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An analogy: interferon and endogenous pyrogen

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AN ANALOGY BETWEEN INTERFERON AND ENDOGENOUS PYROGEN

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by

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TABLE OF CONTENTS

AN ANALOGY: INTERFERON AND ENDOGENOUS PYROGEN

Introduction

For a number of years it has been observed that host resistance to the effects of viral infections could be enhanced by gram negative bacteria and their products,[42] particularly endotoxins. (Endotoxins are high molecular weight lipopolysaccharides derived from virtually all gram negative bacteria. Their exact structure and the factors responsible for their extraordinary toxicity remain unidentified.[98]) Specifically, Groupe[55] in 1956 demonstrated that xerosin, an endotoxin-like material from Achromobacter xerosis, could suppress the neurotoxic effect of influenza virus in mice when given intracerebrally before, but not after, inoculation of virus by the same route. Gledhill reported that serum of mice treated with endotoxin present in growth filtrates of Salmonella typhimurium when inoculated into suckling mice increased resistance to ectromelia virus infection.[49] He also demonstrated similar effects with saccharated iron oxide and concluded that stimulation of the reticuloendothelial system was the mechanism involved.[48] Also, Wagner et. al. were able to increase resistance of mice to eastern equine encephalitis and encephalomyocarditis viruses by administration of endotoxin.[153]

More recently, there have appeared in the literature suggestions that the "non-specific" enhanced resistance to virus infections caused by bacterial endotoxins might be due to the production of the low molecular weight protein moiety called interferon. Indeed, Stinebring

and Youngner[138] and Ho[67] reported that bacterial endotoxins do, in fact, induce the release of an interferon-like material *in vivo* in chickens, mice and rabbits.

It has been known for some time that one of the most sensitive indices of endotoxin activity is its pyrogenic effect in laboratory animals.[50] As little as 0.0001μg per kilogram is capable of producing a detectable pyrogenic response in the rabbit.[90] The mechanism by which endotoxins cause fever has been attributed to the release into the circulation of an endogenous pyrogen,[9,125] which can be differentiated from endotoxin and will cause a fever when injected into a recipient animal.

Thus, because of these observations, viz. that endotoxins induce release of both endogenous pyrogen and interferon, it was the proposal of this thesis to investigate a possible relationship between these two entities using a single inducer-host system.

Interferon: A Review

Although the phenomenon of viral interference had been, upto that time, demonstrated by several investigators,[44,99,117,120] prior to 1957, no one identified an active interfering principle distinct from virus material itself or from part of the host's immunologic response. It remained for Isaacs and Lindenmann to achieve some preliminary characterization of such a substance; they called it "the interferon."[84,85] Using heat-inactivated influenza virus and fragments of chick chorio-allantoic membrane, they found a soluble factor was released that induced interference in fresh pieces of chorio-allantoic membrane. Until this time, all interference had been considered to be a direct effect of whole interfering virus or of virus components.[60] The interferon of Isaacs and Lindenmann was stable in the cold but inactivated at 60°C for 1 hour. It was not dialyzable nor was it sedimented by 100,000 g for ½ hour. Interferon produced in response to influenza A was active against homologous virus, Sendai, Newcastle Disease and vaccinia viruses. It was not neutralized by specific anti-serum and did not agglutinate red blood cells. Their astute initial observations have lead to a whole new field of research and a voluminous literature concerning this substance that, it is hoped, might curtail a wide range of virus infections.

Definition:

There has been considerable difficulty in defining exactly what interferon is; one must resort to listing a number of characteristics

which must be met before identifying a substance as interferon. These may include its source and inducing agent, its mechanism of action and its physico-chemical properties. In general, interferon is the name given to a group of anti-viral proteins produced by vertebrate cells in response to a number of inducing agents, including many viruses. It is distinct from virus material and confers resistance against multiplication of viruses in homologous cells.[77] As will be elucidated later, there have been a number of species of interferons released by various inducing agents and these differ somewhat in their properties. It has been suggested, therefore, that interference be looked upon as a cellular function, rather than as an isolated biochemical phenomenon. The remainder of this section will be devoted to a review of the present knowledge on the identity of interferon.

Assay Techniques:

To date there is no biochemical or immunologic assay for interferon. However, there have been a number of different interferon bio-assays developed. The type most widely used measures the degree of inhibition of virus production by cells treated with the interferon preparation before infection. In general, this can be measured by:(1) a decrease in the yield of virus from the treated cells; or (2) the decreased susceptibility of the infected cells to a virus lesion (e.g., a plaque or cytopathogenic effect). Originally, Isaacs and Lindenmann measured the effect of interferon by reduction of influenza virus hemagglutinin titer.[84] This type of assay has largely been replaced by the more

sensitive plaque reduction assay in which the concentration of interferon that will reduce the plaque count by 50% in a tissue culture monolayer is measured.[148,149] In this system a linear relationship exists between the percentage of plaque reduction and the log of the interferon concentration; therefore, it is legitimate to determine the end point of the assay by interpolation. Although this technique is not as sensitive as some, it has been used widely because of its relative simplicity and reproducibility.[137] Another assay technique used by Hitchcock[63] is an adaptation of the Oxford cup technique. Its principle is that the size of a zone of protection afforded by interferon allowed to diffuse through agar over a sheet of virus-infected cells is directly proportional to the concentration of interferon. This technique is not widely used.

A fourth technique described by Sellers and Fitzpatrick[135] is based upon the degree to which cell cultures are protected against cytopathogenic effect as evaluated microscopically. All these techniques are well reviewed in "Techniques in Experimental Virology" (R.J.C. Harris, ed.).[127]

Interferon-Producing Systems:

Soon after interferon production was described in chick cells infected with inactivated influenza virus, many other inducer-cell systems were shown to produce interferon. Ho listed almost 35 different systems in 1962;[77] their range is extremely wide and has been constantly expanding. Most of these systems employ <u>in vitro</u> cell cultures; however, many <u>in vivo</u> models have also been described.[62,63,82]

Viruses as inducers: Viruses demonstrated to induce production of interferon include both RNA and DNA viruses, a wide range of sizes and both cytolytic and tumor viruses. One may justifiably conclude, then, that the production of interferon is a rather general response of cells to virus infection.

The viruses used as interferon inducers include members of the myxovirus,[27,52,75,139,144] arbovirus,[59,64,145] polyoma,[1] measles,[31] enterovirus,[70] vesicular stomatitis,[29] foot-and-mouth disease,[35] and vaccinia virus groups.[47] An equally impressive number of viruses have been shown to be inhibited by interferon and it seems quite likely that no virus will be found to be completely unaffected.[150] (An exception may be the adenoviruses.[23]) Two essential properties of virus-induced interferons are worth noting: (1) interferons induced by different viruses in a single cell system seem to be identical as far as can be determined; and (2) interferons induced by different viruses in a single cell system show no evidence of virus specificity, i.e., they are not most active when tested against the homologous virus.[150]

Much has been written about the relative effectiveness of the various inducers of interferon. In addition to cell type and environmental factors, titers of interferon are influenced by the infectivity, dose, strain and virulence of the inducing virus. The difference in the relative ability of inactivated and virulent viruses to induce interferon is not understood. The original studies[84,85] showed that UV-inactivated influenza virus is a better stimulator of interferon production than heat-inactivated or fully infectious virus. Although there is a lack

of entirely adequate quantitative data, there is suggestive evidence that infectious avirulent viruses stimulate cells to produce more interferon than do virulent strains.[150] Ho and Enders, in 1959, showed that the attenuated strain of poliovirus is a good interferon inducer.[69] Vaccine strains of Newcastle Disease virus are relatively poor interferon inducers in chick embryo cells, although they are more efficient than the virulent variants.[132] Enders states that attenuated measles virus is a better interferon inducer than the virulent strains.[36]

In distinct contrast to the myxoviruses, inactivated arboviruses and enteroviruses are notoriously poor inducers; whereas, under the same conditions the identical infectious viruses elicit a good response.[69,146] Ho and Breinig[68] reported that heat-inactivated Sindbis virus induced no interferon production but "sensitized" cells released greater amounts of interferon when challenged with infectious virus. One explanation offered is that inactivated virus initiates the production of an incomplete interferon, the synthesis and release of which may be affected by the addition of active virus.[77]

It would thus seem that the capacity to induce interferon production is, at least in part, a property of the virus. This is supported by the studies of Thiry[143] who showed that one characteristic of chemically induced mutant strains of NDV is the capacity to induce higher yields of interferon per infectious particle.

In addition to the differences in the amount of interferon which viruses can induce, they also differ in their sensitivity to the antiviral

action of interferon. (It is not known if these two properties, which may help to determine virus virulence, are in any way related.) As an example, Ruiz-Gomez and Isaacs[132] showed that NDV plaque reduction required almost 30 times more interferon than an equivalent inhibition of O'nyong-nyong virus. Also, Herpes simplex virus is much more resistant to the action of interferon than vaccinia or cow pox viruses grown on chick chorion.[80] Viruses which in general are relatively resistant to interferon action include fowl plague, NDV, Herpes simplex, pseudorabies and adenoviruses. (It is interesting to note that some members of this resistant group, especially Herpes and adenoviruses have been associated with long-term chronic infections in man.) Vaccinia, many arboviruses and rhinoviruses seem to be relatively more sensitive.[14,139]

Non-viral inducers of interferon: A variety of non-viral materials have been reported to be effective inducers of interferon in both *in vivo* and *in vitro* systems. These include heterologous animal nucleic acids,[78,131] rickettsiae,[72] bacteria,[161] yeasts,[88] statolon (a polysaccharide derived from *Penicillium stoloniferium*),[95] hellenine (an antibiotic-like material from *Penicillium funiculosum*),[133] cyclohexamide,[162] *Mycoplasma*,[13] phytohemagglutinin,[156] and, of course, endotoxins.[138] It has been theorized that many materials like statolon and hellenine may be effective as inducers because they, like nucleic acids, are polyanionic macromolecules.[95]

Some of these systems are certainly radically different from the classic virus-cell system and are in general a testament to interferon

production being a more general host response to insult. Isaacs drew the analogy that antibody production is a response to a foreign protein, whereas interferon production may be a response to foreign nucleic acids at the cellular level.[81]

Cells involved in interferon production: In vitro, cells from a wide variety of animal species have been shown to produce interferon; these include chickens, ducks, mice, rats, guinea pigs, hamsters, rabbits, ferrets, dogs, sheep, pigs, cows, monkeys and man. There have been essentially no differences noted to date in fibroblastic, leukocytic and epitheloid lines with regard to interferon production or susceptibility. In vivo production has not been so extensively studied and there is no indication that any specific organ acts as a site for interferon production.

It is generally felt that primary and secondary cell cultures are superior to continuous replicating cell lines for use in interferon assay systems.[28,69,86] The tumor cell lines have been thought to be rather ineffectual producers of interferon,[61] but this may be due to the fact that many of these lines are relatively insensitive even to the interferon produced in the same cells. Ho and Enders[132] noted that interferon produced in HeLa cell cultures infected with attenuated Type 2 poliovirus worked well in primary human amnion or human kidney cell cultures but not in infected HeLa cells. Similar findings were reported by Chang in KB cells[28] and for a human amnion cell line by Mayer.[108] However, that the cell susceptibility may be only relative in these continuous lines was pointed out by Cantell[26] in showing that

HeLa cells did show some sensitivity to the action of homologous interferon, although less than that of primary human thyroid cells. These observations led Isaacs et. al.[86] to speculate that this behavior might reflect general differences in metabolism between tumor and normal cells.

Effect of interferon on cells: The effect of interferon on tissue culture cells has been actively investigated in attempt to elucidate the mechanism of action:

(1) Morphological changes: With most tissue culture systems there has not been observed any significant morphologic change in interferon-treated cells. Wagner and Levy[152] found that Eastern equine encephalitis virus-infected chick embryo fibroblasts treated with interferon had normal architecture and nucleic acid distribution. Pretreated cells were seen to undergo apparently normal cell divisions. The only morphologic changes described are in cultures of human amnion cells.[51] Between 48 and 72 hours after treatment with interferon the polygonal cells became fibroblastic in shape. The cells readily reverted to the original epithelioid type after the interferon-containing medium was removed. The same stimulus produced no change in primary human kidney cells or in continuous cell lines derived from human amnion cells.

(2) Growth rate changes: Baron and Isaacs[12] found that cultures of primary human thyroid cells subject to one hundred 50% inhibitory doses of interferon resisted multiplication of vaccinia virus and continued to

grow and divide normally. They formed confluent monolayers at about the same time as untreated cultures. Paucker et. al.,[122] however, reported that treated L cells in suspension did show some growth retardation when exposed to very high titers of interferon. The significance of the contradictory information is not apparent.

(3) Biochemical effects: The work of Levy et. al.[102,103] lends weight to the data that suggest that interferon has little effect on uninfected cells. They found no alteration in the incorporation of labeled precursors into cellular proteins, phospholipid, nucleotides or nucleic acids.

Properties of Interferon

Biological Properties:

Species specificity-Tyrrell in 1959[144] first observed that calf and chick interferons were relatively species specific in that they were much more active in cell cultures of the homologous species. Later the specificity was described between chick and rabbit cells[87] and even for duck and chick cells.[149] Merigan,[112] using a highly purified preparation, has demonstrated that interferon produced in mouse tissue does not inhibit the replication of interferon-sensitive viruses in chick embryo cells and, conversely, that chick interferon is not active in mouse cultures. However, a number of workers have stated that this specificity may not be quite so absolute. Sutton and Tyrrell[139] reported on their work showing that monkey interferon was active in human and calf tissue, although calf interferon failed to manifest any activity in monkey kidney cells. Curiously enough, Sellers and

Fitzpatrick found just the opposite one-way relationship between calf and monkey interferons.[135] Some more recent studies have demonstrated that mouse interferon exhibits about 5% of its anti-viral activity on phylogenetically related rat embryo and hamster embryo cells but none on distantly related monkey testis or chick embryo cells.[22] The data of Paucker[121] seem to have contributed considerably in resolving the conflict. He reported that interferon activity in heterologous cells was reduced to about 3% of that in homologous systems and that this same fraction of interferon-like activity was not neutralized by anti-interferon antibody. Of course, this raises the question of the variability of specificity of interferons with the degree of purification of the preparation. His data suggest that this anti-viral activity in heterologous species may be attributable to viral inhibitors other than interferon. When the most purified interferons available at this time are used, a strong species specificity has been demonstrated.[103]

Antigenicity-Interferon is quite distinct as an antigen from the virus that induces its production.[85] Several groups of investigators have found interferon to be a rather poor antigen, however. When inoculated into rabbits or chickens either alone or with oil adjuvants or after alum precipitation, chick interferon did not induce the production of interferon-neutralizing antibody.[25] Nagano and Kojima[119] found that a series of injections of rabbit interferon into hens, guinea pigs and two groups of rabbits produced no neutralizing antibody; however, a third group of rabbits developed antibodies as measured in

rabbit skin. They later confirmed the positive observation and also found neutralizing substances in the serum of immunized fowl.[119] Furthermore, Paucker and Cantell[123] have found that after prolonged immunization of guinea pigs with mouse interferon a low titer of antibody was produced. It could be demonstrated only by using very dilute preparations of interferon.

Glasgow[82] does not find it surprising that interferon is a poor antigen and likens it to the polypeptide insulin. Insulins produced in different species vary by only a few amino acids and as a result are poor antigens when injected into heterologous species. Interferons, too, may vary only slightly in their structure and fail to be recognized as foreign proteins by a host.

Physico-chemical properties:

Interferon is a non-dialyzable and non-sedimentable protein.[85] Purified preparations have been found to contain no nucleic acid and only a trace of carbohydrate.[148] The protein, glycoprotein or polypeptide nature of interferon is inferred primarily from the fact that its antiviral activity is greatly reduced or abolished by treatment with proteolytic enzymes such as trypsin, pepsin or chymotrypsin.[24,85,148] On the other hand, it is not affected by treatment with ribonuclease, desoxyribonuclease or neuraminidase. Its ultraviolet adsorption spectrum is characteristic of a protein, i.e., maximum absorption at about 280mμ.

It is stable over a wide range of pH (from pH 2-10);[85] this property has been used to great advantage in eliminating infectious

virus particles from interferon preparations. The protein has been characterized as slightly basic with an isoelectric point at about pH 8.0.[112] It is precipitated in the 60% saturated ammonium sulfate fraction[135] and by acetone or ethanol.[25,119] Glasgow and Habel[47] reported that their mouse embryo interferon was inactivated by ether but Chany[28] observed no effect when an interferon preparation derived from KB cells infected with parainfluenza virus was ether-treated.

Generally the interferons have been found to be thermostable; some of the conflicting reports, however, may be due to pH differences and the stabilizing effects of other proteins in crude suspensions. It is stable on storage at -2°C, -10°C and -70°C; and most workers have found only partial loss of activity at 56°C for 30 minutes.[85] Antiviral activity is lost when preparations are heated at 76°C for 1 hour. Although species differences in heat stability do exist, Chany found that his preparations of human interferon were entirely inactivated after heating at 56°C for 30 minutes.[28] Merigan showed that the heat inactivation curve of his highly purified mouse interferon was significantly different from similar preparations of chick cell interferon.[112]

The molecular weight of interferon has been a much-investigated topic. In 1963, Lampson et. al.[96] studied a highly purified preparation of chick interferon and estimated, by means of high-speed centrifugation, that it had a molecular weight of 20,000-34,000. Rotem and Charlwood[130] carried out studies of the molecular weight of chicken, mouse and monkey interferons by means of sedimentation in

sucrose density gradients along with radioactive markers of known molecular weight. They found each to have a molecular weight close to that of lysozyme with limits of 13,000-25,000.

With the discovery of non-viral inducers of interferon, it was found that some of the materials meeting the standard criteria had a wide range of molecular weights from 20,000 to 100,000.[97,113,114,160] At first it was thought that viruses induced the 30,000 MW interferon and that non-viral inducers, such as endotoxin, led to the release of a high molecular weight preformed interferon. However, statolon induces the synthesis of a 30,000 MW interferon in tissue culture,* and the release of a heavy species molecule into the circulation of animals.[13] Additional findings are that the spleens of mice treated with statolon contain a 30,000 MW interferon and phytohemagglutinin induces the formation in white blood cells of an 18,000 MW variety.[13]

Youngner, Hallum and Stinebring prepared the following list of interferon molecular weights obtained by intravenous injection of the various inducing agents into mice:

Stimulus	Molecular Weight by Sephadex G-100 gel Filtration
Viruses:	
NDV	25,000
Bacteria & products:	
Brucella abortus	77,000
	54,000
E.coli endotoxin	89,000
Mold products:	
Statolon (Penicillium)	90,000
Cyclohexamide (Streptomyces)	41,000

*Only light interferons have been detected in tissue culture so far.[67,138]

It would seem, then, that a wide range of molecular sizes can be recovered from animals in response to various stimuli. It is not known whether the interferons of different molecular weights may be related to each other in the sense of polymers of some subunit or by having an active moiety attached to different protein carriers.

It has been observed that endotoxin-induced release of high molecular weight interferon into the serum is not inhibited in mice treated with cyclohexamide to inhibit protein synthesis.[162] The same results have been obtained in rabbits treated with inhibitors of RNA and protein synthesis;[71] whereas, it is inhibited when virus is used as the inducing agent.[66] It would seem reasonable to conclude, therefore, that endotoxin-induced interferon is probably not produced in the same way as virus-induced interferon, i.e., that endotoxin-interferon does not require the synthesis of a new messenger RNA or protein. These results have been interpreted as indicating that the interferon released in animals upon treatment with endotoxin is not newly-formed, but rather preformed in some cells, perhaps the reticulo-endothelial system.

Mechanism of Action:

A number of observations about the action of interferon were made quite soon after its discovery.[85,152] Ho and Enders,[69] Isaacs and Burke,[79] Vilcek,[145] and Wagner,[149] all demonstrated quite conclusively that it did not act directly on the virus. Grossberg and Holland[54] failed to detect any evidence of a block in release of newly synthesized

particles in poliovirus-infected cells treated with interferon. These data, along with evidence from Isaacs' work[25,80] that there was no accumulation of synthesized but unassembled viral components, strongly implied that the assembly and release phases of virus multiplication were unaffected. Moreover, it has been demonstrated that interferon forms a stable complex with cells which cannot be dissociated by washing or even disrupting the cells.[149] This means that interferon must be fixed to and perhaps even metabolized by the cell as a prerequisite to its antiviral action. Therefore, attention was focused on the more challenging processes involved in the intracellular synthesis of viral protein and nucleic acid.

One of the first suggestions concerning the mode of action was that interferon-treated cell cultures showed increased glycolysis, increased lactic acid production and increased oxygen consumption. These observations are similar to those made of cell cultures exposed to dinitrophenol which inhibits or uncouples oxidative phosphorylation. These presumed effects were found to be due to impurities in the crude preparations of interferon, as there was no evidence for this when the experiments were done with purified interferon.[96] Other evidence against the hypothesis was offered by Zemla and Schramek,[163] who were able to show that interferon inhibits the replication of western equine encephalitis virus under anaerobic conditions. Since oxidative phosphorylation does not occur without oxygen, it is unlikely that uncoupling could be the mechanism.

More evidence began to accumulate about the precise site of interferon action. DeSomer[34] demonstrated that interferon inhibited the synthesis of viral RNA and also Grossberg and Holland,[54] Ho,[65] and Mayer et.al.[109] proved that this inhibition was exerted on infectious viral RNA as well as on whole virus. These data confirmed earlier impressions that interferon acts intracellularly and more specifically on the synthesis of viral nucleic acids and protein.

An interesting observation made by Ho[65] is that over a narrow dosage range the inhibitor of virus plaque formation conforms to first-order kinetics. This has been interpreted as implying that perhaps as little as one molecule of interferon is sufficient to render one cell resistant to virus infection.

A series of experiments described by Taylor in 1964[141,142] added much to the attempt to further define the mode of action. She took advantage of the fact that the antibiotic actinomycin D inhibits DNA-dependent RNA synthesis without affecting viral RNA synthesis in cells infected with Semliki Forest Disease virus. In the absence of interferon, actinomycin-treated and infected cells incorporated tritium-labeled adenosine into viral RNA and the virus multiplied normally. When cells were first treated with partially purified interferon for 5 hours and then treated with actinomycin D overnight and infected with virus, the synthesis of viral RNA and the yield of progeny were markedly reduced. However, if the cells were treated with actinomycin before they were exposed to interferon, viral RNA synthesis and viral replication were not

inhibited by interferon. These data have been construed as evidence that interferon acts by inducing cellular synthesis of a new messenger RNA which, in turn, presumably codes for the synthesis of a new cellular protein. It would seem that this interferon-induced protein appears to be the active component in this inhibition of viral RNA synthesis.

These results have been confirmed by Lockhart[106] who also demonstrated that actinomycin can reverse the antiviral action of interferon for a period of 2 to 3 hours after exposure. Additional weight has been lent to these theories by reports that selective inhibitors of protein synthesis, p-fluorophenylalanine[43] and puromycin,[101] also block interferon action. Thus far, no one has isolated the interferon-induced protein that inhibits viral RNA synthesis but the evidence for its existence seems conclusive.

Very recently, Marcus and Salb[107] have published evidence further elucidating the precise site of interferon action. The authors used a cell-free protein synthesizing system, with Sindbis virus RNA as messenger and ribosomes from normal and interferon-treated chick embryo cells. Using polyribosome breakdown (as evidenced by loss or ribosomal RNA absorbancy and labeled viral RNA from the 250 S region) as evidence of messenger RNA readout and protein synthesis in normal and interferon-treated cells, they showed that interferon inhibits virus messenger RNA translation, while messenger from the cell genome is translated normally. They therefore postulate that the inhibition of viral RNA translation constitutes the primary mechanism of action of

viral interference.

Thus it can be seen that although much about interferon remains to be learned, a tremendous amount of information has been accumulated with ten short years of its recognition.

ENDOGENOUS PYROGEN

Introduction

Fever is one of the most common signs of illness, but until quite recently little has been understood about its pathogenesis. In the mid-nineteenth century pyrogenic agents were found in pus and necrotizing tissues.[19] Later, Hort and Penfold, while studying "injection fevers" found that the pyrogenic agents were in fact bacterial cell contaminants.[73] The most potent of these pyrogens were shown to be soluble materials associated with gram negative bacteria,[134] and for some time it was taken for granted that most clinical fevers were directly related to these pyrogens. The concept of pyrogens of endogenous origin was reintroduced by Menkin who found a "pyrexin" in inflammatory exudates.[110,111] However, his material was later shown to have characteristics identical with bacterial endotoxin and probably was due to gram negative bacterial endotoxin contamination.

The existence of a pyrogen truly endogenous to an animal was first demonstrated by Beeson[15] in polymorphonuclear leukocytes obtained from sterile saline-induced peritoneal exudates. The cells were lysed by shaking with glass beads and the supernatants were shown to be pyrogenic when injected intravenously into rabbits. This pyrogen was clearly different from endotoxin in its heat lability, rapidity of action and the absence of tolerance to repeated injection. However, in screening other rabbit tissues,

Bennett and Beeson[17] were unable to find evidence of pyrogen except in those containing large numbers of leukocytes.

Much of the experimental fever work has been done with gram negative bacterial endotoxins, lipopolysaccharides of about one million molecular weight.[98] It has been quite well demonstrated that the pyrogenic effects of endotoxins are mediated in large part through the release of an endogenous pyrogen, presumably from circulating granulocytes.[17] The release of this pyrogenic substance has also been demonstrated in vitro.

Endotoxins when given intravenously characteristically induce a fever with a latent period of 20-30 minutes.[158,159] When sufficient endotoxin is given, the response is a biphasic fever with peaks at one and three hours.[50] The response of circulating leukocytes varies with the dose of endotoxin: small doses evoke no change or only a progressive leukocytosis without an initial leukopenia; larger doses, however, cause an initial leukopenia during the latency period, followed by a leukocytosis.[8]

Several other experimental fever systems have been shown to involve the release of an endogenous pyrogen:

(1) Gram positive bacteria do not possess the lipopolysaccharide endotoxins that are found in virtually all gram negative organisms.[154] It was thought from very early experiments that gram positive bacteria were not immediately pyrogenic when given intravenously. A fever appeared only after several hours delay when an infection was established.[74] This was shown not to be the case, however, by Atkins and

Freedman in 1963. Large numbers of autoclaved gram positive organisms given intravenously caused biphasic fevers after a latent period of 45-60 minutes. This delay period is significantly longer than that for endotoxins given by the same route. Streptococci have also been shown to contain several non-infectious pyrogenic materials.[30,154]

It should be pointed out that intravenous injection of a number of relatively inert materials also have produced fever with many of the characteristics of gram positive bacteria. These include dextran, methylcellulose, calcium phosphate, sulfur, kaolin, quartz, thorium dioxide, iron oxide and gold.[8]

(2) Viruses have been shown to produce fever in rabbits when injected intravenously.[6,151] The fever associated with virus is somewhat different from endotoxin and gram positive bacteria-induced fever. It has a latent period of between one and two hours rather than 20-30 minutes,[6,151] and is associated with a lymphopenia rather than a granulocytic response.[58] The pyrogenic property of the myxoviruses is abolished by incubation with specific immune serum,[6,151] whereas homologous antibody does not affect endotoxin's ability to produce a fever. It seems probable, then, that the initial mechanism of fever production by virus differs in some basic way from endotoxin fever.

Several investigators have shown that the mediator of virus-induced fever is a pyrogen with the characteristics of the leukocytic

and endogenous pyrogens associated with other systems.[6,93] Recently Atkins et. al.[4] have demonstrated that a myxovirus can cause release of an endogenous pyrogen when mixed in vitro with whole rabbit blood. Rabbit alveolar macrophages have also been shown to release pyrogen in response to whole virus and to a polysaccharide extract of the virus.[3] Although a relatively few viruses have been tested for pyrogenicity, Coxsackie virus has been the only one beside the myxovirus group to induce fever.[92,93]

(3) Another experimental fever system shown to involve the release of endogenous pyrogen encompasses several immunologic mechanisms. Farr et. al.[38,39,40] used repeated intravenous injections of bovine serum albumin to sensitize rabbits to respond with a fever to subsequent challenge with the protein. The febrile response was biphasic and accompanied by leukopenia but differed from endotoxin fever in its longer latency period. Circulating antibodies were shown to be involved in the release of the endogenous pyrogen by passive transfer experiments. Lymphocytes transferred from sensitized to normal animals did not mediate the response.[53] It is probably a fair conclusion that an antigen-antibody reaction causes host cells to release endogenous pyrogen into the blood stream.

Delayed hypersensitivity has also been shown to be a mechanism that releases endogenous pyrogen. Rabbits which have been infected

with BCG vaccine have significant febrile reactions to intravenous injections of old tuberculin.[57] The reaction involves an early granulocytopenia followed by a prolonged lymphopenia which is a characteristic reaction of delayed hypersensitivity.[8] This type of fever is distinguished from endotoxin fever by its latency of nearly one hour. Atkins and Heijn[5] further demonstrated that tuberculin releases endogenous pyrogen in vitro from blood leukocytes of sensitized rabbits, but not from lymph node and spleen cells. Since normal blood cells, incubated in plasma of sensitized donors, were also activated, it was postulated that circulating antibodies sensitize cells (presumably granulocytes) to release endogenous pyrogen on contact with tuberculin.

Fevers have also been shown to be produced with products of other microorganisms. Culture filtrates of Staphylococcus aureus[2] and of several of the pathogenic fungi[21] have been shown to induce febrile responses in normal rabbits. Atkins postulates that these responses are due to sensitization by undetected previous infections of the "normal" animals by homologous or cross-reacting organisms. Bodel and Atkins[20] showed that this phenomenon can be passively transferred with lymphocytes but not serum, and therefore presumably is due to delayed hypersensitivity.

Endogenous Pyrogens

As mentioned previously polymorphonuclear leukocytes in sterile saline-induced peritoneal exudates were first shown by Beeson[15] to release endogenous pyrogen. Subsequent work by several investigators has shown that normal blood leukocytes, both of rabbits[89] and man,[41] contain essentially no pre-formed pyrogen. Leukocytes release pyrogen only in response to a number of stimuli as previously described. It had been thought for some time that only granulocytic leukocytes were involved in the release of endogenous pyrogen. However, recently Atkins has demonstrated that mononuclear macrophages which line the respiratory tree of rabbits can be stimulated to release pyrogen by several agents.[3]

Endogenous pyrogen is characterized by the rapidity of onset of fever production when it is injected intravenously. Unlike other pyrogens that produce fever only after a variable period of latency, endogenous pyrogen produces an immediate, quickly rising fever with a 5-10 minute lag. Another characteristic is the failure of an animal to develop significant tolerance to repeated injections of endogenous pyrogen. This is in distinct contrast to most exogenous pyrogens.[8] Atkins and Huang,[6] however, described a very interesting phenomenon that may be related to tolerance. Large doses of leukocytic pyrogen produce an immediate, biphasic fever with peaks at one and three hours. Repeated injections of such large doses leads to the eventual disappearance of the second fever peak but never the first.

Atkins believes that this may be explained as a direct action on the thermoregulatory centers causing the first peak, while the pyrogen causes the release of the recipient animal's own endogenous pyrogen to cause the second fever peak. The mechanism of partial tolerance seems to involve only the release of pyrogen from the recipient's cells.[116]

Species specificity:

The question of species specificity has been raised with regard to endogenous pyrogens, although little quantative data is available. Petersdorf and Bennett in 1957[124] described their experiments with sterile peritoneal exudates from dogs and rabbits. The exudates contained pyrogens that were identical to previously described leukocytic pyrogens in that they produced immediate fevers in homologous animals. Of note in their early experiments is that, although the exudate pyrogen was quite active in the homologous animal, it was entirely inactive in a heterologous system, i.e., rabbit pyrogen was not active in dogs, nor was canine pyrogen active in rabbits. Their conclusion was that species specificity might be an important character of endogenous pyrogens. Later experiments by the same investigators show that canine serum endogenous pyrogen does cause prompt monophasic fevers in rabbits.[157] However, since they could not demonstrate a dose-related response, they concluded again that the pyrogens are species specific. It would seem that this is not necessarily the most logical conclusion from their experimental results as published.

More recently Bodel and Atkins[20] have shown that human blood leukocytes, after incubation with endotoxin derived from Proteus vulgaris or with heat-killed Staphylococcus albus cells, release a potent pyrogen that produces fever in rabbits and is distinct from contaminating bacterial pyrogen.

Thus, the question of species specificity is not resolved at this time and awaits the outcome of more extensive experiments.

Naturally occuring pyrogens:

Soon after the discovery of granulocytic pyrogen, Bennett and Beeson[17] reported on their efforts to find pyrogen occuring in rabbit tissues other than leukocytes. In short, they were unable to find evidence of any pyrogenic material in extracts of normal or infarcted kidney, spleen, heart or lung. Likewise, no febrile response could be elicited from extracts of erythrocytes, lymphocytes or macrophages from peritoneal exudates. Extracts of acute inflammatory cutaneous Shwartzman and Arthus lesions produced fever when injected intravenously. The authors postulated that the large number of polymorphonuclear leukocytes in these reactions could explain the fevers observed.

Later, Snell and Atkins[136] attempted again to demonstrate an endogenous pyrogen in normal rabbit tissues, largely because there are clinical and experimental fevers in which granulocytes do not seem to be implicated. Indeed, by using larger quantities of tissue extracts than had been used before, they found that skeletal and abdominal muscle, diaphragm, liver, kidney, heart, lung and spleen

all yielded detectable pyrogen which in all but one case (kidney) produced fevers much like granulocytic pyrogen. These responses were clearly shown not to be attributable to endotoxin contaminants and most unlikely due to sequestered granulocytes in the homogenates.

Physico-chemical properties:

Because exact quantitation is difficult by the bioassay for endogenous pyrogen, purification and biochemical characterization has been quite difficult. Rafter et. al.[129] in 1960 described some characteristics of the pyrogenic component of sterile rabbit peritoneal exudates. It was shown to be a non-dialyzable protein which is precipitated by perchloric acid and extractable with phenol; it is soluble in 50% methanol and 33% saturated ammonium sulfate. At that time they had achieved approximately 50-fold purification by a combination of chemical and chromatographic techniques. This partially purified material contained less than 1% carbohydrate, was resistant to periodate oxidation and was unaffected by butanol extraction. They also showed that this material contained at least two components when tested by immunophoresis in Ouchterlony gel plates.

In some later studies, Hadley et. al.[56] described their pyrogen obtained from serum of rabbits treated with intravenous Newcastle Disease virus. They achieved a 25-fold purification of the serum pyrogen by successive acid and ethanol precipitations. It was found to be non-dialyzable and at least partially protein in nature in that it

was destroyed by pepsin and trypsin. It was extractable by phenol but not by butanol and partially inactivated by periodate oxidation. As can be seen, this virus-induced pyrogen has some characteristics in common with Rafter's granulocytic pyrogen, but because of the relatively crude preparations, no more exacting comparison can be drawn.

Recently, Rafter et. al.[128] have reported on their further modified technique of purification and some further characterization of the leukocytic pyrogen. It appears to be a lipo-protein complex having a molecular weight between 10,000 and 20,000 by sucrose gradient centrifugation. They offer several lines of evidence that it contains an essential lipid component: (1) inactivation by cuprous ions; (2) lability in solutions of pH 8.5 and above: and (3) loss of pyrogenicity after extraction with acid-isooctane. Its solubility in 66% methanol and the enhancing action of ethanol in freeing it from sonicated cells are cited as evidence of the presence of exposed lipid groups at its surface. The authors believe that the demonstration of an essential lipid component adds weight to the hypothesis that leukocytic pyrogen is derived from cellular membranes.

Atkins has shown that protein synthesis is necessary for the action of endogenous pyrogen.[3] However, essentially nothing else is known about its mechanism of action. The site of action has been presumed to be the brain since the response to intracarotid injection is greater than to intravenous injection.[94]

MATERIALS AND METHODS

Interferon

Virus Strains: A stock of a rabbit kidney cell-adapted Sindbis virus (Egypt AR-339) was obtained by inoculation of 0.1cc of a 10^{-1} dilution of the original stock onto 5-day monolayer cultures of chick embryo fibroblasts. The tissue culture medium was harvested after 48 hours and made up to a 10% solution of bovine serum albumin. The stock originally titered to 10^{9} $TCID_{50}$ per ml. on tube cultures of primary rabbit kidney cells. The virus was stored in 1 ml. aliquots at -70°C until used.

Vesicular stomatitis virus (Indiana) was prepared by inoculating 0.1 ml. of a 10^{-2} dilution of the original virus suspension intracerebrally into suckling mice. The mice brains were harvested in 48 hours and homogenized in a solution of 10% bovine serum albumin. The virus was distributed into 1 ml. aliquots and stored at -70°C until used. The original titer of the virus was 10^{8} $TCID_{50}$ per ml. on tube cultures of primary rabbit kidney cells.

Tissue Culture Media: Two types of tissue culture media were used:

"Hanks' Growth Medium"- Hanks' salt solution base with 0.5% lactalbumin hydrolysate, 5% fetal calf serum, phenol red indicator and approximately 200 units of penicillin and 200 μg of streptomycin per ml. Final pH 7.6-7.8.

"Earle's Maintenance Medium"- Earle's salt solution base with 0.5% lactalbumin hydrolysate, 5% calf serum, phenol red indicator and approximately 200 units of penicillin and 200 μ g of streptomycin per ml. Final pH 7.6-7.8.

Overlay medium- 0.5% lactalbumin hydrolysate in Hanks' balanced salt solution, 5% calf serum, sodium bicarbonate, 1.5% agar (Special Agar-Noble, Difco Labs.), antibiotics, and neutral red (1.5% of a 1:1000 solution per 100 ml.)

Primary rabbit kidney tissue cultures: Two to 3 kg. white New Zealand rabbits were killed by air embolism and the kidneys were removed asceptically. The capsules were removed and the kidneys were washed in phosphate-buffered saline. The cortices of the kidneys were cut off and minced into small fragments. The tissue was transferred to a 125 ml. trypsinizing flask and 50 ml. of pre-warmed 0.25% trypsin (Flow Laboratories) in Earle's balanced salt solution was added to the flask. The material was stirred at a moderate speed by a magnetic stirrer for approximately 5 minutes. The supernatant cell suspension was decanted into an Erlenmeyer flask containing 50 ml. of chilled Hanks' balanced salts solution and kept cold. The trypsinization was repeated until the kidney tissue was exhausted; this usually required about 4-5 aliquots of trypsin.

The cell suspension was then centrifuged in 200 ml. centrifuge bottles at about 1000 RPM in an International PR2 centrifuge at 4°C. The supernatant solution was decanted and the packed cells were

resuspended in 50 ml. of Hanks' growth medium. The cell suspension was filtered through 6 layers of sterile gauze and a cell count was done on the resulting suspension. The cell concentration was adjusted to 4 X 10^5 cells per ml. and dispensed (1 ml. into 16 mm culture tubes; 10 ml. into 3 oz. prescription bottles).

Three days after seeding, the original medium was replaced with an equal volume of Hanks' growth medium. This was done earlier if the medium became acid. Monolayers were usually formed in 7 to 10 days and were maintained until used with Earle's maintenance medium.

Interference Assays: Two types of interference assays were used, CPE-reduction in tube cultures and a plaque reduction method in the prescription bottles.

(1) Tube cultures of primary rabbit kidney cells were prepared as outlined above. The solution to be tested for interferon activity was freed of inducing virus in one of two ways. The sample was heated for 1 hour at 56°C and then centrifuged to removed precipitated matter; or it was dialyzed against 0.2 M KCl-HCl buffer at pH 2.0 for 24 hours at 4°C. The pH was readjusted to 7.4 by re-dialyzing against Earle's balanced salts solution for an additional 12 hours. These methods have both been demonstrated to inactivate the viruses used but not the interferon.[64,104] The former was usually used because of its simplicity and brevity.

Serial 2-fold dilutions were made of the solution to be tested. The maintenance medium was then decanted from the tube cultures and

0.5 ml. of the tested material was added to each tube. The material was incubated at 37°C for 8 to 12 hours and then a challenging dose of 1000 $TCID_{50}$ of Sindbis virus in 1 ml. was added and allowed to adsorb for 4 hours. Assays were run in quadruplicate. Controls consisted of using 1 ml. of Earle's maintenance medium instead of the virus. After the virus adsorption 1.0 ml. Earle's maintenance medium was added and the cultures were incubated at 37°C. They were checked at 12 hour intervals for the development of cytopathogenic effect, and the titer of interferon was considered to be the highest dilution at which 50% of the cultures showed inhibition of CPE as interpolated by the Reed-Muench method.[100]

Because the plaque reduction type of assay has been found to be a more sensitive indicator of interferon activity than the CPE reduction method, it was also tested. Confluent monolayers of the rabbit kidney cells in 3 oz. prescription bottles were usually attained 10 days after seeding. Here, too, 2-fold serial dilutions of the material to be tested were made and 0.5 ml. aliquots of the solution were incubated at 37°C for 8 to 12 hours with the cell sheets. A challenge dose of 30-100 PFU of the Sindbis virus was added to each bottle and allowed to adsorb for 4 hours. The cultures were then overlaid with plaquing medium and incubated upside down in a totally dark environment. The highest dilution of the test material which reduced the number of plaques to 50% of the inoculum as determined by controls was to be considered to be the titer of interferon. The plaquing technique did not work, probably because the concentration of neutral red was toxic for the cells.

Endogenous Pyrogen

General: Pyrogenicity assays were done in rabbits in order to be able best to compare interferon and endogenous pyrogen in a homologous system.

Male and female albino rabbits weighing 3-5 kg. were used both as donors and recipients of materials to be assayed for pyrogenicity.[57] They were all caged individually in an air-conditioned room and temperatures were recorded in an adjacent room maintained at 65 to 70° F.

All glassware and needles were sterilized and made pyrogen-free by dry heat at 170°C for 2 hours. Commercial pyrogen-free Cutter physiologic saline solution was used throughout. All materials were given to the rabbits intravenously by the marginal ear veins.

Temperature recording: Temperatures were recorded with rectal thermisters with a Foxboro scanning switch fever recorder. Rabbits were restrained in metal or wooden stalls with openings for the head and tail. Before use, rabbits were trained by being restrained for at least one 5 hour period. They were not given food or water during an experiment. Only rabbits whose temperature did not exceed 40.5° C and did not vary by more than o.3° C during the hour before injection were used. Temperatures were recorded every 15 minutes for the duration of the experiment. Some animals that had remained afebrile or had rapidly regained a normal temperature were given a second dose if they remained stable for at least 1 hour.

EXPERIMENTAL AND DISCUSSION

It must be stated at the outset that a satisfactory series of experiments were never completed to resolve the questions originally posed. However, an explanation of what was attempted and why the experiments were not successful will be presented in this section.

The general experimental approach in assessing a possible relationship between viral interference and endogenous pyrogen was to compare these two phenomena in a single host system so that any conclusions drawn would be unaffected by questions of species specificity. For this reason, and the fact that a well-controlled pyrogen assay was readily available, the rabbit was chosen as the source of host cells. It was proposed to do reciprocal experiments, i.e., to test material known to contain interferon for its pyrogenicity and to test material shown to contain endogenous pyrogen for its interfering properties.

Ho[66] describes a reliable assay using primary rabbit kidney tissue cultures with vesicular stomatitis and Sindbis viruses as inducing and assay virus, respectively. It was thought that this would be a suitable system to employ in investigating this problem.

Two major experiments were attempted:

(1) Monolayer cultures of the rabbit kidney cells in 3 oz. prescription bottles (approximately 10^6 cells per bottle) were washed twice with 10 cc of the pyrogen-free tissue culture medium. They

were then infected with 0.2 ml. of a 10^{-1} dilution of the VSV stock which was allowed to adsorb for 30 minutes. Ten ml. of the pyrogen-free medium was added and the cultures were incubated at 37°C for 12 hours. At that time about 50% of the cells were observed to have detached from the glass in both uninoculated control bottles and infected cultures. There was no sign of any specific cytopathogenic effect. The medium was decanted and the virus was heat-inactivated as described earlier. It was then clarified by low-speed centrifugation and injected into rabbits to test for pyrogenicity. Both samples from uninfected controls and infected cultures were found to elicit no febrile response in the recipient rabbits.

When this material was assayed for interferon by the tube dilution technique using Sindbis as a challenge virus, no interference was demonstrable.

(2) An attempt was made to assay material known to contain endogenous pyrogen for virus interfering activity. Rabbit alveolar macrophages were suspended in a phosphate-buffered balanced salt solution at pH 7.2. They were incubated overnight at 37°C with purified tuberculin protein. The cellular material was removed by centrifugation and the supernatant was demonstrated to cause an immediate monophasic fever in rabbits.

Serial 2-fold dilutions were made of this supernatant and 0.5 ml. aliquots were added in replicates to tube cultures of the

rabbit kidney cells after the medium was decanted. These were then incubated for 8 hours and then challenged with 1000 $TCID_{50}$ of the VS virus (Indiana) in 0.1 ml. The virus was allowed to adsorb for 30 minutes after which 1.0 ml. maintenance medium was added. There was no interference demonstrable during the ensuing 3 day period. See Table I.

Table I. Interferon Assay: CPE in rabbit kidney cell cultures challenged with 1000 $TCID_{50}$ VSV (Indiana) read on scale 1 to 4.

Material assayed	Control	Undil.	1:5	1:10	1:20	1:40	1:80
8 hr	0	0	0	0	0	0	0
16 hr	0	0	0	0	0	0	0
24 hr	1	2	1	0/1	1	2	1
48 hr	3	3	3	3	3	3	3
72 hr	4	4	3	4	4	4	4

On many attempts, the major difficulty lay in trying to establish a positive interferon assay. The first problem encountered was with the cell cultures themselves. Early in the course of experimentation the cells were found to grow quite slowly compared to other cell systems and the shortest time in which monolayer cultures were achieved was 10 days. At this time, the cell layer was confluent but rather

thin when compared to chick embryo fibroblast and rhesus monkey kidney cultures of the same age. Variations in types of media and serum concentrations during the early growth phase of the cultures were found to be ineffectual in achieving a more rapid growth or heavier monolayers.

Another problem that was encountered early was that in order to assay for endogenous pyrogen, any material tested by intravenous injection had to be free of any contaminating pyrogens, either infectious or non-infectious. Screening of a number of commercially available tissue culture media showed that they were invariably pyrogenic for rabbits, although sterile by culture. The fever pattern produced was quite typical of an endotoxin-like pyrogen and undoubtedly resulted from endotoxin contamination during commercial preparation since all materials used in our laboratories were pyrogen-free.

In an attempt to solve this problem, an Earle's balanced salts solution-base pyrogen-free tissue culture medium was prepared from basic ingredients that were demonstrated to be pyrogen-free. This involved 2 hour autoclaving of components of the salts solution separately (as described by Hsiung[76]) and similar treatment of lactalbumin hydrolysate. Pyrogen-free rabbit serum was used in place of calf serum. This type of medium was never found to be satisfactory in that, although it was not pyrogenic, it was incapable of maintaining the cell sheet for the time required for interferon production. After about 6 hours incubation in this medium,

uninfected cells could be observed to be degenerating. (An explanation might be that the essential polypeptide and amino acids in the lactalbumin hydrolysate were destroyed by the long period of autoclaving.)

A manner of eliminating the problem of pyrogenic tissue culture media might be to use rabbits for the pyrogen assay that have been made tolerant by repeated IV injections of endotoxins. Rabbits treated this way have been shown to be unresponsive to endotoxins but to remain responsive to endogenous pyrogens.[8] Other difficulties, loss of virus stocks and contamination, occurred before this could be attempted.

It is felt that because a positive assay for interferon was never achieved, no valid conclusions could be drawn from these two unsatisfactory experiments. They could not be repeated because of a very distressing gradual decline in the Sindbis virus titer, even though the virus was stored at -70°C in 10% bovine serum albumin. Bacterial and fungal contaminants of the tissue cultures also were perplexing problems.

In conclusion, the problem posed is a challenging one, but probably soluble with some further refinement of experimental design and technique.

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