

2/18/87

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Dear Dr. Pybus,

It was a pleasure to hear from you again. Your hypothesis that clotrimazole and metronidazole may differentially affect T cells and macrophages, respectively, is thought-provoking. Apparently somehow I received a false impression that in patients treated with clotrimazole the same apparent cytotoxic effects was observed on macrophages as you had reported for metronidazole. I was motivated to comment because this may be a very important observation, and I think that my remarks are still apropos. To paraphrase myself, I am skeptical to believe at this point that metronidazole is simply cytotoxic for macrophages, primarily because such a generalized toxic effect on macrophages would have profound consequences. Rather, I interpret your observation that fewer macrophages are found in the joint effusions of patients after drug treatment as resulting from effects of metronidazole on the inflammatory process within the synovial tissue, with the consequence that there is a reduced discharge of macrophages from that tissue into the synovial fluid, and not resulting from macrophage toxicity.

Because of the potential importance of this observation we have begun to study the "macrophagicidal" activity of metronidazole. Macrophages were isolated from the peritoneal cavity of mice, and cultured for two days in the presence of various concentrations of metronidazole and clotrimazole, then quantitated. The results were as follows:

Table 1. Effects of Clotrimazole and Metronidazole on Macrophage Viability

Drug	Concentration (μ molar)	% of Control	+/- S.D..
None (control)	-	100%	4%
Solvent	-	101%	3%
Clotrimazole	0.3	102%	1%
Clotrimazole	1.0	102%	6%
Clotrimazole	3.0	96%	9%
Metronidazole	0.3	106%	2%
Metronidazole	1.0	97%	3%
Metronidazole	3.0	87%	3%

As you can see, we observed no significant toxic effects of metronidazole or clotrimazole on macrophages at physiologic doses.

Of course a caveat in conducting in vitro studies with metronidazole is that in the intact animal, metabolism of the drug may be required. Perry Chapdelaine believes that the quality of the bacterial flora is important. On the other hand, metronidazole is useful in the treatment of various parasitic protozoan infections (e.g. *Trichomonas vaginalis*, *Giardia lamblia*, *Entamoeba histolytica*), and is effective in vitro against these cells in the absence of bacteria (Wittner et al . , 1977, *Ann. Trop. Med. and Parasitol.* 64:19-27). We will follow up on your observation further, however, by a variety of means including studies in intact animals.

We have examined clotrimazole for additional effects on macrophages. You may recall that when blood monocytes arrive at a site of tissue inflammation they undergo differentiation into macrophages,

and contribute to the inflammatory response in part by the production of complement proteins. The initiation of synthesis of one particular complement protein, C2, is used as a "marker" of monocyte differentiation into macrophages. Differentiation in the synovium is stimulated by gamma-interferon, produced by helper T cells. We therefore tested clotrimazole for its ability to inhibit C2 synthesis by monocytes stimulated by prefabricated gamma-interferon. As you can see, only the highest concentration of clotrimazole (100×10^{-6} M) inhibited stimulation of C2, a 100-fold higher concentration than that which effects helper T cells.

Table 2. Effect of Clotrimazole on gamma-Interferon Induced C2 Production (Index of Monocyte to Macrophage Differentiation)

Clotrimazole Concentration (micromolar (μ M))	Molecules of C2 Produced per 100,020 Monocytes* ($\times 10^{-8}$)	
0.00	4.23	+/- 0.27
0.03	4.17	+1- 0.27
0.16	4,17	+1- 0.42
0.80	4.1.3	+/- 0.43
4.00	3.46	+/- 0.26
20.00	4.21	+/- 0.26
100.00	2.64	+/- 0.18

*Background C2 production of uninduced monocytes (no gamma-interferon added to medium) was 1.24 +/- 0.14 [Insert Table on C2]

We are in the process of repeating this experiment with metronidazole, tinidazole, and levamisol (Most of our experimental procedures are worked-out initially using only clotrimazole, then repeated with the other drugs.

We are also in the process of testing the effects of clotrimazole on other macrophage functions, most notably their capacity to process and present antigen to T and B cells, and IL-1 production. From your letter it seemed that you were of the impression on we had already studied IL-1 synthesis. We have not, although these experiments will be initiated shortly. Measurement of IL-1 activity involves a complex bioassay, and only recently have we received the indicator cell line which is IL-1 responsive. A previous shipment of these cells from the American Type Culture collection was not viable; therefore we have had to wait for ATCC, to thaw a vial of the cell line in their laboratory, grow them up, and ship them to us.)

Although it would appear from the two sets of experiments conducted on macrophages with clotrimazole (described above) that at least parts of your hypothesis holds up, i.e., that clotrimazole does not affect macrophage functions, I think that these studies are still preliminary and that additional experiments must be conducted before firm conclusions can be reached. Antigen presentation and IL-1 production are two very important macrophage functions in terms of their relevance to chronic inflammation.

We have studied further the immunomodulatory effects of clotrimazole using the mixed leukocyte culture-cytolytic T lymphocyte (MLC-CTL) system detailed previously. Two of the more significant observations are as follows: The first is that clotrimazole is capable of inhibiting at least two stages in CTL development -- both proliferation of naive CTL precursors, and also differentiation of activated CTL precursors subsequent to proliferation. Second, once activation and fully mature, clotrimazole failed to inhibit the cytolytic activity of CTL effector cells. These observations suggest that clotrimazole is effective in suppressing activation of yet unstimulated or immature cells in an area of inflammation (e.g., newly recruited cells in the site), but may not affect the functions of inflammatory cells already in operation.

Our studies in the MLC-CTL system indicate that in vitro clotrimazole has immunosuppressant activities. We have extended our observations on the immunosuppressive effects of clotrimazole to another system, one involving activation of T cells by mitogenic lectins. Mitrogenic lectins, or mitogens, (e.g., Concanavalin A, phytohemagglutinin) are polyclonal activators, stimulating nearly all mature T lymphocytes regardless of their antigenic specificity. Thus, they induce a more generalized [effect] which

activates only those clones of T cells with receptors for the given antigen. Antigen-driven generation of CTL in MLC and mitogen-driven responses show similar requirements for cellular interactions between CTL precursors, helper T cell and macrophages, however. Similar to the results obtained on antigenic (clonal) activation of CTL and helper T cells, polyclonal activation of CTL and helper T cells functions with Concaravalin A was inhibited by clotrimazole (Figures 1 and 2).

Finally we come to your question of the relevance of my observations to those of Dr. Franson's. Lymphokines such as IL-1, IL-2, IL-3, and gamma-interferon are distinct from the prostaglandins and leukotrienes. The former are all proteins, the latter are lipid mediators. Arachidonic acid is the precursor of the wide variety of prostaglandins and leukotrienes. It is derived from membrane phospholipids by the action of phospholipase A (PLA). The prostaglandins and leukotrienes appear to be of considerable importance in the inflammatory process. They are produced by most, if not all, of the inflammatory cells, and many of the anti-inflammatory drugs are known to inhibit events associated with their synthesis. Clotrimazole does so by inhibition of PLA . Much of the actual tissue damage in rheumatoid arthritis is from the infiltrating inflammatory cells. I have previously reviewed for you the contributions of the various cellular and lymphokine components to the pathogenesis of rheumatoid arthritis.

A similarity in Dr. Franson's and my findings is that in either set of processes examined, clotrimazole appears to act at an early stage. It affects the initial step of prostaglandin/leukotriene production by inhibition of PLA2, and the early stages of T lymphocyte activation (induction of CTL, stimulation of lymphokine production), but not the effector functions of T cells and lymphokines (target cell killing CTL, effects of lymphokines such as IL-2 and IL-3 on their "target" cells).

Many investigators are still trying to piece together the complex interactions between prostaglandin/leukotriene, lymphocytes, macrophages and other inflammatory cells. Both systems are an integral part of the inflammatory reaction, and the efficiency of clotrimazole may stem from the fact that it has dual activity. Since the intracellular, biochemical mechanisms of clotrimazole are not entirely understood, however, it is too early to tell whether the effects in both systems are mediated by the action of clotrimazole through a common messenger

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cc: Mr. Perry Chapdelaine, Sr.

I trust that I have satisfactorily covered the issues about which you had inquired and have provided you with further food for thought. I will keep you informed of our progress.

Sincerely yours,

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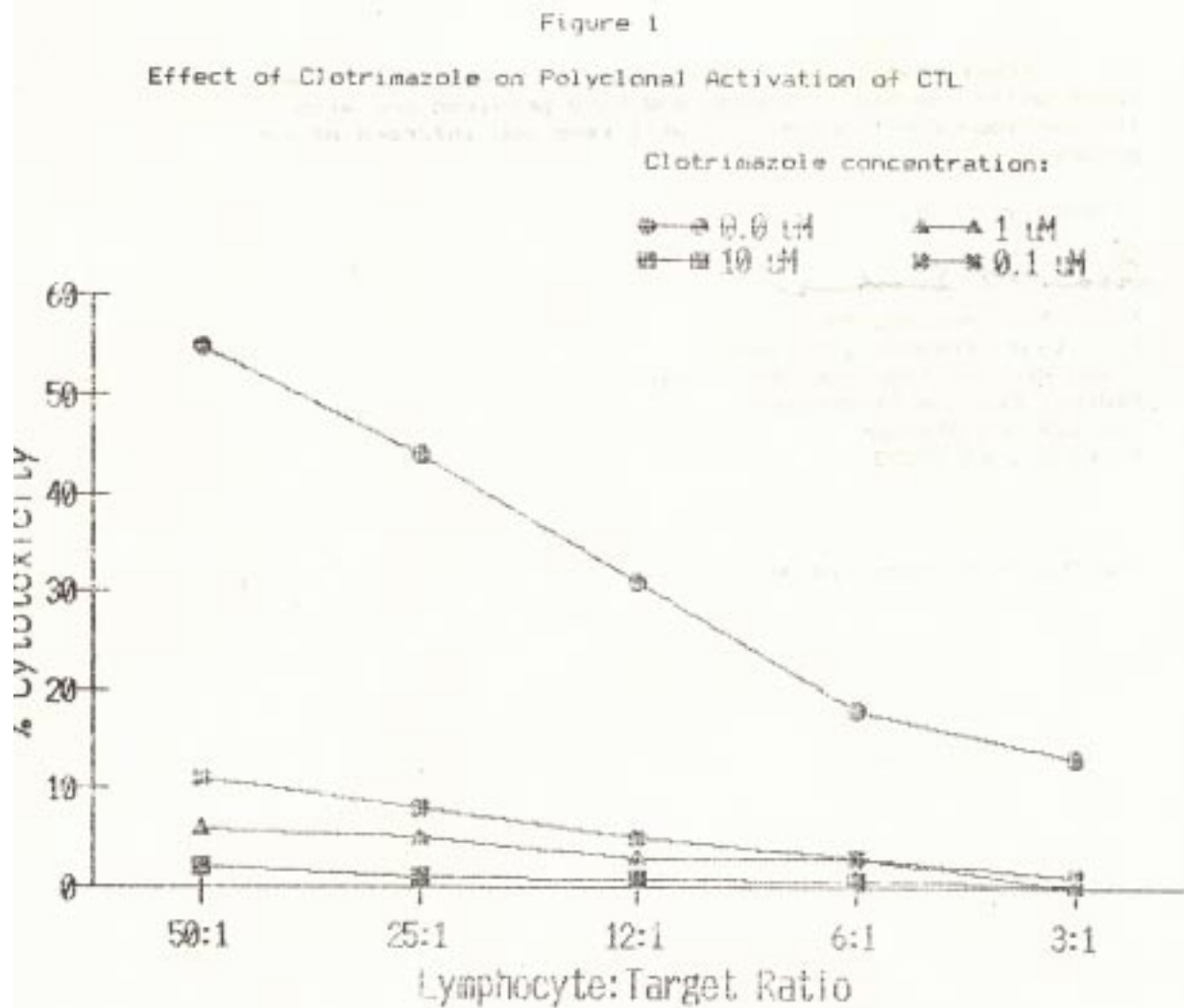


Figure 2

Effect of Clotrimazole on Helper T Cell Polyclonal Proliferation

