Research Report from Brian Susskind, Ph.D. (11/6/86)

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To: Perry *A.* Chapdelaine, Sr. Executive Director/Secretary
Rheumatoid Disease Foundation

Dear Mr. Chapdelaine:

This will be my report which you requested summarizing our work for the first year of the grant and a progress report on our new initiatives. Already there is more to say about the latter than the former.

We began work on the project in mid-October, 1985. 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 arthritics, and during the past two months from malignant tissues. The procedures followed are detailed in the original grant proposal and in my interim progress report to Dr. Pybus dated 4/7/86. We have been unable to identify or isolate pathogenic free-living amebae from surgical specimens of thirty-four rheumatoid synovia, three breast carcinomas, three colon carcinomas, one primary liver carcinoma, and one melanoma. We have not yet received any lymphosarcoma tissue. Most of these patients are treated by the medical oncologists with chemotherapy. What surgery is warranted, the amount of tissue removed is small and the pathologists have been reluctant to spare any for research purposes. I do not think this is an issue, however, because in his book, *The Causation of Rheumatoid Disease and Many Human Cancers - A New Concept in Medicine*, Roger Wyburn-Mason states that the organism he claims to have isolated was found "from all of a large number of human and animal malignant tissues examined" (page 121), and on page 281, "limax amebae are present in all the tissues of human malignant disease, especially in the solid tumors" (carcinomas).

At present, we are proceeding with studies to evaluate whether the usefulness of clotrimazole in the therapeutic management of rheumatoid arthritis is based on modification of immunologic functions. I think that a brief overview of the immunopathology of rheumatoid arthritis may help to bring into focus the rationale for our approach.

Although the antigenic stimulus that initiates the immune response in rheumatoid arthritis remains unresolved, chronic inflammation clearly underlies the pathogenesis of the disease. The immune response in rheumatoid arthritis involves a complex network of interactions between cells of the immune system - B lymphocytes, T lymphocytes, macrophages, and polymorphonuclear cells, and their products. B lymphocytes give rise to plasma cells, which produce antibodies (e.g., rheumatoid factor). T cells are divided into several functionally distinct subsets. T-helper cells collaborate with B cells in the production of antibodies. Cytotoxic T lymphocytes destroy antigens by contact-mediated lysis, and are also dependent upon T-helper cells. A third subset, T suppressor cells, have as their function to keep immunologic responses under control. Macrophages are required to process and present antigen to the T cells in a form that is immunogenic.

Macrophages contribute to the pathogenesis of rheumatoid arthritis in a variety of other ways. Macrophages and polymorphonuclear cells become activated in response to factors (cytokines) released by the antigen-stimulated T-helper cells, becoming more aggressive in terms of phagocytosis and the release of enzymes and products which inflame the surrounding host tissues. Activation of the complement system by rheumatoid factor results in recruitment of macrophages and polymorphonuclear cells from the blood and also stimulates their activation. Macrophages in the blood are immature (termed monocytes) but mature in the tissues under the influence of the T-helper cells. The mature tissue macrophages produce components of the complement system, thus completing a circuit that serves to amplify the inflammatory process.

Macrophages, B cells, and T cells communicate with each other by a variety of short range chemical messengers, referred to in general as "cytokines." Among the principle cytokines involved in rheumatoid arthritis are Interleukin-1, Interleukin-2, Interleukin-3, and interferon. Macrophages are the source of Interleukin-1, and T-helper cells synthesize the others.

The antigenic stimulus that initiates the immune response is presented by the macrophages along with Interleukin-1 to the T-helper cells. The T-helper cells begin to produce Interleukin-2, a T cell growth factor, and proliferate. Interleukin-3 and interferon from the T-helper cells, in turn, stimulate the further growth and maturation of the monocytes/macrophages, and excite them to greater activity. Interleukin-1 from macrophages and T-helper cell cytokines (Interleukin-2, B cell growth factor and B cell maturation factor) are required for differentiation of B cell into plasma cells. Thus, in my view, it is the macrophage/T-helper cell axis that initiates, amplifies, and perpetuates the inflammatory process by influencing the accumulation and activation of immuno-inflammatory cells within the synovium, the synthesis of rheumatoid factor, activation of complement, and the subsequent production of many other inflammatory products (histamine, prostaglandins, etc.). Furthermore, macrophage-derived Interleukin-1 is an important mediator of the inflammatory process with effects on cells outside of the immune system per se, stimulating, for example, prostaglandin and collagenase production from synoviocytes, endothelial cells, osteoclasts, and chondrocytes, resorption of bone, and the fever response in the hypothalamus. (Interleukin-1 is the "endogenous pyrogen.")

In normal individuals, T-suppressor cells moderate the development and limit the force of the the inflammatory response to antigen so as to minimize damage to "bystander" host tissues. In rheumatoid arthritis, however, the numbers of T-helper cells are elevated in the synovium, while T-suppressor cell numbers are reduced. This suggests that an immunoregulatory defect may result in a chronic, hyperactive T-helper cell response.

Since the chronic inflammatory response in rheumatoid arthritis appears to result from a deregulated immune response, the role of clotrimazole may be to correct the derangement. Levamisole, a related imidazole compound, is a known immune response modifier which affects both T-helper cell and T-suppressor cell functions, and has been demonstrated to be clinically useful in rheumatoid arthritis. Clinical benefits are observed only after several months of treatment, however, which is in contrast to the more rapid effect of clotrimazole avowed by the Rheumatoid Disease Foundation. Attention should therefore be focused on defects within the immunoregulatory pathways that can be brought under control with clotrimazole.

Since the pathogenesis of rheumatoid arthritis involves a complex network of interactions among cells and products of the immune system, there are many sites where the drug could act. Our approach is twofold. First, to isolate segments of the immune system and measure the modification of their responses by the addition of clotrimazole. This is most readily accomplished *in vitro*. Second, *in vivo* studies with the drug using

animal models of rheumatoid arthritis since these reflect the whole interactive system. In this way we will gather evidence that not only may support the Foundation's claim of the efficacy of clotrimazole, but also develop credible hypotheses that might explain the drug's therapeutic mechanism.

We have begun to analyze the effects of clotrimazole in several systems that allow us to assay immune cell functions and interactions. Historically, one of the most useful systems for the *in vitro* study of the immune response has been the generation of cytotoxic T lymphocytes in mixed leukocyte cultures . Mixing leukocytes from one individual (or strain of inbred animals) with irradiated leukocytes from a genetically different individual (and thus "antigenic") results in the generation of cytotoxic T lymphocytes capable of killing cells bearing the incompatible tissue antigens. Development of cytotoxic T lymphocytes requires cooperative interactions with T-helper cells and macrophages, and involves the production of Interleukin-1, Interleukin-2, and interferon.

We have found that clotrimazole inhibits the generation of cytotoxic T lymphocytes in mixed leukocyte cultures at dosages from 10 to 1 micromolar (Figure 1). While plasma level of 10 micromolar clotrimazole would probably have severe side-effects, 1 to 2.5 micromolar concentrations are well within the therapeutic dose range. From the discussion above, it should be apparent that the immuno-suppressive effect of clotrimazole on the cytotoxic T lymphocyte response could be due to a direct action of the drug on the cytotoxic T lymphocytes themselves, or alternatively, upon the T-helper cells or macrophages.

We have assessed the affect of clotrimazole on the T-helper cells. The large majority of cells that undergo proliferation in the mixed leukocyte cultures are the T-helper cells, and it is well established that by assaying the proliferative response of cells in mixed leukocyte cultures, one is measuring T-helper cell activity (this is called a "mixed lymphocyte reaction in the vernacular of the immunologist). As shown in Figure 2, clotrimazole at concentrations down to 0.1 micromolar inhibited the T-helper cell mediated mixed lymphocyte reaction.

Clotrimazole may have its effects on cytotoxic T lymphocytes and T-helper cells by inhibiting the production and/or activity of the various cytokines. We therefore tested whether clotrimazole would interfere with stimulation of primed lymphocytes in the presence of pre-formed cytokines. In contrast to its effects on the generation of a primary cytotoxic T lymphocyte response or T-helper cells activation in a naive leukocyte population, clotrimazole at 1 micromolar does not inhibit the ability of sensitized T cells to react with prefabricated Interleukin-2 (Figure 3). Furthermore, clotrimazole also fails to inhibit the proliferation of leukocytes which utilize Interleukin-3 as a growth factor (Figure 4). We are in the process of testing the effects of clotrimazole on the ability of lymphocytes and macrophages to respond to Interleukin-1 and interferon as well as testing the alternative hypothesis, that clotrimazole affects the synthesis of cytokines. With answers to these questions we will know whether inhibition of the cytotoxic T lymphocyte response and the mixed lymphocyte reaction is exerted at the level of the macrophage, T-helper cell, or possibly both. In summary, physiologic concentrations of clotrimazole have been found to suppress at least two T lymphocyte functions: the generation of cytotoxic T lymphocytes (Figure 1) and the antigenic stimulation of T-helper cells (Figure 2) in mixed leukocyte cultures. The utilization of Interleukin-2 and Interleukin-3 by primed leukocytes was not inhibited. Decreased production of cytokines may contribute to the inhibition of the cytotoxic T lymphocyte and T-helper cell responses, although this is not entirely established by the experiments conducted to date. We also have plans for experiments to determine the effects of clotrimazole on B cell activation, differentiation of monocytes into macrophages, and the synthesis of complement proteins by macrophages.

It could be expected from our data that immuno-suppression of lymphocyte functions is the predominant effect of clotrimazole in rheumatoid arthritis. This would be in accord with the hypothesis that the chronic inflammation in rheumatoid arthritis is due to an inappropriate, hyperactive immune response, and with the observed therapeutic benefits of immuno-suppressive drugs such as corticosteroids, azathioprine, and cyclophosphamide.

An alternative possibility is that activation of T-suppressor cells may be responsible for depression of the cytotoxic T lymphocyte response and mixed lymphocyte reaction. In rheumatoid arthritis, T-suppressor cell function which normally would control inflammation in the affected joints is known to be depressed. If clotrimazole stimulates T-suppressor cell function, the immunologic balance may be restored. We will investigate whether increased activity of T-suppressor cells plays a role in clotrimazole-mediated immuno-suppression.

We have also conducted tests of levamisole, tinidazole and metronidazole in the same systems as clotrimazole (Figures 5 - 8). In short, these compounds either had little or no effect, or a stimulatory effect, at doses of clotrimazole which were significantly immuno-suppressive. Levamisole, in reports where it has been found to have an effect on immunologic responses, is generally an immuno-potentiator. One therefore would not expect its mechanism of action in rheumatoid arthritis to involve blocking the activities of immuno-inflammatory cells. However certain drugs, including levamisole and cyclosporin A (the new "miracle" drug which has had great impact on the success rate of organ transplants and is currently being tested in rheumatoid arthritis), possess condition-dependent immuno-stimulatory and immuno-suppressive activities. Therefore clotrimazole, levamisole, tinidazole and metronidazole may yet be found to subserve similar immuno-modulatory mechanisms in rheumatoid arthritis. Further studies are necessary to determine if clotrimazole exerts modification of the inflammatory response at one or more specific sites, and if it acts as a general immuno-suppressant or as an immuno-potentiator under selective conditions. Hence, the paramount importance of correlating *in vitro* data with an experimental in vivo system in order to determine which effects are relevant to the drug's therapeutic activity. Complete understanding of the clotrimazole's immuno-modulating activities will also lead to the design of more effective protocols.

I think that we are entering an exciting phase in our research. I have endeavored to provide a comprehensive picture of the significance of this line of investigation. Where there are points that require clarification, I will be glad to elaborate further.

Please keep me informed of developments with the Foundation and the prospects for continued support in 1987. I think that

we will delay the animal studies until I hear from you. We can conduct more *in vitro* experiments on limited resources, and there are still questions to be answered using these systems, although the *in vivo* studies ultimately become necessary.

Figure 1: Effect of Clotrimazole on Cytotoxic T Lymphocyte Response

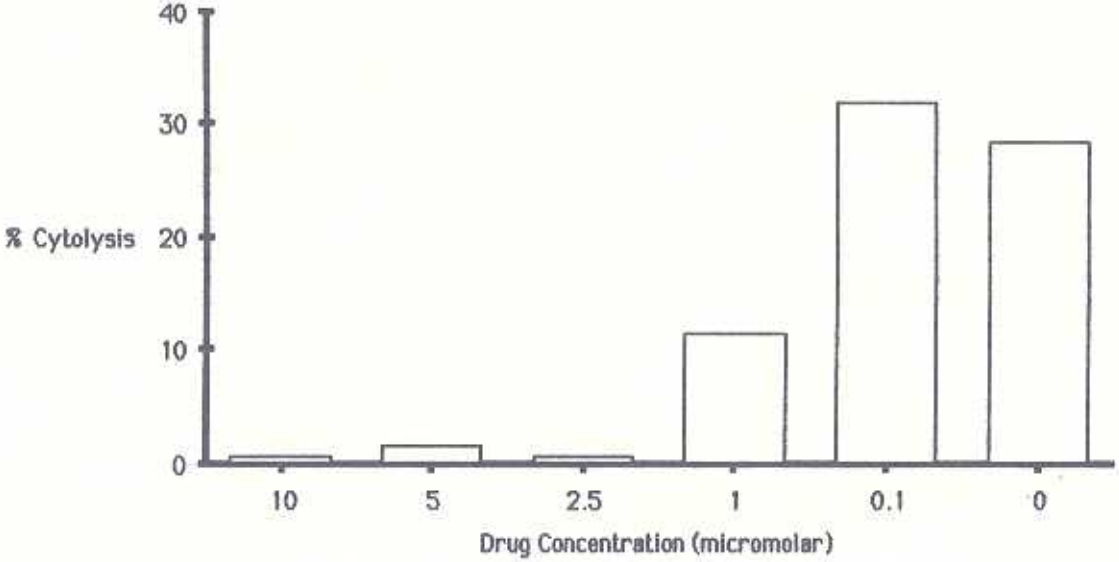

Figure 2: Effect of Clotrimazole on Mixed Lymphocyte Reaction

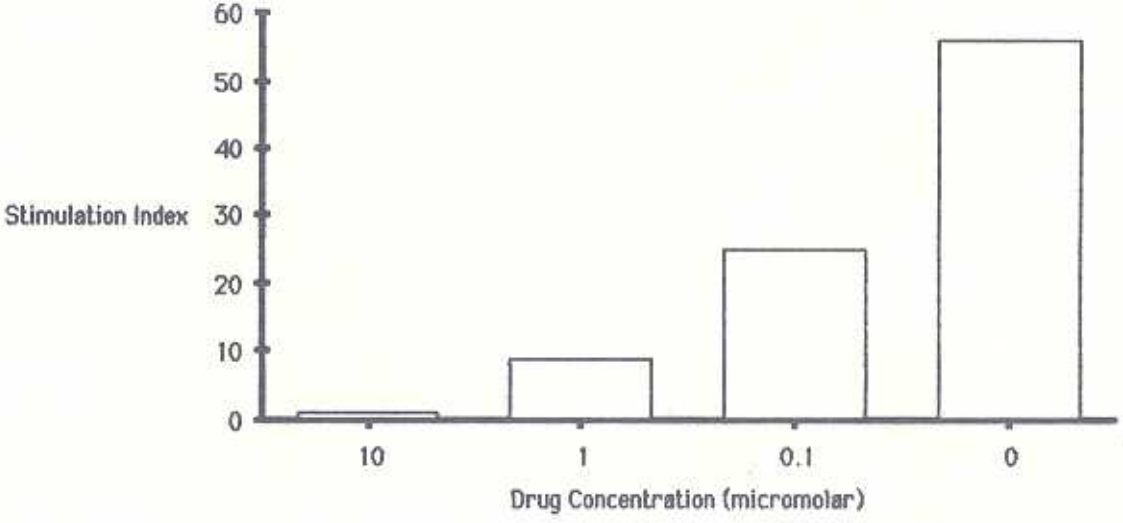

Figure 3: Effect of Clotrimazole on Response to IL-2

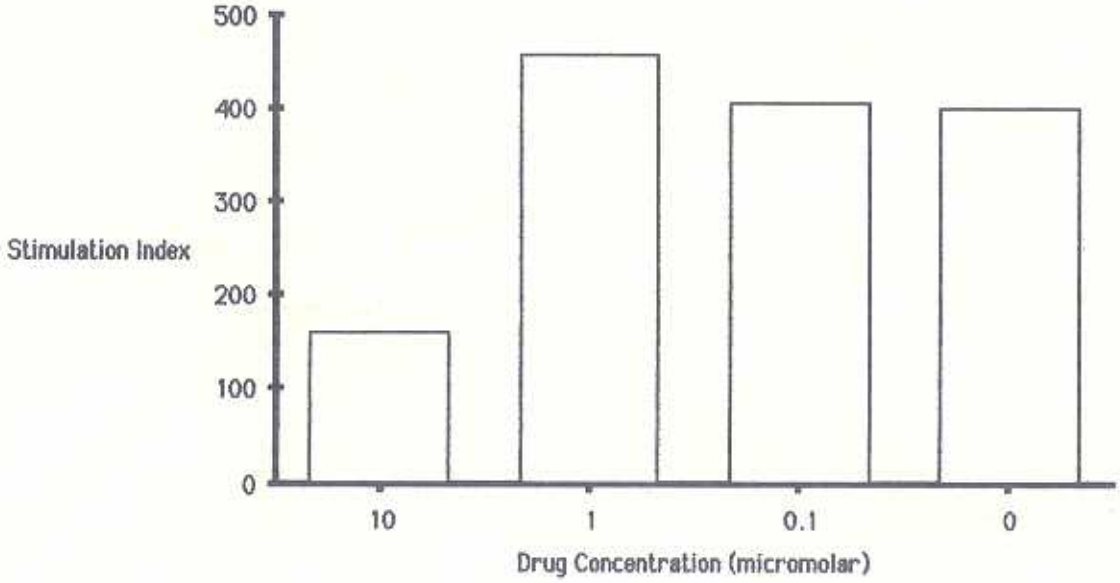

Figure 4: Effect of Clotrimazole on Response to IL-3

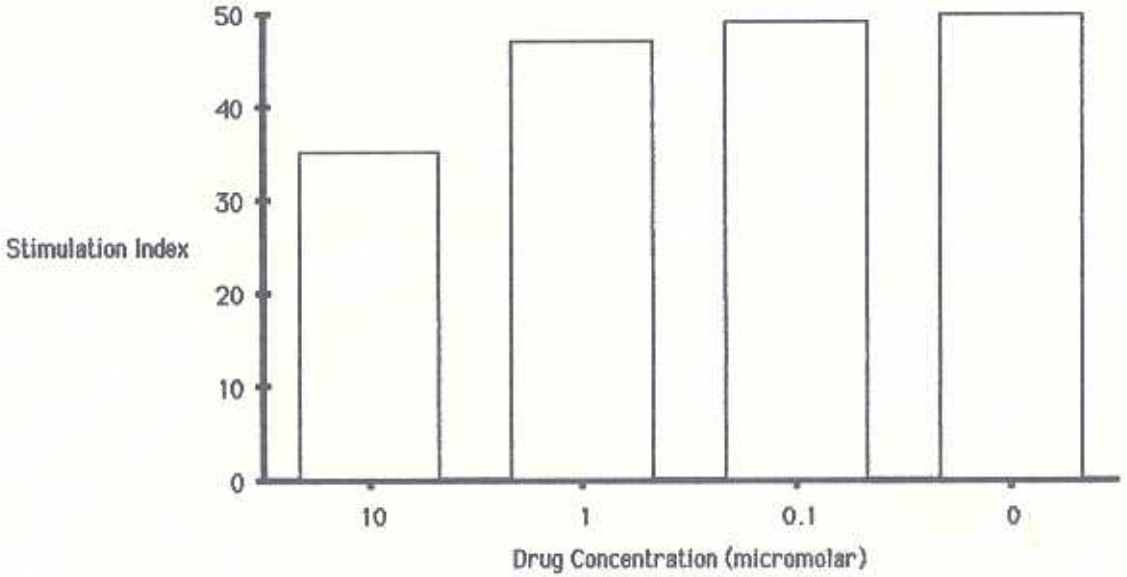

Figure 5:
Cytotoxic T Lymphocyte Response

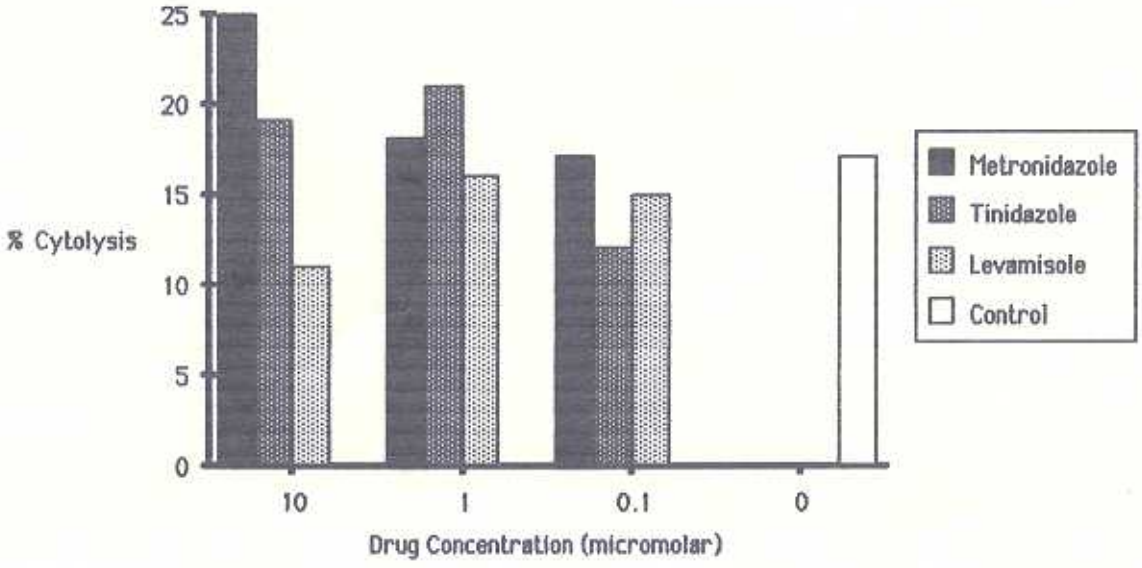

Figure 6:
Mixed Lymphocyte Reaction

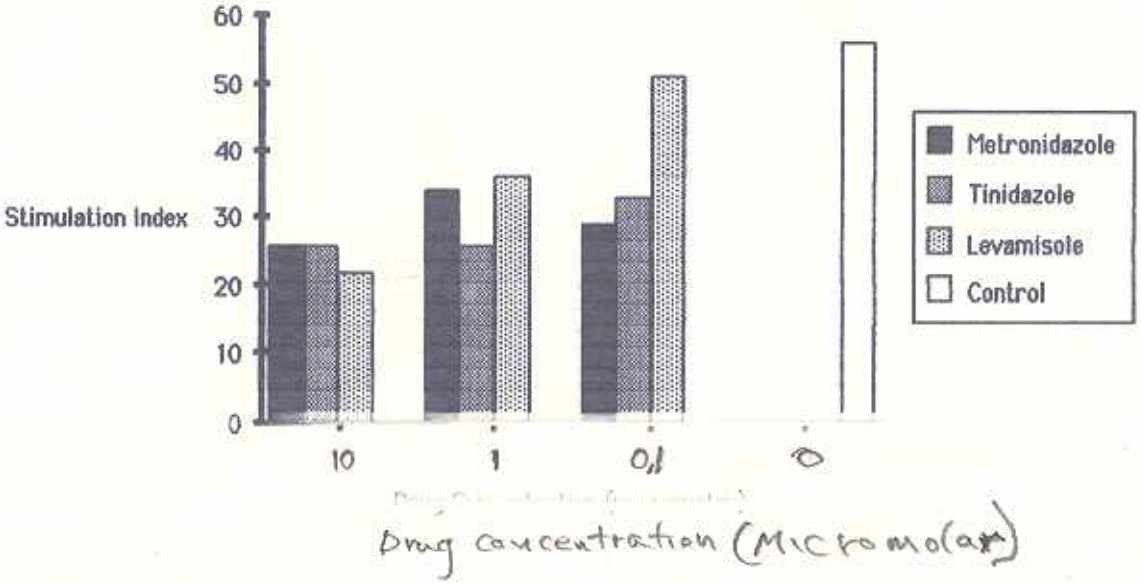

Figure 7: Response to IL-2

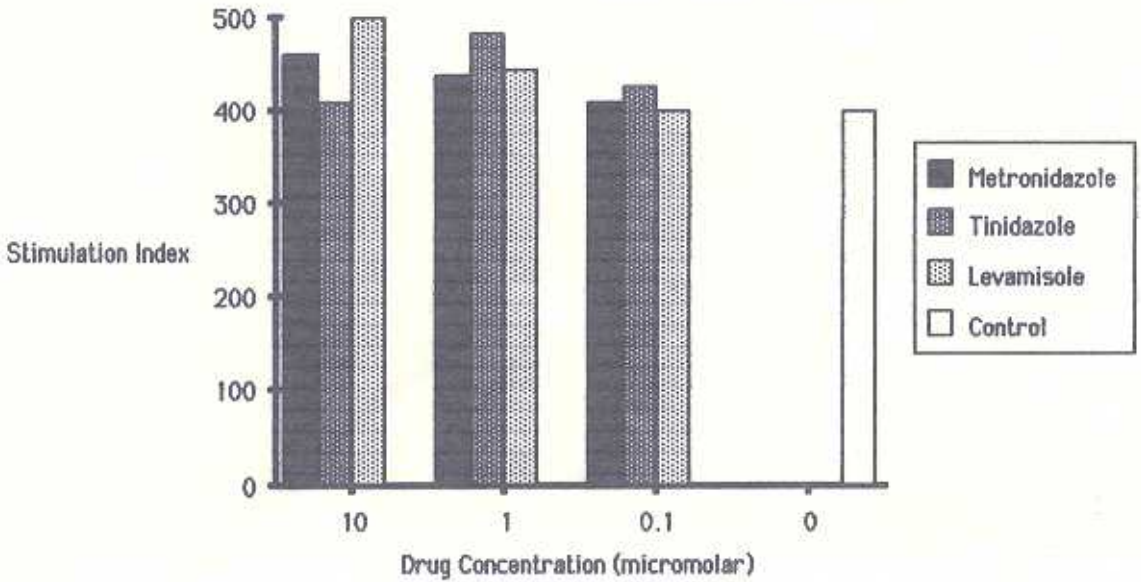

Figure 8:
Response to IL-3