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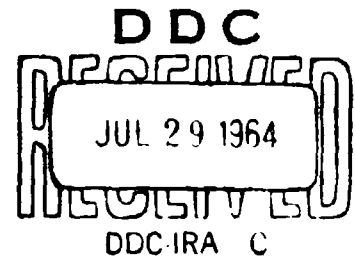
First Annual REPORT

Covering the Period

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Title: Chemical, Ultrastructural and Clinical Studies on  
a Pyrogen Isolated from Leukocytes



Prepared by

Katherine E. Fritz

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The results of the first twelve months of study of leucocytic pyrogen under this contract can be summarized as follows.

In the area of production, isolation, and characterization of the pyrogen, two reproducible techniques for production of crude pyrogen have been developed. The first method includes producing a sterile peritoneal exudate in rabbits by the injection of 250-300 ml. sterile 0.9% NaCl, harvesting this exudate, separating the leucocytes by centrifugation, and stimulating them to release pyrogen by incubation with a bacterial endotoxin. In the second method 100 ml. of human blood is obtained by venipuncture, the erythrocytes sedimented with 3% Dextran, the supernatant separated, the leucocytes recovered by centrifugation, and incubated with bacterial endotoxin. The pyrogenic solutions from either source are then either freeze-dried or dialyzed overnight against 20% polyvinyl-pyrrolidone after which they can be stored as a "stock pile."

These techniques have been applied to more than 200 rabbits and over three liters of human blood to provide the crude pyrogen used in these studies. The crude pyrogen has been purified by application to DEAE and phosphorylated cellulose columns, its pyrogenicity and protein concentration being determined at each step by rabbit assay and chemical analysis.

The purification and biochemical and biophysical characterization of both human and rabbit pyrogen have been approached by column chromatography, electrophoretic and electron microscopic methods. The use of DEAE and phosphorylated cellulose columns as in the purification of rabbit pyrogen has now been extended to the purification of human pyrogen, with promising results. In addition, Sephadex chromatography is being incorporated into the purification procedure. The addition of Stubbings preparative electrophoresis equipment makes possible the accumulation of much larger quantities, relatively, of highly purified pyrogen. At first glance it might seem that these procedures are straightforward and should have been adapted with considerable ease. But the whole purification process has been plagued at every step by contamination with bacterial endotoxins, and separate procedures have had to be developed to circumvent this contaminating pyrogenicity of large amounts of distilled water, DEAE and phosphorylated cellulose columns, Sephadex, and the preparative electrophoresis. For instance, it has been found that Sephadex columns autoclaved at 250°F for 90 minutes are nonpyrogenic.

Using ferritin and ovalbumin conjugated to ponceau red as models, a procedure for separation of proteins on the basis of molecular weight using Sephadex has been developed. Also worked out with these models is a promising technique for eluting a protein from a single band of a cellulose acetate electrophoresis strip, negatively staining it, and examining it in the electron microscope. This may well prove to be an essential tool in the characterization of the most highly purified pyrogen.

The search for an assay animal other than man for response to human pyrogen has been successful. The first animal tried was the laboratory rat and it was found that one strain of rat did respond to large doses of human pyrogen. However, a second strain was found to be unresponsive, and this finding, coupled with the technical difficulties involved in using rats, led to the abandonment of this approach. The rabbit, however, is a relatively easy animal with which to work so seemed a good choice, should it prove responsive to human pyrogen. Massive doses of human pyrogen were found to cause a minimal febrile response in an untreated rabbit. However, intravenous injection of a suspension of trypan blue one day prior to the injection of

human pyrogen is found to enhance this response greatly. The question of whether the rabbit response is due to leucocytic pyrogen or to the very small amount of bacterial endotoxin used to stimulate the human leucocytes in the course of preparation of the crude pyrogen was raised. It has been definitely established by several means, such as the leucocytic pyrogen being inactivated by heating at 100°C for 5 min., the type of febrile response and finally the production of typical response in second-day endotoxin-tolerant rabbits, that human leucocytic pyrogen can be assayed in rabbits whose reticulo-endothelial system has been blocked by injection of trypan blue. However, it must be emphasized that the response of individual rabbits is variable and such assays should be carried out in triplicate.

The relation of the structure of the leucocytic pyrogen molecule to its biologic activity is currently under investigation. It has been found that 1 ml. of a certain crude rabbit pyrogen preparation will elicit a good response in a rabbit. Since previous investigation has shown purified pyrogen to be composed of spherical units (perhaps molecules) of approximately 50 A diameter which exhibit a very decidedly osmiophilic area in the center of the sphere, the possibility that this osmiophilic area may be the site of pyrogenic activity seems worth investigating. The approach used thus far is to add  $\text{OsO}_4$  in varying concentrations to see at what level the pyrogen is no longer active. Correlated with this is electron microscopic study of negatively stained preparations of the pyrogen solutions used for assay.  $\text{OsO}_4$  in  $10^{-7}\text{M}$  concentration does not inhibit the activity of the pyrogen. Electron micrographs of this preparation show that at least some of the pyrogen particles have reacted with  $\text{OsO}_4$  to demonstrate the densely osmiophilic centers.  $10^{-5}\text{M}$   $\text{OsO}_4$  does not inactivate the pyrogen, while with  $10^{-3}\text{M}$   $\text{OsO}_4$  all pyrogenicity is destroyed. Investigation along this line is proceeding with the objective of estimating the  $\text{OsO}_4$  to pyrogen molecular ratio at which pyrogenicity is destroyed.

Work toward the delineation of the site of action of leucocytic pyrogen has been limited to establishment of a procedure for preparation of maraglas embedded hypothalamus sections for electron microscopic study. This was undertaken because there is less tissue damage with this medium, and because it permits microscopic study without the use of a substrate on the supporting grid. Application of this technique to the study of the site of action is dependent on the successful production of an antibody to the pyrogen, and is thus necessarily being deferred until such time as antibody is available.

Immune studies of leucocytic pyrogen and related subjects have met with varying degrees of success. A major accomplishment has been the successful establishment of a tissue culture laboratory in previously undeveloped space. This laboratory is proving to be a tool of major significance.

Less successful have been the varied attempts to produce antipyrogen antibody in rabbits. To date no detectable antibody to human pyrogen has been produced in rabbits, which is attributed to two main causes: the first, a possible chemical similarity to rabbit pyrogen which would render human pyrogen less "foreign" than some other proteins and thus less likely to evoke an antibody response; and second, the extremely small quantity of pyrogen protein available as antigen.

Hoping to circumvent these difficulties, mice are currently being immunized with both rabbit and human pyrogen. Amounts of protein as small as 10  $\mu\text{g}$  have been proven to be antigenic in mice, so it is hoped this method

may prove successful. The possibility of similarity to a mouse leucocytic pyrogen cannot be ruled out until the results of the current attempts at immunization are known.

Of unexpected interest have been the results of studies of virus-like particles in leucocytes from healthy human donors. Leucocytes from fifteen healthy donors have been examined by a negative-staining technique and all have contained some morphologically virus-like particles, varying in size, shape and number. Since it is known that in the few instances in which it has been studied, the protein coat of virus particles is the pyrogenic component, while the nucleoprotein fraction is nonpyrogenic, the finding of virus-like particles in leucocytes from healthy humans is of significance and makes attempts to identify these particles as virus, either by immunologic or by replicative techniques imperative. Moreover, one cannot overlook the broader implications of such a "viral flora" with its possible relationship to leukemia and other forms of malignancy, and the mechanism of immunity.

Much effort has been spent perfecting negative-staining methods for tissue culture cells as well as for peripheral leucocytes and these procedures are now fairly reproducible. Further, leucocytes have been grown in tissue culture repeatedly with variations in techniques to find the most effective method for culture. In the course of this experimental work two findings have emerged. One is that the treatment of a leucocyte suspension with fine magnetic iron particles followed by separation of phagocytic cells by use of a magnet somehow stimulates the growth of leucocytes in culture and the appearance of one type of virus-like particle. Secondly, the addition of  $10^{-8}$  M colchicine to growth medium for three hours stimulates the release of a different type of virus-like particle into the growth medium. Further, similar particles can be demonstrated in cells of a culture and in the pellet from centrifugation of the growth medium from the cell culture at  $10^4,000$  g for 1 hr. Similar particles have been seen in three different cultures made at different times from one donor. Parallel light microscopy studies are being conducted.

For the coming year the production, purification and characterization of pyrogen will continue, extending and amplifying the work reported. The attempts to produce detectable antibody will be continued vigorously. It is planned that in view of the possible significance of the presence of virus-like particles in human leucocytes from healthy donors, greatest emphasis will be placed on investigations of this phenomenon. Leucocytes will be grown in culture, and antibody from known common viruses, labeled with fluorescein or uranium, used in an attempt to identify these particles as virus immunologically. Likewise the growth medium from leucocyte cultures, especially after colchicine will be used as an inoculum to HeLa cells, secondary human amnion and other cell lines in attempts to produce demonstrable infection by particles derived from leucocytes. If particles from the leucocyte cultures of the one donor currently under study can be successfully used to infect other cell lines, the study will be broadened to include other healthy donors. Also a significant quantity of the particles will be grown in order to attempt identification and also to use in pyrogenicity studies. The experience gained this year in the evolution of procedures applicable to this study should be of tremendous help in furthering these objectives.