Interferon Induction by Viruses

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ABSTRACT Interferons are proteins of cellular origin capable of conferring virus resistance to vertebrate cells. Most cells do not produce interferons except in response to proper stimulation. Clearly, the stimulation of interferon production encompasses two phenomena. When stimulated, some cell systems produce their interferons by synthesizing new proteins. Other cell systems do not require the synthesis of new proteins to produce interferons, and still other cell systems may produce interferons by both means. Before much can be learned from the detailed study of the nature of the molecules which stimulate interferons, the type of phenomenon which the stimulus induces must be identified. Chick embryo tissues apparently make interferons by synthesizing new proteins. Many viruses stimulate interferon production in chick embryo tissues. Data available suggest that neither the protein nor nucleic acid moieties of the added virions act as inducing molecules. Also, double-stranded replicative form is probably not responsible. It is suggested that the inducer molecule may be cellular in nature and may be produced in response to a wide variety of insults among which are viral infections.

The word "induce" is defined in the dictionary to mean (a) to call forth or bring about by influence or stimulation or (b) effect, cause; to cause the formation of. This and several succeeding papers will consider the bringing about of interferon formation by various means of stimulation. The first two papers are to discuss the formation of interferon resulting from viral stimulation. Because I am first, and because I have such an authority on viral induction of interferon following me, I feel I might serve a worthwhile purpose by discussing my understanding of what we, who work in the field, are talking about when we mention interferon induction.

Interferon or, more aptly, interferons are a class of proteins capable of inhibiting virus replication in vertebrate cells (1). Interferons are of cellular origin, and their production may be stimulated by a fairly wide range of materials (1-3). They may be produced by cells in culture, or they may be recovered from the serum or extracts of tissues of animals which have received an effective stimulus. As mentioned, interferons are proteins, and different interferons have different molecular weights (4), this will be discussed in
some detail in the paper to be presented by Dr. Fantes. However, I wish to emphasize that if one is dealing with an inducing system that produces interferons with different molecular weights, one cannot be at all certain that he is not dealing with a heterogeneous cell population and, therefore, a heterogeneous response.

To be considered interferons, the suspected antiviral substance must be shown to be a protein of cellular origin and must inhibit the growth of viruses in cells through some intracellular action requiring both cellular RNA and protein synthesis. The antiviral effect must not result from the direct inactivation of the virion or from nonspecific toxic effects on the cells. The resistant state invoked by the inhibitor must be against a range of unrelated viruses (1).

It is by use of their biological activity (i.e. the ability to inhibit viruses) that interferons are assayed. The amount of interferon in a solution is determined by finding the greatest dilution of that fluid which will bring about a predetermined amount of viral inhibition (i.e. inhibit the number of plaques by 50% and inhibit the yield of virus by 50%, or inhibit viral cytopathological effects). To study interferon induction, the experimental design is simple. It consists of adding a material called the inducer to vertebrate cells (animals; the inducible system) and measuring the fluid surrounding the cells (serum) at several succeeding intervals of time for the amount of antiviral activity present. When a new inducible system is first tested for interferon production, the material in the fluid must be characterized and found to be an interferon.

There are two facts that I want to emphasize. One is that interferons are proteins. The other is that most inducible systems so far studied do not spontaneously produce interferons but do so only in response to an added inducer. Notable exceptions have recently been reported (5-10), and we may soon have to admit that not all interferons require induction. Considerable confusion exists when we talk about interferon induction because two different phenomena are clearly involved, both of which result in the production of interferons. Some interferons result from the de novo synthesis of new protein molecules (5, 11-13). Some interferons do not require new proteins to be made and, therefore, result from preformed proteins (14-17). The name by which we refer to the latter phenomenon is "release" of interferon. It is not yet clear whether interferons which can be released are present as interferon molecules and need only to be unbound or whether they exist as some type of precursor molecule and require some kind of transformation to become active as an interferon. However, if we are to make any sense at all of the nature of the induction process, each investigator must define which phenomenon is occurring in the system he has chosen for study. Perhaps the most confusion arises from studies carried out in inducible systems in which both phenomena probably are occurring (14, 16-18).

To the present time, cultures of chick embryo cells are probably the best
studied interferon producing system for which there are adequate data to show that \textit{de novo} synthesis is the only means by which interferon is produced (4, 12, 19, 20). The evidence that interferon is produced only as a result of \textit{de novo} synthesis in cell cultures of chick embryo tissues is as follows: (a) chick embryo cell cultures make interferon of only one molecular weight variety; (b) production of interferon requires DNA-dependent RNA synthesis; and (c) production of interferon is immediately inhibited by the addition of a protein inhibitor (i.e. puromycin) at all times during its production.

I am not saying that there are no other systems to study the induction of the \textit{de novo} synthesis of interferon; I am saying that all of the proper experiments to show that interferon results only from \textit{de novo} synthesis have seldom been done in other systems.

I would now like to present some hypothetical, diagrammatic models illustrating our beliefs of the phenomenon by which cells are induced to \textit{de novo} synthesize interferon.

All the models assume that the genetic information for interferon production resides in the genome of the cell and that interferon is not normally produced because the expression of that genome requires molecules not normally present. In the model which I believe represents the thinking of most interferon workers, we have borrowed directly from the Jacob–Monod Model for the genetic regulation of enzyme synthesis in \textit{Escherichia coli} (21). This is shown in Fig. 1. We hypothesize that the gene for interferon production is prevented from expressing itself by the presence of a repressor molecule, the product of still another cellular gene called a regulator gene. In this model, the inducer stimulates the inactive or repressed interferon gene to a state of activity by interacting with the repressor molecule. Once stimulated, the interferon gene is actively transcribed and produces new messenger RNA molecules. They in turn are translated by the cell’s normal protein synthesizing system and result in the production of new interferon.

I would feel considerably more comfortable with this model if there were some evidence for either a regulator gene or a repressor molecule.

I would like to provide two alternative models that should be considered.

\begin{figure}
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\includegraphics[width=\textwidth]{figure1.png}
\caption{Hypothetical scheme of interferon induction. (a) Added molecules are the inducers; (b) derepression is required.}
\end{figure}
The first is but a slight alteration of the previous model and is shown in Fig. 2. Here, instead of adding a specific inducer molecule, we add an agent capable of stimulating a biochemical response in the cell, which results in the production of the specific inducer molecule. Although this is adding another unknown to our thinking, I believe a number of observations on virus-induced interferon production indicate that we cannot discard this possibility. I will mention them later. The second alternative model, shown in Fig. 3, proposes that the inactive gene requires a positive stimulus for activation. This model has to be entertained as long as no evidence for a regulator gene or a repressor exists. In all models, there still exists the alternative choices between stimulation by (a) added inducer molecules, (b) inducer molecules of viral origin generated during viral replication, and (c) cellular-inducer molecules made in response to the added stimulus. It is the choice between these possibilities that I wish to consider as I discuss the viral induction of interferon formation in cell cultures of chick embryo tissues.

There exists in the literature several suggestions that molecules which are part of the added virions may act as inducer molecules. Incubation of adenoviruses types 5 and 12 (22, 23) and Newcastle disease virus (24) with trypsin destroyed their ability to induce interferon even though the viruses remained infectious. This suggested that a protein component of the virion might be responsible for the induction observed. However, attempts to stimulate interferon production with isolated fractions from purified adenoviruses...
proved unsuccessful, and the authors concluded that intact virions were required (23).

Quite a large number of viruses stimulate interferon production in chick embryo tissues even when they are exposed to ultraviolet irradiation or heating and thereby are rendered unable to replicate (25, 26). In fact, Newcastle disease virus is only effective in chick embryo cells when inactivated (27, 28), and influenza viruses are rendered considerably more effective inducers (29). It is noteworthy that only mild ultraviolet irradiation may be employed since the overzealous use of this means of rendering the virus unable to replicate also destroys their ability to stimulate interferon production. Also, the ability of some viruses to stimulate interferon production is lost when they are rendered inactive by ultraviolet irradiation. Members of the group A arboviruses generally stimulate relatively high yields of interferon in chick embryo cells. However, all lose their inducing ability after exposure to sufficient ultraviolet irradiation to render them inactive. Also, Semliki Forest virus, a group A arbovirus, lost its ability to stimulate interferon production when it was inactivated by exposure to hydroxylamine under conditions which should only have affected its nucleic acid (30). Thus there has grown a general acceptance of the idea that an intact viral genome not too badly damaged is required for viral stimulation of interferon production. I know of no published data which describe attempts to stimulate interferon production by RNA, which was extracted from viruses rendered inactive by ultraviolet irradiation, but which still are competent interferon stimulators.

However, from some of the considerations just mentioned, Alick Isaacs, the discoverer of interferon, formulated the concept that foreign nucleic acids might be capable of stimulating interferon production; and he and others demonstrated that RNA from a variety of sources was able to stimulate the production of interferon by chick embryo tissues (31–33). However, relatively large amounts of RNA were required, low titers of interferon were usually produced, and frequently relatively long periods of time were required for significant amounts of interferon to accumulate. Thus as so often happens, a considerable body of information fell quietly into the scientific junkyard.

However, our attention toward RNA as an inducer of interferon was refocused in 1967 when it was shown that double-stranded RNA from a variety of sources stimulated interferon production when inoculated into rabbits (34–37). At first, only double-stranded RNA was thought effective (34–37). However, so-called single-stranded RNA has since been shown to be effective if it contains sufficient amounts of stable secondary structure (38, 39). Data on these points will be presented in greater detail later. I mention this finding with double-stranded RNA here because of the impact it has had on the thinking about how viruses induce interferon production.

Most of the work on the viral induction of interferon formation has utilized RNA viruses (25, 30) even though both active and ultraviolet inactivated
DNA viruses have been shown to stimulate interferon production in chick tissues (22, 40, 41). It has now been established that most, if not all, RNA-containing viruses form ribonuclease resistant, double-stranded species of RNA during their replication cycle. Therefore, based on the above finding concerning the ability of double-stranded RNA from synthetic and viral sources to stimulate interferon production, it has been widely inferred that the formation of such a structure during viral replication is responsible for the induction of interferon.

I would like now to present some experimental data obtained from efforts to test this hypothesis experimentally.

Burke et al. (30) infected chick embryo cells for 1 hr at 37°C with Semliki Forest virus and then shifted the incubation temperature from 37°C to 42°C. Under these conditions, Semliki Forest virus did not replicate, and the authors reported that they could not detect viral RNA or viral proteins. Nevertheless, the cells produced interferon. The authors have conservatively stated, however, that they could not guarantee that the input viral RNA had not been converted to a double-stranded form during the hour at 37°C.

We have used a slightly different approach. We also used cultures of chick embryo cells as our inducible system. As inducers we used several temperature sensitive mutants of Sindbis virus which differ with respect to their ability to replicate their viral RNA at 42°C (42, 43). Sindbis virus replicates in chick embryo cells under our laboratory conditions to produce several thousand plaque-forming units per cell of virus in about 8–10 hr (43). Viral RNA synthesis can be demonstrated by the use of uridine-3H and actinomycin D. Viral RNA synthesis commences at about 1½ hr after infection and reaches a maximum rate at about 6 hr. Double-stranded RNA can be demonstrated. However, synthesis of viral RNA was not sufficient to insure the production of interferon. Thus three different temperature-sensitive mutants of Sindbis virus produced at the nonpermissive temperature 83, 25, and 17%, respectively, as much RNA as the wild type parent virus; but in all cases they induced the production of less than 5% as much interferon (44). We have more recently taken the direct approach and tried to demonstrate interferon production in chick embryo cells with the mixture of the synthetic polynucleotides, polyinosinic acid and polycytidylic acid (poly rI:rC). We have found that we are able to inhibit almost completely the multiplication of Sindbis virus by incubating cultures of chick embryo cells overnight with 1 μg/ml of poly rI:rC. To obtain this degree of inhibition, however, we found we needed to include a polyanion in addition to the poly rI:rC. We chose protamine sulfate because it was nontoxic to the cells after overnight incubation and had no effect on viral multiplication. It was effective at any concentration greater than 10 μg/ml. Under these conditions (i.e. with 1 μg/ml of poly rI:rC and 25 μg/ml of protamine sulfate) we have been unable to find any interferon. We have attempted many times to induce interferon by using
poly rI:rC at a concentration of 10 μg/ml with added protamine sulfate (200 μg/ml) and a few times by using DEAE-dextran (100 μg/ml). We occasionally found interferon but never more than 1 unit. We are also unable to find interferon in the cells after freezing and thawing them, and we conclude that interferon is not produced but is retained inside the cells. Colby and Chamberlin (38) also were unable to use interferon production as a measure for interferon inducing capacity of double-stranded RNA in chick embryo cells because they too only occasionally recovered interferon and then only in small amounts. I have therefore concluded that double-stranded RNA is a poor inducer of interferon in chick embryo cells even though it is effective in promoting those cells to a state of viral resistance.

In summary, I can find no evidence sufficiently compelling to permit the generalized conclusion that double-stranded RNA, produced during the replication of animal viruses, is responsible for their ability to induce cells to synthesize interferon. I am completely convinced that double-stranded RNA from viral as well as from synthetic sources can stimulate interferon production in a number of systems, but I’m not yet convinced that there is proof that the interferon produced as a result of such stimulation results from de novo synthesis. Investigators have been equally unable to demonstrate that viral protein can act as inducers. This leaves me unable to answer with any assurance the question of how viruses induce interferon. At the moment, the model that best explains the observations of interferon induction by a wide assortment of viruses, microorganisms, and synthetic materials is that which proposes that the cell provides the inducer molecules in response to a wide range of stimuli. The answer will only be forthcoming when we know more about the control of protein synthesis in vertebrate cells.

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Discussion from the Floor

Dr. Anne L. R. Pidot (Dartmouth Medical School, Hanover, N. H.): Dr. Lockart. It has been demonstrated in rabbits that there is a marked difference in the effectiveness of poly IC in inducing interferon in cell systems as compared to the whole animal. Is this true in your chick embryo as well?

Dr. Lockart: I can't answer that question because we can't get poly rI : rC to induce interferon sufficiently well in chick embryo tissues.

Dr. Pidot: Have you infected the intact chick embryo? You're using chick embryo monolayers, aren't you?

Dr. Lockart: Yes. We're using monolayer cultures derived from chick embryos. We haven't done anything with intact embryos.

Dr. A. K. Field (Merck Institute for Therapeutic Research, West Point, Pa.): In answer to Dr. Pidot's question concerning interferon induction in chick embryo by poly rI : poly rC, we tried induction by injection of 10 µg poly rI : poly rC into the allantoic cavity. Allantoic fluids harvested from eggs 0, 2, 6, and 18 hr postinjection contained no evidence of interferon when titrated for protection of chick embryo cell cultures against infection by vesicular stomatitis virus.

Also, intravenous injection of up to 105 µg poly rI : poly rC into adult chickens failed to stimulate detectable antiviral substance in serum extracted 2 hr postinjection. However, wing-web injection of poly rI : poly rC did yield 40% protection against Rous Sarcoma virus infection in chicks.
Dr. Lockart, in your work did you find intracellular interferon in poly rI : poly rC-induced chick embryo cell cultures?

Dr. Lockart: No, I said we did not.

Dr. Field: As for induction of primary rabbit kidney cell cultures by poly rI : poly rC, we see production of cell-associated interferon accompanied by continuous release of large amounts of interferon into the surrounding medium.

Dr. Lockart: The point I've been trying to make in the whole talk is the need to understand the system you are working with. In this respect, I'm going to take to task rabbit kidney cell primary cultures. For example, it has not been demonstrated that the interferon that is made in that system is de novo synthesized, although I think some may be. It's quite possible, and one should look at the data that Dr. Vilček has published recently that both kinds of stimulatory phenomena are happening in these cultures. Thus I think there is the so-called "release" phenomenon and also de novo synthesis.

Now, for any inducer including poly rI : poly rC one should define which induction phenomenon he is talking about.

Dr. Field: Yes, I agree. We must define what is involved in the process of induction. The primary rabbit kidney cell system is complicated by not containing a single cell type. The induction process in such cultures may involve de novo interferon synthesis as well as some other mechanism of interferon production. We do know that induction of interferon in this cell system by poly rI : poly rC can readily be inhibited by pretreatment of cells with actinomycin D. We also find that cycloheximide, when added 2 hr after induction of cultures with poly rI : poly rC substantially inhibited interferon production. Total inhibition of interferon production was not obtained, but neither was total inhibition of protein synthesis by the concentrations of cycloheximide used.

Dr. Lockart: I just want to add one more remark. I think the distinction between the two phenomena is quite important, if one wants to inject reality to the whole situation. The problem is screening compounds to be interferon inducers. Let's say that the true explanation—and this will have to be hypothesis of course because I don't know the answer and I don't think the experiments have been done—of the release phenomenon is primarily a response to some kind of toxic effect. If your screening system allows you to pick up the release phenomenon, you may be screening for more and more effective toxic agents. And if you inject it into an animal, it will still work. You'll get large amounts of interferon in the serum, but you may be screening in the wrong direction. If you want to screen for an interferon inducer, which induces de novo synthesis, then one has to very carefully decide that the screening system that he's dealing with is actually screening for that kind of an inducer. Therefore, it makes all the difference in the world whether you're dealing with a mixed system.