04/07/86

Dr. Paul K. Pybus
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The Rheumatoid Disease Foundation
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181 Church Street
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Dear Dr. Pybus,

I am in receipt of your letter of 1/27/86 and subsequent correspondence of 3/17/86. I apologize for the delay in my reply. I am glad to take this opportunity to report on our progress to date.

We began work on the project in mid-October. A June 1 start-up date was requested; however, funds for the research were not received until September. To recruit and train a technical person took a month and a half. Attempts were begun immediately thereafter to isolate pathogens from the synovium of rheumatoid arthritis patients by following the accounts given by Wyburn-Mason. To answer the question that must be foremost in your mind, no, we have thus far been unable to identify or isolate pathogenic free-living amebae from rheumatoid synovium. Nonetheless, I feel that we have made considerable progress toward the aims of our proposal.

A complete work-up of the samples which we receive entails: 1) Preparation of a thermophilic filtrate (see below (*) for a description of our modification of the technique employed by Wyburn-Mason). 2) Inoculation of the thermophilic filtrate and of minced synovial tissue into various media generally used for the isolation and growth of pathogenic free-living amebae (e.g., agar with bacterial lawns, liver infusion agar, Nelson's medium, Balamuth's medium, 1:1 Nelson's:Balamuth's media). 3) Examination of the filtrate and of H&E fixed sections of tissue for amebae by light microscopy. 4) Examination of frozen sections of tissue for amebae by immunofluorescence. (High titer antisera directed against *Naegleria fowleri* and against *Acanthamoeba polyphaga* were raised in rabbits). At the time your first letter arrived we were completing the work-up on our first eight samples. Another nine are now in process.

It is important to note that our procedures for the detection and propagation of pathogenic free-living amebae have been validated using positive controls. *Naegleria fowleri* and *Acanthamoeba polyphaga* will grow in the culture media and under the conditions of temperature and atmosphere we employ. The antisera we use for immunofluorescence will agglutinate these amebae, and the immunofluorescence protocol used detects *Naegleria fowleri* in sections of brain from experimentally infected mice.

Several reasons could account for our negative findings thus far. Patients sampled may be devoid of the etiologic agent as a result of prior therapy, yet pathogenesis may continue, perhaps as an autoimmune sequela. It may be necessary to obtain samples from patients with earlier stages of the disease, although it is probable that only synovial fluid, and not the preferred synovial tissue, would be available from these patients. Culture conditions may not be suitable for growth of the pathogen. However, we have used the same culture media as Wyburn-Mason, plus additional types of media, and his reports do not indicate that the organism isolated was particularly fastidious. Still, it may be necessary to try different culture media (e.g., using mammalian tissue culture cells as an associate food source or embryonated eggs) and conditions. And although species within the genera *Naegleria* and *Acanthamoeba* demonstrate close antigenic relationships and are detectable with our antisera (we purposely immunized rabbits intramuscularly since this route potentiates cross-reactivity which would be advantageous for detecting an unspecified organism in either genera), the organism described by Wyburn-Mason may belong to a different genus or a non-cross-reactive species. Obtaining pure cultures of the pathogen from patient material will greatly facilitate demonstration of the organism *in situ*.

Alternatively, Wyburn-Mason could have misinterpreted his observations. The organism he described may have been a environmental contaminant (both *Naegleria* and *Acanthamoeba* form cysts) or ameboid cells of host origin (e.g., fibroblasts, macrophages or neutrophils). This latter possibility seems unlikely since human cells will not grow under the culture conditions reported by Wyburn-Mason, and the nuclear morphology of neutrophils is totally beyond the realm of description given by Wyburn-Mason for the putative pathogen. I might add that I have been impressed by the number of artifacts one might misconstrue when looking for an amorphous organism such as an ameba. Also, an interesting artifact which was detected about the time of your first letter was the presence of L forms (cell wall variants of bacteria) in four of the cultures plated on bacterial lawns. Fresh colonies do resemble animal cells and may assume ameboid shapes. We realized that these were not amebae, however, because of a lack of intracellular organelles, and systematically proved that they were mutants which arose within the population of *E. coli* used as an associate food organism. (Pursuing this observation to its ultimate conclusion contributed to the delay in my response to your initial inquiry.) Still, this episode reinforces the need for prudence, considering that L forms have previously been implicated in the etiology of rheumatoid disease.

For the immediate future we will continue our efforts to detect pathogenic free-living amebae in synovial tissue from

rheumatoid arthritics. I am informed by Mr. Chapdelaine that Dr. Robert Neff of Vanderbilt University and Dr. Kwang Jeon of the University of Tennessee are making similar attempts. This is important for two reasons. First, any positive findings would have to be independently confirmed, and this would be expedited by having the machinery already in place; second, because of our diverse backgrounds, the procedural courses will vary somewhat from one another and this should enhance the possible detection of an organism. When one of the investigators is thoroughly convinced of a positive finding, it should be immediately reported to the Foundation so that the others may duplicate the successful protocol and offer corroboration.

I am also anxious to hear about findings from the double blind clinical trial of clotrimazole being conducted at the Bowman Gray School of Medicine. The assertations from you, Mr. Chapdelaine, and Dr. Simoons about successful remissions and cures of rheumatoid arthritis using clotrimazole are encouraging. but must be considered anecdotal until validated by evidence from this type of investigation. If we continually are unable to identify pathogenic free-living amebae in clinical specimens, I plan to test clotrimazole in an animal model of arthritis. I have already discussed suitable models and protocols with a rheumatologist and a pharmacologist here at MCV. We would conduct animal trials using two models - collagen induced arthritis in the rat, and the MRL/1 mouse strain. The rat model is the prototypic model use by the drug industry in screening new agents. The MRL/1 mouse model is a more recent development. These mice spontaneously develop inflammatory arthritis, IgG and IgM rheumatoid factors, and antibodies to types I and II collagen, thus exhibiting morphologic and immunologic similarities to human disease. These models would help ascertain the efficacy of clotrimazole, and once established, allow for elucidation of its mechanism of action.

If you have further questions regarding our progress and future aims, I will be happy to reply promptly.

Sincerely yours,

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* Several modifications in the type of apparatus described by Wyburn-Mason to prepare the thermophilic filtrate were deemed necessary. First we have eliminated the used of the membrane with 0.5 micron diameter pores. We found that *Naegleria* and *Acanthamoeba* would migrate through a 5 micron pore filter but not through a 0.5 micron pore filter. Also we find that the funnel-in-a-water bath arrangement is not conducive to good sterile technique since collecting the filtrate requires running it through the cylindrical portion of the funnel which has been immersed in the water bath. The apparatus which we have designed and feel is more suitable for maintenance of sterile conditions consists of the following: A stainless steel iodine cup (6 oz.) is held recessed and slightly suspended within a stainless steel cylindrical sleeve. Lying on top of the iodine cup is a piece of 400 mesh stainless steel screen, held on the outer edge between two concentric metal rings. The screen is removable, and the whole apparatus can be autoclaved. In practice, the iodine cup is filled with sterile Page's saline, the minced tissue is placed on the mesh screen, and the screen is replaced on top of the iodine cup. The apparatus then can be lifted by the outer sleeve and placed in a water bath to a depth such that three-fourths is immersed (but well below the lip of the iodine cup). A sterile beaker is placed on top of the minced tissue and filled with ice. The weight of the beaker causes the mesh screen to protrude into the iodine cup, thus bringing the saline in contact with the tissue. The 400-mesh screen holds the tissue, and for the most part only motile cells pass through, into the saline. After a 2 hour incubation, the apparatus is removed from the water bath and the saline pipetted from the iodine cup. The fluid collected as well as washings from the iodine cup and the underside of the mesh screen are collected, gently centrifuged as described by Wyburn-Mason (250 RPM for 15 min), and the material collected ("thermophilic filtrate") inoculated into various media for growing pathogenic free-living amebae. Since the specimen is somewhat exposed during the incubation period, we carry out the procedure in a bio-containment hood to assure that there is no air-borne contamination. If you are interested, I will send photographs and a schematic drawing of the apparatus.