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Dear Dr. Pybus:

Your letter of 8 October 1987 arrived nearly one month ago. My apologies for not having written sooner. Besides keeping busy with the research, teaching, and administration, in the past month I have had to hire and train a replacement for the technician who was working with me on the rheumatoid disease project.

You will be pleased to know that we have made substantial progress since the last time I wrote. As you may recall, at that time I expressed the opinion that investigation of clotrimazole using *in vivo* models would be important for documenting the effectiveness of clotrimazole. Therefore during the past four months one of the major objectives was to evaluate the potency of clotrimazole in the rat adjuvant arthritis model.

In vivo studies. To review the model, injection of Freund's complete adjuvant (mineral oil containing cell protein from mycobacteria) into rats produces a synovitis similar to that of rheumatoid arthritis. Acute inflammation in the injected paw develops within 24 hours; arthritis in the uninjected paws, immunologically-mediated by T cells, is detectable by day 12, and achieves a maximum response by days 16 - 21. This is one of the most useful model for pharmacologic evaluation of drugs used to treat chronic inflammation and arthritis because drug inhibition in this model correlates well with clinical effects in man.

We have tested two treatment protocols. In the first, arthritis in rats was induced by subplantar injection of complete Freund's adjuvant into the left rear foot pad. Three dose levels were performed of the effects of clotrimazole administered daily by mouth from the day of adjuvant injection - a 100 mg per kg dose (MKD), 33 MKD, and 10 MKD. Body weights of the animals and edema in the uninjected right paw were followed. Indomethacin at 3 MKD was used for comparison, as a positive control. Drug effectiveness was assessed on the basis of the volume of the uninjected right rear paw, measured on day 18 after the beginning of the test, the peak of the arthritis. The results were:

Protocol #1

<u>Drug</u>	<u>Dose</u>	<u>% Inhibition</u>
Clotrimazole	100 MKD	57.1*
Clotrimazole	33 MKD	76.2*
Clotrimazole	10 MKD	47.6*
Indomethacin	3 MKD	71.4*

* Significantly less than the untreated control animals at $p < 0.05$.

Body weight was not affected by the clotrimazole; therefore antiinflammatory effects were not due to gross toxicity from the drug.

In the second protocol, arthritis was established in the usual manner and allowed to proceed untreated until day 18 when animals were divided into treatment groups. One group was continued without further treatment, one group received 3 MKD indomethacin daily, and three other groups received 100 MKD, 33 MKD, and 10 MKD clotrimazole daily. Drug treatment continued daily for 11 days. This protocol tends to exaggerates potency differences among drugs, although the rank order of drugs is usually the same in this test as in therapeutic trials in man. Measurements for uninjected paw edema were made on day +30 post adjuvant injection.

Protocol #2

<u>Drug</u>	<u>Dose</u>	<u>% Inhibition</u>
Clotrimazole	100 MKD	45.4*
Clotrimazole	33 MKD	34.7*
Clotrimazole	10 MKD	35.4*
Indomethacin	3 MKD	211.1*

* Significantly less than the untreated control animals at $p < 0.05$.

Thus, clotrimazole also was found to have a favorable effect if treatment was delayed until the arthritis had been well established. Indomethacin, however, was significantly more potent than clotrimazole in the established disease model.

We have also continued to carry out *in vitro* evaluation of potential modes of action of clotrimazole, as described below.

In vitro studies.

Background. First to review some of our previous results most relevant to the discussion that follows: We have found clotrimazole to suppress at least two T lymphocyte functions - the production of IL-2 and IL-3 by T-helper cells, and antigen stimulated generation of cytolytic T lymphocytes (CTL) in mixed lymphocyte cultures.

Macrophage competence for antigen presentation is unaffected insofar as Ia expression and IL-1 production. The results therefore indicated that clotrimazole acted directly on the T-helper cell, inhibiting IL-2 and IL-3 synthesis. This would in turn inhibit the development of CTL.

In vitro results and planned future experiments.

Suppressor cells. A possible alternative mechanism was that clotrimazole stimulated suppressor cells that would then play the central role in immunosuppression of T-helper cells and CTL. However, our investigation revealed no evidence of increased suppressor cell activity.

In addition to serving as an accessory to CTL activation, T-helper cells also collaborate with B cells in the production of antibody, e.g., rheumatoid factor. Suppressor cell function in the affected joints of rheumatoid arthritis patients, which normally would control autoantibody production, is known to be depressed. If clotrimazole stimulates suppressor cells that function in B cell immunoregulation, the immunologic balance would be restored. Experiments are in progress to test whether clotrimazole acts to correct the suppressor cell defect that regulates the autoimmune B cell response.

Interleukin 4 (IL-4). We have recently determined that clotrimazole does not inhibit production of IL-4, a B cell growth and differentiation factor. Like IL-2 (the T cell growth factor), IL-4 is a product of T-helper cells. Interestingly, different subsets of T-helper cells produce IL-2 and IL-4, which may explain the differential effect of clotrimazole on their production. This may be an important observation and is being pursued.

Oxygen radicals. Recall that we were also studying effects of clotrimazole on the generation of oxygen radicals such as superoxide anion. Radicals cause tissue damage at an inflammatory site through oxidation of lipids and by activation of phospholipases (e.g., phospholipase A2 (PLA2, studied by Dr. Franson), resulting in cell membrane dysfunction, microvascular damage, and the release chemotactic factors that attract macrophages and neutrophils into the inflammatory site.

We have now shown in two separate assay systems for measurement of superoxide anion that clotrimazole at 1 micromolar (approximately 0.3 micrograms per milliliter) inhibits production of detectable superoxide anion by 20 to 30%. You might be interested to know that allopurinol and oxypurinol have also been shown to inhibit superoxide anion production (Moorhouse et al., 1987, FEBS Lett. 213:23), since these are components of alternative therapies advanced by the Rheumatoid Disease Foundation.

In further experiments we will determine whether the inhibition is by blocking the formation of superoxide anion, or whether clotrimazole acts after the radical has formed, e.g., like superoxide dismutase, to break down the noxious agent.

It has recently been shown with cimetidine that copper ion (Cu(II)) may dramatically increase cimetidine binding to imidazole receptors in tissues, and that such complexes exhibit much more superoxide dismutase-like activity than previously reported copper complexes (Kimura et al., 1986, *Inorg. Chem.* 25:2242). A colleague of mine here at MCV has evidence that copper ion is important for pharmacologic activities of cimetidine *in vivo*. The same could be expected of other imidazole compounds, e.g., clotrimazole. Therefore it is imperative that we determine the potencies of clotrimazole-copper complexes.

Antiinflammatory effects vs. immunosuppressive effects.

In vitro dissection of the effects of clotrimazole on various components of the inflammatory process has suggested several potential mechanisms for the therapeutic effect of clotrimazole *in vivo*. It could be expected from our data so far that the predominant pharmacologic effect of clotrimazole on rheumatoid arthritis is due to immunosuppression of lymphocyte functions, or antiinflammatory effects on macrophages and neutrophils. Clotrimazole apparently has a different mechanism of action from the slow acting antirheumatic drugs (SAARD), e.g., gold salts and penicillinamine. A predictive test for drugs with SAARD-like activity is the test for inhibition of differentiation of monocytes to macrophages on the basis of complement protein 2 (C2) production. As previously reported, clotrimazole was without effect in this assay. This result recently has been confirmed and similarly, negative results were obtained with metronidazole and tinidazole.

Therefore, the next assessment to be made is whether the dominant mode of action for clotrimazole is immunosuppressive (e.g., inhibition of T-helper cells and CTL) or antiinflammatory (e.g., inhibition of superoxide anion production and of PLA2 activity). For this evaluation we will return to the rat adjuvant arthritis model. Treatment with the drug will only be given in the nonestablished disease phase, e.g., days -1 to +3 around the time of adjuvant sensitization. Antiinflammatory drugs reduce inflammation when administered therapeutically (after established disease), compared to immunosuppressants which will inhibit when administered prophylactically (during the sensitization phase). It is possible for a drug to express both antiinflammatory and immunosuppressant activities by these definitions, and the relative contributions of the two mechanisms to the overall therapeutic efficacy of the drug would require further experimentation.

After completion of the additional studies outlined in this report, I will provide a comprehensive final report to the Rheumatoid Disease Foundation. Furthermore, the results will be submitted for publication to a reputable scientific journal. Since it is the standard in science that new findings first be accepted for publication before any public disclosure is made, I have recently communicated to Perry Chapdelaine my strong belief that results from these interim reports must not be publicized in the meantime. To make premature disclosures is not to abide by proper protocol, and moreover, may make acceptance in a peer-reviewed scientific journal that much more difficult. Perry has expressed his understanding and agreement, and I trust that you concur.

Best wishes for a happy and healthy holiday season.

Sincerely yours,



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