue culture cells as protectors of virus in the course of infection of animals or tissue cultures.

3. The application of modified organotypic tissue culture as a supplement for the classical undifferentiated tissue culture.
4. The use of clumping phenomenon for demonstration of virus in cells which do not exhibit overt cytopathogenic changes.

The tests contained in "1" and "2" are suggested on the basis of our findings with dengue virus in KB tissue cultures. These findings are part of our results of a research project supported by N.I.H. The mechanism of our observations has not been established but the findings appear to be interesting from both a basic and applied point of view. In brief, it was found that cytopathogenic changes were induced more efficiently and more readily when tissue cultures were inoculated with infected intact cells than with infected lysed cells or with cell-free virus obtained from another source. One of our working assumptions is that this labile virus may be afforded protection from the environment by the intact cells and we would therefore like to determine whether other labile viruses, such as herpes simplex, measles and LGV could also be "enhanced" in their survival and capacity to cause cytopathogenic changes by the addition of cells. The purpose then would be to create infectious "nuclei" or "complexes" composed of any one of these viruses and appropriate cells, and then compare these "nuclei" with cell-free virus as regards survival under various conditions of storage, efficiency of initiating infection at limiting dilutions, and the speed and extent of CP in tissue culture. Obviously, the cells comprising the virus-cell "complexes" will have to be relatively resistant to the virus (allowing adsorption of virus but showing either no cytopathogenic changes or delayed changes) as fully susceptible cells would give rise to only temporary "complexes" (with destruction of the cell, the virus would again become a free virus). The original discovery was made by Miss Wellings of this laboratory.

Should virus-cell complexes prove more resistant to environment and more efficacious in initiating infection in tissue culture, this procedure would be of inestimable value for the purpose of shipping specimens containing labile viruses and would therefore contribute to improving the chances of virus isolation. I am sure that you are aware of the fact that in some viral infections the lability of the virus makes it essential to take extreme precautions in collecting and handling specimens, sometimes requiring that the specimen be inoculated at the patients bedside.

The procedure listed under "3" has been recently put into use in our laboratory by Dr. Burstein and his associates.
This group has obtained survival and growth of tissues from hamster vagina, hamster cervix and brain. The technique is a modification of the classical technique developed by Fell. It employs open chambers composed of millipore filters on plastic rings placed into petri dishes containing medium and incubated in a box with an approximate atmosphere of 5% CO₂ as regulated by Pardee's buffer. In this technique one can see elements of tissue retaining their natural morphology and topography as well as outgrowth of cells from these fragments.

Such organotypic culture may be combined with a monolayer of cells of an established culture which can act as an indicator system for virus that is being sought in the organ maintained in the culture. Thus, one has a double opportunity for detecting virus, either in the original culture or in the superimposed detector culture. This technique should be of value in demonstrating viruses which may be masked by antibodies or which are held in check by inhibitors or by other factors in the original tissue. By special manipulations such as the inclusion of coverslips one can prepare cultures which are useful for histologic preparations, for direct observations, as well as for fluorescent microscopy.

The clumping phenomenon referred to under "4" was discovered by Miss Ann Beasley in our laboratory, in her work with dengue virus. Specifically, she found that carrier cells infected with dengue virus exhibit a clumping phenomenon when brought into contact with dengue immune serum on the surface of soft glass bottles. This phenomenon is not obtained in other types of bottles. This discovery has assumed special interest as in the system employed infectious dengue virus as measured in mice is demonstrable only in low concentrations. Thus, this clumping phenomenon may be useful in demonstrating other viruses which may be present in low concentrations in tissue cultures and we are proposing therefore, to utilize this procedure for determining whether other viruses which present problems in isolation and detection may be demonstrated by the use of this phenomenon. Here again, we would like to use the LGV virus, herpes zoster, and other viruses which may be considered suitable for this purpose.

We estimate that the direct cost of phase "A" of the methodology contract would be approximately $22,000.

AREA B: A comparison of diagnostic procedures in relation to efficacy and feasibility of detecting viruses at different stages of infection. Two models would be used for this purpose. One would represent the virulent infection terminating in severe or fatal disease of the animal...the other would represent a chronic or latent infection with the animal not demonstrating any
gross evidence of illness. The first model can be created from among many viruses including coxsackie in the intact mouse and encephalitic viruses injected into the brain of adult mice, etc. For the second model, one can use any one of several myxoviruses inoculated intranasally into hamsters or into guinea pigs or a virus producing a latent infection in mice. In the first model, animals will be sacrificed during one or more intervals during the incubation period, at the time of onset of symptoms, at the time of height of symptoms, at the time of death.

In the second model, where obvious symptoms are not anticipated, certain specified intervals for sacrificing of the animals will be established so as to include the early stages of infection, a stage or stages when virus would be expected to be at its highest level and thereafter, several stages lasting for a period of several weeks. Appropriate tissues, the type to be determined by the nature of the virus, will be removed and subjected to the following types of study:

1. Pathologic examination.

2. Assay for virus in tissue culture or in any other suitable system.

3. Fluorescent microscopy employing non-specific fluorescence provided by such reagents as acridine orange.

4. Specific fluorescence employing tagged antibody.

5. The CF test employing as the antigen, the tissue removed from the animal.

The above tests have been employed with various degrees of success and with varying frequency in diagnostic procedures. Moreover, some of these have been a part of research investigations with specific viruses in certain hosts. I might state that the last mentioned procedure has been used for a long time in the rapid diagnosis of smallpox using crust material from the patient as antigen for CF test. My associates and I have previously shown that liver and spleen of parakeets infected with psittacosis virus can be used in a similar fashion, when, because of certain conditions and circumstances, it is not possible to isolate the virus from such organs.

The proposed work would compare the relative merits of the various procedures. What is more important yet, it will for once provide the means of determining which of these procedures has its greatest value in different stages of infection and under conditions of overt or inapparent infection. Such knowledge is not available. The direct cost of this area of work is expected to be approximately $20,000.
AREA C: Determination of the effect of PPLO on the isolation and cultivation of viruses in tissue culture. Viruses, especially those recently obtained from patients, will be inoculated in small amounts into tissue cultures known to be free of PPLO, as well as into similar cultures deliberately contaminated with these micro-organisms. The ease of isolation of the agents and the time required for, as well as the extent of, cytopathogenic changes will be compared. Moreover, the resulting virus cultures would be sub-inoculated, similarly, so as to see whether any enhancing or inhibiting effects by the PPLO may be noted. Similarly, PPLO free preparations, as well as preparations with PPLO will be inoculated by various routes into animals known to be susceptible to the viruses in question. The results in such animals will be compared.

The significance of this study is quite obvious. It is well recognized that many laboratories experience contamination of tissue cultures by PPLO, yet very little is known about the possible effects of PPLO on the events taking place during virus isolation, virus cultivation, virus passage, or the sub-inoculation into animals. Such a methodological investigation would determine whether the PPLO contaminants represent a purely academic problem, or whether they are of a real significance in the virus diagnostic laboratory as regards isolation, identification and maintenance of viral agents.

We believe that this institution is well suited to carry out such an evaluation inasmuch as a member of the staff has had considerable experience with PPLO and will be available for this phase of the work. The estimated direct costs required for this phase of the work amount to $11,000. If limited to tissue culture, and to $16,000. If carried out in both tissue culture and animals.

The work listed in the areas "A", "B" and "C" can be carried out in a systematic and methodical fashion with scheduled infections and well-designed experiments. The proposal under area "D" deals with similar studies but employing human clinical material specimens collected from sizeable groups of patients, preferably during epidemics and outbreaks of certain viral infections. Thus, the actual execution of the experiments would be at the mercy of time and circumstances. Essentially, area "D" would cover diagnostic work employing elements listed under areas "A", "B" and "C" making use of various available procedures enumerated under those areas, but relying principally on fresh specimens obtained from patients. For this study to be successful, we would need the services of a full-time clinician or epidemiologist. Experience has shown that the procurement of full-time clinicians for this type of study is not an easy
matter. However, because of circumstances of political nature involving Cuba, this area has a large number of physicians and scientists, some from the faculty of the University of Havana, who are available for recruitment into such a study. It is difficult to state the exact amount of direct funds required for this type of study, but one could offer an intelligent guess and say that the cost, including a salary for the clinician, materials, supplies, animals, etc. would be in the vicinity of $21,000. This is a sizable amount of money which one would hesitate to request unless one were assured of an outbreak or an epidemic in the immediate future. I am not sure just how one would proceed in activating such a project, but I have two suggestions: One is that only the amount required for the salary of the clinician be provided immediately so that the clinician is available in the event of an outbreak; the other is that this type of contract be held in suspension pending the occurrence of an outbreak and, should such an outbreak develop, funds for the initiation of the study be immediately available inasmuch as many outbreaks are likely to last for only a short period of time. If one has to await the slow turning of the wheels of the machinery required for initiation of a contract, one may be too late in availing himself of the materials in such an outbreak.

These essentially are my thoughts, suggestions and proposals for implementing my ideas with regard to methodological work covered by a contract or contracts for the purpose of making some inroads into the improvement of procedures, techniques and approaches for the isolation, and detection, and sorting of viruses. I hope that you will give me your reaction at your earliest convenience.

Best regards.

Cordially,

/s/  M. Michael Sigel, Ph.D.
Professor - Department of Microbiology
University of Miami School of Medicine

Research Director
Variety Children's Research Foundation

MHS:rk

CC: Mr. William A. Taylor
Administrator - Variety Children's Hospital and Research Foundation

Dr. Harvey Scudder

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