BACTERIOLOGICAL REVIEWS
Vol. 28, No. 4, p. 367-381 December, 1964


# IDENTIFICATION AND "INDUCTION" OF INTERFERON[1]

MONTO HO

*Department of Epidemiology and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania*

INTRODUCTION ..... 367
INDUCTION OF INTERFERON IN CLASSICAL INTERFERON-PRODUCING SYSTEMS ..... 368
*Biochemical Mechanism of "Induction"* ..... 368
*Interferon Induction and Virus Virulence* ..... 371
INTERFERON INDUCTION IN VIVO ..... 375
IDENTITY OF VIRAL INHIBITORS ENDOGENOUS TO CELLS ..... 376
SUMMARY ..... 378
LITERATURE CITED ..... 379

## INTRODUCTION

Interferon is a viral inhibitor which acts by inhibiting intracellular replication of virus with no direct inactivating effect on virus (reviewed in 25, 22, 77, 6, 35, 62). The precise site at which it acts is unknown. Much evidence points to the site being very early in the intracellular replicative cycle (9, 10, 49). Since its discovery in 1957 (36), it has been produced under a large variety of conditions. These have usually involved a system consisting of cells in culture or an intact host, and a virus "inducer" of interferon. [The words "inducer" and "induction" are used here in the generic sense. They mean an agent which "brings about" interferon or the phenomenon involving "bringing about" interferon. They are not used in the special biochemical sense (39).] The virus inducers used have been either infective virus, so that virus was produced along with interferon, or virus partially or completely inactivated by such agents as heat or ultraviolet irradiation. An interferon induced in chick embryos by infective type A (WS) influenza and isolated from allantoic fluid has been highly purified (47). It is characterized as a small basic protein with a molecular weight of about 20,000 to 34,000. It has high specific biological activity, since as little as 0.004 $\mu$g possessed detectable viral inhibitory effect. The physicochemical aspects of this work have been confirmed by Kreuz and Levy (45) using CsCl gradient centrifugation, and by Merigan using Sephadex column chromatography (53).

Taken together, the source of interferon, its mode and mechanism of action, and its physicochemical properties should generally define what interferon is. The identifying properties of interferons, classified as either biological or physicochemical, are as follows. Biological properties: (i) produced by cells; (ii) does not directly inactivate virus; (iii) inhibits replication of virus and infectious nucleic acid intracellularly; (iv) more effective in species of cells from which it was produced, i.e., relative "species specificity"; and (v) not activated by antibodies against virus. Physicochemical properties: (i) small nondialyzable protein molecule inactivated by proteolytic enzymes including trypsin; (ii) relatively stable to heat; (iii) stable in high and low pH; and (iv) no proteolytic or nuclease activities. These characteristics are not exclusive enough to predict authoritatively whether a specific viral inhibitor which possesses some or most of the properties listed is or is not interferon. This lack of precision in its definition may be illustrated by the fact that 31 scientists interested in problems of interferon met informally to discuss this very problem at the 1964 meeting of the American Society for Microbiology in Washington, D.C. They could not and would not produce an inclusive definition of what more precise conditions must be met for a substance to be called interferon.

Extensive work on the physicochemical aspects of interferon obtained from one particular interferon-producing system also has its limitations. There may be physicochemical differences

[1] A contribution to the Symposium on "Current Progress in Virus Diseases" presented as part of the program for the Centennial of the Boston City Hospital, 1 June 1964, with Maxwell Finland serving as Consultant Editor, and John H. Dingle and Herbert R. Morgan as moderators.

between different types of interferons obtained from different sources. Indeed, crude interferon from primary mouse-cell cultures has been found to be more heat-labile than chick interferon. In contrast to other types of interferon, partial inactivation occurs at 56 C (43, 4, 25; Kono, Postic, and Ho, *unpublished data*); this has been the case when purified interferon made from mouse-cell cultures was compared with that from chick-cell cultures (53). Other differences have been reported, such as ether sensitivity. Thus, interferons made in chick chorioallantoic membrane (51) and in continuous mouse-cell lines are reportedly inactivated (17), but one type made in KB cell cultures induced by parainfluenza type 3 (8) and another made in HeLa cells induced by measles virus are not (12). Comparative studies of various types of interferons have been reported (53, 18), but generally these studies have not been numerous or complete. There is no *a priori* reason why an interferon will not be found which may have completely different physicochemical properties from the ones described. Interferons may belong to a relatively homogeneous group of proteins such as antibodies do, but they may also belong to a very heterogeneous group, and even the possibility that an interferon may be found that is nonproteinaceous cannot be excluded.

The main biological characteristics, such as those listed above, would appear to be more reliable. But each of the characteristics listed is subject to modification following advances in knowledge. For example, one of the most interesting properties of interferon, its "species specificity," holds up well generally and is an extremely useful identifying property of interferon (73, 25, 18). But exceptions are readily found, and specificity is certainly not absolute (1, 66, 25). Another essential property of interferon would appear to be its lack of direct effect on viruses, and there are no accepted exceptions to the necessity of meeting this criterion. However, there are viral inhibitory substances which are difficult to exclude on this basis, and the possibility cannot be excluded in the future that, under appropriate conditions of production or by physicochemical alteration, the interferon molecule will be found to inactivate virus particles or viral nucleic acid (see last section).

The problem of identifying interferon has assumed some importance, because it has now reportedly been produced under conditions radically different from the classical cell-virus systems. The newer systems are distinguished by the variety of nonviral inducers of interferon, including nucleic acid from animal tissues (34, 63), rickettsiae (32), bacteria (80), and yeasts and plants (40, 67); and statolin, which is a polysaccharide (42). Interferon in these systems was produced in cell cultures or in the intact host. For example, it was induced in chickens by brucella (80), in mice by yeast ribonucleic acid (RNA) (67), and in mice by pneumococci (W. D. Hann, *unpublished data*).

Another important consideration in any final definition of interferon, as well as in the understanding of its significance, is the possibility that interferon may be present in untreated cells in the absence of any known inducer. There are already reports that interferon may be extracted from the chick embryo and from medium overlay of chick-embryo cell cultures (R. Z. Lockart, *unpublished data*).

In the absence of complete publications, it is difficult at this stage to evaluate some of the data cited above. One conclusion seems warranted: perhaps it is not premature to exclude from the definition of interferon any provision that it must be induced by a virus or a component of virus (25). As to what these various inducer substances have in common, if anything, and whether there is endogenous interferon, it would be profitless to speculate at this stage. In addition, a certain lapse in time is desirable before an overall analysis is attempted.

What I would like to do in the following pages is not to add to the many general reviews of interferon, but to review specifically the problems of interferon induction and identity from certain arbitrarily selected points of view, bearing in mind some of the questions raised by new data. First, I shall consider the nature of induction of interferon in classical models of interferon formation in cell culture. Then I shall consider some problems of induction of interferon in the intact animal, and finally I shall consider viral inhibitors endogenous to cells as a specific problem in the identification of interferon.

## INDUCTION OF INTERFERON IN CLASSICAL INTERFERON-PRODUCING SYSTEMS

### *Biochemical Mechanism of "Induction"*

By "classical" interferon-producing systems,

I mean those systems in which the production of interferon is induced by infectious or inactivated virus. Since the inception of studies on interferon in these systems (25, 22, 77, 6, 35), it has been evident that interferon was different from viral particles in terms of such properties as revealed by centrifugation and antigenic characteristics (25). Therefore, the assumption has been that interferon is a cellular protein, the biosynthesis of which may not be directly related to viral replication. Until recently, proof of this on a biochemical basis was lacking; even now the relationship of the virus inducer and intracellular events of virus infection to interferon formation is still unclear.

The most promising recent approach to this problem has been to attempt to study the conditions under which virus replication may be dissociated from interferon production, and thereby to gain insight into the differing determinants of the biosynthesis of these two macromolecules.

There were scattered reports in the literature prior to 1963 which purported to show the effect of various types of chemical inhibition on interferon formation. Thus, Burke and Isaacs mentioned that proflavine did not inhibit interferon formation by influenza virus in chick chorioallantoic membranes (7). I also found this to be the case, using proflavine in a chick-cell culture system with infective Sindbis virus as interferon inducer (Ho, *unpublished data*). I found, in addition, that proflavine in nontoxic doses failed to inhibit Sindbis virus replication. In the absence of further data, these results, including my own, are difficult to evaluate. Generally, in studies with antimetabolites, further data are desirable. These should include controls which demonstrate that particular concentrations of the antimetabolite used are exerting the desired antimetabolic effect on the one hand, and that they do not produce nonspecific toxicity and cell death on the other. The first type of control may be obtained in terms of a biochemical measurement such as isotope incorporation, or in terms of inhibition cell or virus replication, depending on the antimetabolic effect in question. The second type of control demonstrating the absence of nonspecific toxicity is more difficult to establish, especially if both virus and interferon formation are inhibited by an antimetabolite. Reversibility of the antimetabolic effect may be one method (68).

Another type of available information is represented by what has been reported on the inhibitory effect of steroids on interferon synthesis (41). Studies along this line have added to the literature of the biology of steroids, and have increased our understanding of the effect of steroids on the replication of viruses, but they are of limited value in explaining the mechanism of interferon induction since the mode of action of these steroids is not precisely known. These remarks apply also to studies on the effect of certain carcinogens, such as 20-methylcholanthrene, on interferon synthesis (12).

Both of these objections have been overcome by use of actinomycin D. In the case of this antimetabolite, its mechanism of action is precisely known, and adequate controls may be readily established (61, 33). Wagner (76), using Eastern equine encephalomyelitis (EEE) virus in L cells, and Heller (20), using Chikungunya virus in chick-embryo cell cultures, found (i) that interferon readily produced in the absence of actinomycin is completely inhibited in its presence, and (ii) that one possible result of inhibition of interferon is that virus replication in such cultures is frequently enhanced. This is especially dramatic in the case of EEE virus infection of L cells, since ordinarily this virus does not replicate to high titers in these cells, presumably because of interferon formation in this system.

Knowledge of the mechanism of action of actinomycin D renders selective inhibition of interferon synthesis apart from virus formation by this agent highly significant. It is generally accepted that actinomycin D interferes directly with the function of deoxyribonucleic acid (DNA) by combining with the guanine basis on the DNA double helix (60). Hence, it inhibits synthesis of messenger RNA and, therefore, cell genome-directed protein. The replication of RNA viruses and specifically of the above-mentioned arboviruses is not affected by this antimetabolite (61), implying that these viruses replicate without any participation of cellular DNA. The conclusion is then inescapable that the formation of interferon, unlike that of RNA virus, is directly or indirectly controlled by the cell genome. This is the best evidence so far that interferon synthesis is demonstrably distinct from that of viral protein synthesis (Fig. 3).

Ho et al. (28) have also found that actinomy-

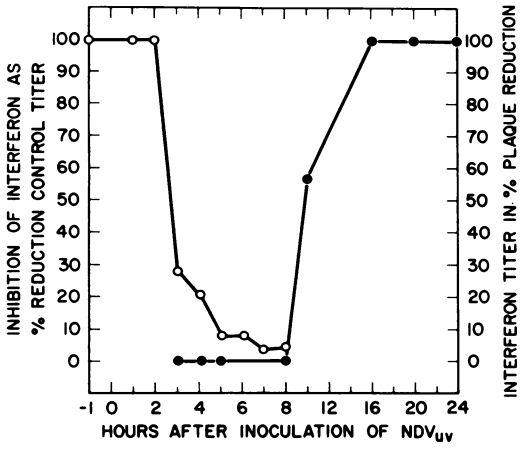


FIG. 1. *Course of production of interferon after adsorption of $NDV_{uv}$ in chick-cell cultures and periods during which actinomycin D is effective in inhibiting interferon production.* Symbols: ●, *course of extracellular interferon formation after a 2-hr period of adsorption of $NDV_{uv}$;* ○, *inhibition of interferon production. All cultures received $NDV_{uv}$ for 2 hr, but, at the times indicated, actinomycin D (0.5 μg/ml) was added for 1 hr. Cultures were all collected 18 hr after inoculation of $NDV_{uv}$, and the interferon titers were obtained and compared to that of fluid from a culture that was not treated with actinomycin D.*

cin D inhibits interferon formation in chick-cell cultures, with ultraviolet-inactivated Newcastle disease virus ($NDV_{uv}$) as an inducer. The merit of using $NDV_{uv}$ as inducer is that interferon is formed in the absence of significant viral replication. This eliminates any complicating effect of infective virus replication on interferon formation, including the uncontrolled increase of interferon inducer due to repeated cycles of virus formation. With this system, it is possible to estimate the time of interferon messenger RNA synthesis following adsorption of $NDV_{uv}$, and to test whether or not continuous messenger RNA synthesis takes place prior to the release of interferon.

These problems were approached in our laboratory by attempting to pinpoint the time, with respect to the inoculation of inducer, at which interferon formation is inhibited by actinomycin D (28). Interferon becomes detectable after about 8 hr of incubation following a 2-hr adsorption of $NDV_{uv}$ (Fig. 1). The titer reaches its maximum rapidly and does not appreciably increase in the course of the next 24 to 48 hr. If one tries to ascertain the time either before or after adsorption of $NDV_{uv}$ when actinomycin inhibition of interferon formation is effective, one finds that this is represented by a fairly short 2-hr period after the adsorption of $NDV_{uv}$. It is also effective if added for 1 hr prior to the adsorption of $NDV_{uv}$. The important point is that at 3 hr after the adsorption of $NDV_{uv}$ the necessary nuclear transcription for interferon synthesis is already complete, and actinomycin is at this time, and thereafter, no longer effective in inhibiting interferon formation. One explanation may be that messenger RNA necessary for interferon production is not continuously synthesized after exposure of the cells to $NDV_{uv}$. Such synthesis appears to be a "one-shot" affair.

However, these experiments with actinomycin D do not reveal the precise role of such messenger RNA. They do not answer the question whether interferon is a newly snythesized molecule or one that is slightly modified from a precursor. Although I have no concrete evidence on this point, there is evidence that new protein synthesis is necessary for effective induction of interferon. This has been shown by the demonstration that puromycin, which is a specific inhibitor of protein synthesis (79), will prevent production of interferon (28). Such a result is consistent with the notion that interferon represents newly snythesized protein, but it could also mean that the new protein is merely a necessary intermediary for interferon release.

Superficially, the notion that interferon formation resembles induced enzyme synthesis in which there is derepression of the expressibility of a genic function is very attractive (20, 76). In both cases, a protein normally undetectable in cells is released in response to an inducer. And, in both cases, this process is mediated by cell messenger RNA. Furthermore, the fact that the inducer for interferon is a virus, or perhaps viral nucleic acid, may be significantly related to the fact that the target of interferon action is inhibition of virus or virus nucleic acid synthesis. Beyond this, however, it would be imprudent to carry the analogy very far.

Indeed, there are aspects of interferon induction which still remind one more of the peculiarities of virus synthesis than of induced enzyme synthesis. In induced enzyme systems, synthesis continues in the presence of the inducer (39); in interferon induction, the presence of $NDV_{uv}$ inducer in cell cultures for 2 or 18 hr makes little difference in the total amount of interferon

produced; it is about the same (Ho, *unpublished data*). This is similar to the nature of virus infection in that the amount of virus inoculated or the length of cell exposure to virus does not determine the amount of virus replicated. The continued presence, then, of inducer beyond a certain period is ineffective in further induction. Admittedly, this resemblance between virus and interferon synthesis may be only superficial, because the continued presence of $NDV_{uv}$ does not necessarily imply a constant rate of adsorption and penetration of inducer and, therefore, an effective intracellular accumulation of inducer. However, alternatively, these data may signify that a cell can only make one quantum of interferon irrespective of increased accumulation of inducer. In other words, either a cell may make more interferon if more inducer could get in the cell, or it is possible that even if the inducer penetrated the cell, no more interferon could be made. Experiments testing these points are possible. These would include studies on the adsorption of both viral and nonviral inducers, or perhaps induction of interferon in a cell-free system in which the problems of adsorption of inducer may be circumvented.

There are some reported data on interferon induction which bear on the relationship of dose of inducer to the amount of product, but they are not quantitative or complete enough to be evaluated. For example, Burke and Isaacs (7) showed that a second crop of interferon may be induced with heated influenza virus in fragments of chick chorioallantoic membrane. However, this may be due to cells that did not adsorb any inducer the first time. Attempts to make further crops of interferon production would have been interesting. Heller (20) shows a graph representing "linear" accumulation of interferon in chick-cell cultures exposed to infectious Chikungunya virus. It is not clear whether this increase represents more induction with increased exposure to inducer or whether it may be due to progressive increase of infectious virus, and, hence, increasing numbers of cells exposed to inducer. Continued measurement of interferon titers beyond the 24 hr charted by Heller may reveal a sloping off of the "linear" accumulation of interferon; this would then represent the point at which the maximal number of cells are infected or induced. In general, it would probably be better to use noninfectious inducers to test these points.

In summary, it is probably premature to describe with any precision the mechanism of interferon induction. There are many models which could suit interferon induction. These include examples of specific "inducers," such as antibody formation and induced enzyme synthesis. There are also nonspecific "stimuli" which increase cellular metabolic activity, as exemplified by increase in the aerobic and anaerobic glycolysis and other biochemical parameters which attend phagocytic activity of white cells or their exposure to endotoxin (11). Possibly, interferon synthesis and release are a response to such "stimuli." [Recent evidence that bacterial endotoxin indeed induces interferon-like viral inhibitors in chickens, mice (W. R. Stinebring and J. S. Youngner, Nature, *in press*), and in rabbits (M. Ho, Science, *in press*) supports this possibility.]

### *Interferon Induction and Virus Virulence*

The next problem I would like to consider is why certain viruses induce the production of interferon while others do not. I will not review here all the possible factors involved but will concentrate on the relationship of interferon induction to virus virulence. There have been attempts to correlate interferon production negatively with (i) virulence of the virus inducer (16), (ii) ability of the virus inducer to grow optimally at high temperatures (64, 65), and (iii) the growth of virus inducer at high pH (13). The details of these purported correlations have been reviewed recently (35, 77), and they hold only in a very general way. However, it is probably unwise to dismiss the validity of these generalizations on the basis that they have not been tested in large enough groups of cell-virus systems, and that exceptions to at least some of them are readily found, because these ideas recur as long as the significance of interferon formation remains undefined.

Let us consider the concept that avirulent viruses are better interferon inducers than virulent ones. First of all, the term "virulence" is ambiguous. Does it apply to manifestations of human disease or does it apply to a specified cell or host system in question? There are examples where either sense of the term could be employed. Considering virulence in the anthropocentric sense, a strain of avirulent poliovirus (RMC) produces interferon in human amnion and

kidney cells, and a virulent strain will not (27; Ho, *unpublished data*); an avirulent or vaccine strain of measles virus produces more interferon than the virulent parent strain (16). It is also interesting that arboviruses that are virulent for humans, such as EEE or Japanese B encephalitis viruses, produce less interferon in chick-cell cultures than do the avirulent or less virulent Sindbis, Chikungunya, and Mayaro viruses (Ho and Mahdy, *unpublished data*; 21, 75, 25). Looking at virulence from the aspect of the particular cell culture under study, one might define a virulent virus as one which replicates quickly and optimally with concomitant total destruction of cells. Under this definition, the examples of polioviruses apply again, since RMC poliovirus does not destroy human kidney cells in culture and induces interferon formation, and wild strains are destructive and do not induce interferon. Sindbis virus does not fit this concept, because it replicates to high titers in chick-cell cultures and is cytopathic, while inducing interferon. Isaacs also cites the example of Kumba virus which destroys cells and yet produces interferon (35, 65).

An interesting model of the complex relationship between properties of a virus and its capacity to produce interferon is Newcastle disease virus (NDV). In the infectious form, NDV in chick-cell cultures is a good example of a type of virus which is not an interferon inducer. It is "virulent" in chick-cell cultures. Parenthetically,

TABLE 1. *Virus and interferon formation in chick embryos infected with NDV*

| Inoculum | Virus titer | Interferon titer* | | | |
|---|---|---|---|---|---|
| | | 1:1 | 1:4 | 1:16 | 1:64 |
| 24-hr harvests (embryos alive) | | | | | |
| $10^{-0}$ | $7 \times 10^{7}$ | 60 | 0 | 0 | 0 |
| $10^{-3}$ | $1 \times 10^{8}$ | 0 | 0 | 0 | 0 |
| $10^{-5}$ | $7 \times 10^{7}$ | 0 | 0 | 0 | 0 |
| 48-hr harvests (embryos dead) | | | | | |
| $10^{-0}$ | $7 \times 10^{7}$ | 100 | 100 | 75 | 40 |
| $10^{-3}$ | $3 \times 10^{8}$ | 100 | 100 | 91 | 57 |
| $10^{-5}$ | $3 \times 10^{8}$ | 100 | 99 | 79 | 20 |

* Results are expressed as per cent inhibition of 500 PFU of EEE virus.

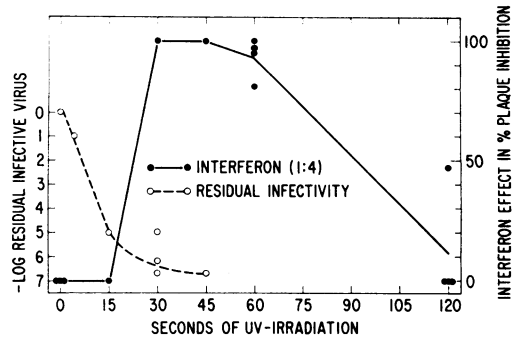


FIG. 2. *Interferon-producing capacity of ultraviolet-irradiated NDV virus pellet. Pellet material was ultraviolet-irradiated with a 28.75-w lamp with a maximal emission of 2,537 A placed at 7.6 cm. Residual virus was titrated by inoculating 10-fold dilutions in duplicate chick cultures and observing for plaque formation. Interferon was titrated in serial fourfold dilutions on duplicate chick cultures, challenged with EEE, and observed for plaque reduction. The values plotted are those of a 1:4 dilution. Points shown are the means of duplicate values; where more than one point is shown, these represent values obtained in separate determinations.*

it multiplies at low $O_2$ tension (5) and requires high temperature for optimal replication (64). The behavior of NDV in mouse-embryo cell cultures is also consistent with the "virulence" theory. The virus does not produce lysis in these cells, and replication is minimal. Hence, it may be considered avirulent in this tissue. And, unlike the situation in chick cells, NDV induces interferon in these cells (43, 65). On the other hand, the situation is more complex in the embryonated chick egg. NDV induces interferon in this host, for which it must be considered "virulent" (64). Virus replication begins in 2 hr and reaches its maximum in 8 to 10 hr. No interferon is detectable at this time. But 48 hr after inoculation the interferon titers become appreciable; at a time when the embryos are dead (48; Ho, *unpublished data*, Table 1). This is an example of a lethal viral infection being associated with interferon production. Similar systems may be gleaned from the literature (77).

Although infectious NDV is not an inducer of interferon in chick-cell cultures, it can be made into one if it is irradiated by ultraviolet light (Fig. 2). Irradiation for 30 to 60 sec under described conditions is optimal, but excessive irradiation destroys this inductive capacity.

Inductive capacity appears to be a biological property of the virus particle which, along with others such as hemagglutination or enzyme activity, is susceptible to physicochemical inactivation.

It appears from these vagaries of NDV as an interferon inducer as if the virus particle is basically capable of inducing interferon, but the reactions accompanying infectivity in certain cell systems counteract this induction capacity.

There is evidence from recent studies on the biochemical effects of the virus infection which bear on this point. These studies promise to supply a biochemical definition of the whole problem of virulence, and may also provide a reason why virulent viruses do not induce interferon. From the work on Mengo virus (2), poliovirus (30), and others (31), it is apparent that certain virus infections are associated with severe inhibition of cellular biosynthetic mechanisms. Since interferon synthesis depends on such mechanisms, it too may be inhibited in the course of virulent infection. Conversely, if the infective cycle does not take place, as when $NDV_{uv}$ is used, the inductive function inherent in the virus particle may proceed undisturbed. These hypotheses may also explain the late induction of interferon by NDV in chick embryo. Possibly by 48 hr, at a time when the embryo may be moribund, certain interferon-producing cells may finally become exposed to NDV particles that have become inactivated by this time, and may act as interferon inducers. Similarly, it is also possible that the inability of other "virulent" viruses, such as wild polioviruses, to induce interferon is related to their inhibitory effect on cell synthetic mechanisms. The "inverse interference" of Lindenmann (50), where infectious virus added after the inducer consisting of inactive virus inhibits interferon production, may also be explained by the cell inhibitory effect of infectious virus.

The parameters of classical interferon induction unfortunately are not entirely exhausted in terms of the NDV model. Certain problems are brought out by another popular class of inducers, the arboviruses. Here, infective virus is more effective than the inactivated (25). Usually, the inactivated virus induces no detectable interferon (for exception, see Gifford and Heller, quoted in 35), but may render cells resistant to superinfection (26, 52). In addition, there is a phenomenon called "priming": if inactivated virus is added to cell cultures prior to infective virus, it will prime interferon production. That is, interferon is produced where none is produced in the absence of the primer, or it may potentiate or increase interferon production when infective virus alone is effective as an inducer (26, 52).

It appears then that, in the case of some viruses, infective virus is the better inducer, and inactive virus may serve as a "primer." There are two possible explanations for infective virus being a more effective inducer. First, the inducing capacity of the virus may be as sensitive to physicochemical inactivation as is infectivity, or more so. This would make it impossible to dissociate the inducing property from the infective by inactivation. Second, viral replication is associated with a large intracellular accumulation of virus; therefore, effective inducer may also accumulate inside the cell. Infection may be a way of getting a large amount of inducer inside cells, and a relatively ineffective inducer may not be operative unless such accumulation following cellular infection takes place. To account for the "priming" phenomenon, it is possible that inactive virus acting as a "primer" functions as an incomplete inducer by forming intracellular interferon which is not released, but which can act within the cell in which it is formed. This "direct inhibitory effect" of the primer has the effect of slowing down the infective cycle of an additional infective virus, and permitting interferon synthesis and release to take place. The data

TABLE 2. *Effect of virus infection ($V_a$) on chick-cell cultures "primed" with inactivated EEE ($V_i$)*

| Infectious virus added ($V_a$) | Interferon production | | Direct inhibition of infectious virus $\left(-\log \frac{V}{V_c}\right)$† |
|---|---|---|---|
| | No $V_i$ | $V_i$* | |
| EEE | 9‡ | 100 | 2.0 |
| Sindbis | 54 | 100 | 2.0 |
| VSV | 19 | 85 | 1.2 |
| NDV | 24 | 36 | 0.0 |

* Cultures preincubated 5 hr with EEE inactivated by ultraviolet light and 37 C.

† V = titer of virus in cultures pretreated with $V_i$ . $V_c$ = titer in untreated cultures.

‡ Activity in per cent plaque inhibition of EEE virus at 1:4 dilution of culture fluid in chick-cell cultures.

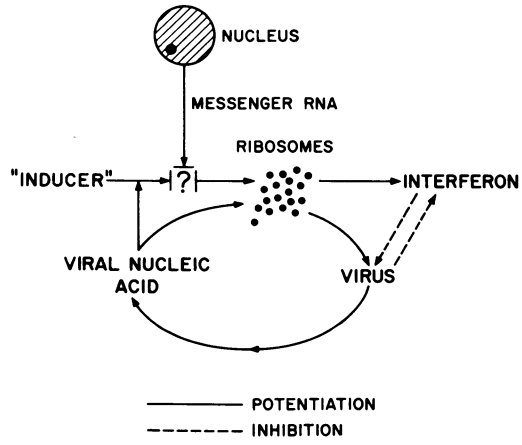


FIG. 3. *Induction of interferon and intracellular interrelationships between virus replication and interferon formation.*

presented in Table 2 (Mahdy and Ho, *unpublished data*) may constitute indirect evidence that the "primer" produces a *forme fruste* type of intracellular interferon. The primer ($V_i$ in Table 2) consisting of ultraviolet-inactivated EEE virus can prime interferon formation by EEE, Sindbis, and vesicular stomatitis viruses. These three viruses are known to be susceptible to interferon, and, hence, presumably to the hypothesized intracellular interferon formed by the primer. On the other hand, the addition of NDV does not result in production of interferon in the "primed" cells. This may be explained by the well-known fact that NDV is not inhibited by interferon in chick cells (38, 64). Hence, it is not inhibited by the primer, and it infects and lyses primer-treated cells without significant interferon production. These data are, of course, only suggestive and are not conclusive.

The intracellular events leading from induction to interferon formation, and the effect of virus and virus replication on these events, are diagrammed in Fig. 3. These designated interrelationships explain the apparently conflicting effects which may issue from superficially similar circumstances, and constitute a complex intracellular dynamic situation. This situation may be recapitulated as follows. (i) Infectious virus may either inhibit or enhance interferon formation: it inhibits by a disruptive action on cellular synthetic mechanism; it augments by providing inducer. (ii) Intracellular interferon, presumably formed by "primers," or incomplete interferon inducers consisting of inactivated virus, can inhibit viral infectivity, inhibit the cell-disruptive influence of infective virus, and thereby further interferon formation. However, if intracellular interferon is present in excess, it may prevent interferon formation by preventing successful viral infection, and thereby preventing accumulation of sufficient viral inducer.

The potential merit of these hypotheses is that they suggest certain problems which may be attacked experimentally. First, is there a correlation between viral virulence, virus disruptive effect on cell synthetic mechanisms, and effectiveness of the virus as an interferon inducer? This question is amenable to biochemical solution. Our model would predict that though some lytic or "virulent" infections, such as poliovirus in primate cells, are totally disruptive to cell synthetic mechanisms, other infections which are equally destructive morphologically, such as Sindbis virus in chick-cell cultures, would not be so disruptive biochemically, since interferon is produced in them. Second, these hypotheses postulate the existence of an intracellular interferon which is biologically active, but which is "incomplete" in that it is not released from the cells. This type of interferon is formed by "primers." The basis for assuming that unreleased interferon may be incomplete is that there is very little intracellular accumulation of interferon if one follows both extracellular and intracellular titers of interferon in chick-cell cultures induced by ultraviolet-irradiated NDV (28). At no time in such studies was intracellular interferon found to be of higher titer than extracellular. The results suggest that interferon is released as soon as it is formed. On the other hand, there is data showing that once interferon is adsorbed to chick cells, it is no longer elutable (75). The reason for this situation is unknown. It may be due to a lack of sufficient quantity of interferon to be detected, or, alternatively, in accordance with our hypothesis, once interferon is adsorbed, it reverts to an "incomplete" form which is not readily released.

These considerations are subject to experimental scrutiny. First, if adsorbed interferon becomes "incomplete," its release may be effected by addition of a "complete inducer" consisting of an infectious virus, in a manner similar to releasing interferon after cells have

been "primed." Second, the mechanism of "priming" may also be studied from various vantage points. If intracellular "incomplete" interferon is formed, it too should be inhibited by such agents as actinomycin D.

If the concepts of incomplete intracellular interferon and incomplete inducers of interferon are accurate, certain obscure types of viral interference may be explained. One of these may be a type of "infection interference," a situation in which an infective virus may be the interfering agent in the absence of appreciable amounts of interferon (24).

## INTERFERON INDUCTION IN VIVO

Baron and Buckler (4) described the appearance of high titers (up to 1:300) of interferon 1 to 4 hr after intravenous injection of large doses of NDV and Sindbis virus in mice. This method of inducing interferon is important, because (i) interferon appears at a time before any evidence of viral multiplication has occurred, (ii) it takes place in the intact animal, and (iii) the titers are of a higher range than those obtained from most other in vitro work (25). It is not yet possible or desirable to review the full significance of this phenomenon at this time, especially with respect to its significance as a biological defense mechanism (3). I will, however, review two problems with respect to interferon induction which this work brings out: (i) the difference between interferon formation in vivo and in vitro and (ii) the source of this rapidly appearing interferon.

The primary difference between interferon formation in cell cultures and in animals after an intravenous inoculation is one of speed. This

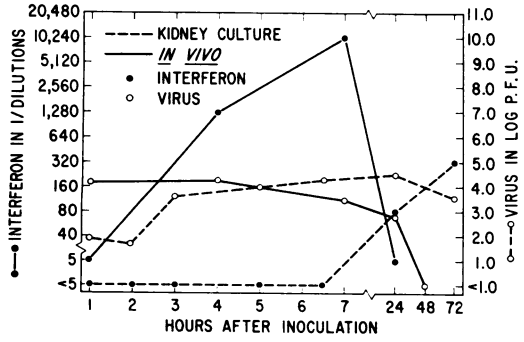


FIG. 4. *Interferon and virus formation by Sindbis virus in rabbit cells in vivo and in vitro.*

TABLE 3. *Interferon formation in rabbit-cell suspensions*

| Incubation time | Virus (log)* | Spleen ($10^{7.7}$ cells)† | Liver ($10^{7.0}$ cells) | White blood cells ($10^{6.3}$ cells) | Kidney ($10^{7.6}$ cells) |
|---|---|---|---|---|---|
| *hr* | | | | | |
| 1 | 6.2 | <1:10‡ | <1:5 | 0 | 0 |
| 3 | 6.1 | 1:80 | 1:20 | 0 | |
| 5 | 6.0 | 1:160 | 1:20 | 0 | 0 |
| 7 | 6.0 | 1:640 | 1:20 | 1:40 | |
| 10 | 5.7 | 1:640 | 1:20 | | |
| 23 | 5.0 | >1:640 | 1:40 | >1:40 | 1:80 |

* PFU per 0.1 ml of splenic cell reaction mixture.

† Cells per milliliter.

‡ Dilution at which 50% VSV plaque reduction in rabbit-kidney cell cultures was observed.

is well demonstrated by contrasting interferon formation in rabbit-kidney cell cultures and in rabbits with large doses of Sindbis virus as inducer. After an intravenous dose of about $10^9$ plaque-forming units (PFU) of Sindbis virus in a 4-kg rabbit, circulating interferon is detectable in 1 hr, and reaches its peak in 7 hr (Fig. 4). On the other hand, in a cell culture of about $10^6$ rabbit-kidney cells exposed to $10^7$ PFU of Sindbis virus, interferon formation does not start until 7 to 24 hr after infection, and virus replication is evident in 3 hr. The latter in vitro data is consistent with most of the data in which interferon formation has been found to lag behind virus replication (75). There may be exceptions. For example, Chikungunya virus seems to be able to form interferon in about 3 hr in chick-cell cultures (20). But what is of interest in Fig. 4 is the difference in speed between in vivo and in vitro interferon formation with one specified cell-virus system (rabbit—Sindbis). The circulating interferon detected in the intact rabbit does not seem to follow any significant virus replication, and seems to be the primary effect of the inoculum. This rapidity of response suggests that there are specialized cells in the intact animal which are especially rapid interferon formers. Possibly, this property of host cells may be dependent on cell differentiation and is lost when cells are cultured in monolayers.

To test this hypothesis, the course of interferon formation in suspensions of cells from

various organs of a rabbit was tested (44). Sindbis virus was added to a concentration of $2 \times 10^7$ PFU per milliliter, and the cell-virus mixtures were incubated under agitation at 37 C. Samples were titrated for interferon effect by inhibition of plaque formation of VSV on rabbit-kidney cell cultures. Interferon was detectable in 3 hr in cell suspensions derived from liver and spleen, but its appearance in kidney and white blood cell suspensions was much later (Table 3). The time of appearance of interferon in kidney-tissue cells is suggestive of what occurs in rabbit-kidney primary explant monolayer cultures.

These results suggest that there is a common property of liver and spleen cell suspensions. The most obvious carrier of this common property is the reticuloendothelial cells present in both tissues. Possibly, the rapidity with which Sindbis virus inducer is taken up is related to the phagocytic function of the reticuloendothelial system (54). Experiments using thoratrast to blockade this system in rabbits suggest that some of this rapidly appearing interferon is inhibited (44).

Another possibility is that rapid interferon formation represents release of preformed intracellular interferon. This is especially so in view of the large variety, and therefore the possible nonspecificity, of interferon inducers (see Introduction). One can easily rule out a simple mechanism of release, since it is not possible to release anything close to the amount of inhibitory material by simple mechanical lysis of tissue cells. In addition, experiments with actinomycin D in both mice and rabbits show that rapidly appearing interferon can be inhibited, as it is in monolayer cultures of cells from these animals (28; Kono and Ho, *unpublished data*). This is indirect evidence that interferon formation in vivo is no different than in cells cultured in vitro. It is, of course, entirely possible that precursors of interferon exist in liver and spleen, which are closer to the final intact interferon molecule than those in monolayer cultures, thus accounting for the rapidity of release.

Finally, the titers reached in the blood stream of the rabbit and in cultures of liver and spleen cells are higher than those obtained in monolayer cell cultures. This is also true with Sindbis virus inducer in the mouse system. Circulating interferon is readily detectable after an intravenous inoculation, but interferon is barely found in mouse-cell cultures infected with Sindbis virus (3; Ho and Postic, *unpublished data*).

It appears from the above that there may well be a difference in the capacity of cells to synthesize and release interferon. It would be of great interest to identify the most efficient cells in this regard, to study the underlying reasons for this difference, as well as to determine whether this capacity is in any way related to any other function of host resistance, such as phagocytosis and antibody formation. With respect to the precise biological significance of rapidly induced interferon in the host, very little that is definite can be said at this time. One would expect such interferon to be more efficacious in preventing viral spread than interferon formed after virus replication (77). Preliminary evidence with Sindbis virus in mice suggests that, indeed, interferon may operate by preventing early viremic death (56).

## IDENTITY OF VIRAL INHIBITORS ENDOGENOUS TO CELLS

The report that many different substances stimulate interferon formation, as well as the rapidity of in vivo interferon formation after intravenous viral and bacterial (80) inoculation, suggest the obvious possibility that interferon is endogenous to cells. Such interferon may be preformed without any inducing agent, or it may be constantly formed in response to an occult inducer of either viral or some other unknown origin.

The possibility that interferon-like inhibitors may exist in uninfected cell systems has been recognized since early interferon studies. It has been the experience of most workers in the field that control fluids in interferon titrations derived either from uninfected cell culture overlay or from extracted cells are occasionally, but frequently erratically, inhibitory to viral action (see, for example, Table 9, 27; Gresser, *private communication*). This is true of fluid overlays of uninfected continuous or primary cell cultures, chick allantoic fluid, and extracts of normal organs. Viral inhibitory activity of these fluids is usually low. It has undoubtedly been explained away in various ways and dismissed as a vagary of laboratory determinations.

Recently, however, Tsilinsky (69, 70, 71, 72), in a series of four papers, described in some detail a viral inhibitor found in the fluid overlay and

cell extracts of five continuous human cell lines (HeLa, ERK, KB, Detroit-6, and FL), and one monkey cell line (CMH). This inhibitor has a combination of properties suggestive of both interferon and cell receptors. It resembles interferon in that it is digestible by trypsin, it is fairly heat-stable (60 C, 2 hr), it can be adsorbed on cells, and it is not lost on washing. On the other hand, it resembles cell receptors in that it is localized primarily in the cytoplasmic fraction and is sedimentable at 22,000 $\times$ $g$ for 2 hr, suggesting a "corpuscular diameter of 200–600 m$\mu$." When applied to monkey-kidney cell cultures, it produces an apparent increase in adsorption of ECHO virus from 55 to 76%. It has antigens of cellular protein, since it is neutralized by anticellular serum. Unfortunately, no definitive test for its direct viral-inactivating capacity was made, and no tests were performed on nonprimate cell cultures to test its species specificity. Somewhat similar, though less complete, findings with various types of continuous cell lines have been reported by Pumper (57) and Pellegrini (55).

In a study of cell receptors, Quersin-Thiry (58, 59) also mentioned that "various receptor material can apparently leak out into nutrient fluid during the growth of normal, non-infected HeLa cultures." Since these receptors are by definition direct inactivators of virus, a strong possibility exists that her material and that described by Tsilinsky are identical, although data for direct comparison are not available.

There are also two considerations made in her work on virus receptors of HeLa cells which are especially relevant in any attempt to distinguish between receptor and interferon. Both increase the difficulty of such a distinction under certain circumstances: (i) the kinetics of direct viral inactivation, which is the hallmark of receptors, is reversible and accompanied by "reactivation" of adsorbed virus especially upon dilution of the virus receptor mixture. Of the four viruses tested for this property [NDV, Western equine encephalitis (WEE), polio (Mahoney), and polio (MEF1)], this was true of NDV and WEE. (ii) There are no physicochemical properties common to all receptors (59). Hence, certain receptors for viruses are heat-labile (polio), and others are heat-stable (NDV); some are inactivated by trypsin (polio, WEE), but others are not (WEE). Recent evidence confirms the idea that there may be chemical differences between receptors of even closely associated viruses. It was found that HeLa cell receptors to Coxsackie $B_3$ and poliovirus Type 1 differed in their susceptibility to the proteolytic enzymes, chymotrypsin and trypsin. Chymotrypsin inhibited attachment of $B_3$ but not poliovirus, and trypsin inhibited adsorption of poliovirus and not $B_3$ (81). In general, it appears that studies of receptors to different viruses by use of different cells have not reached the completeness with which receptors of primate tissues for poliovirus have been characterized (29, 31). It would, therefore, be premature to generalize from these studies to other models.

In a recent attempt to detect endogenous interferon in 11-day-old chick-embryo cell homogenates, I found a virus inhibitor, similar to that described by Tsilinsky and others, in the absence of any inducer (Ho, *unpublished data*). Some of my results in studying this "endogenous inhibitor" may be summarized to further illustrate the problems in distinguishing virus receptor from interferon (Table 4).

This inhibitor was similar to interferon, since it could adsorb on cell cultures and was not completely removed by repeated washings. Furthermore, it was resistant to 56 C and was not dialyzable. Studies on direct inactivation of EEE virus were frequently equivocal, but there was usually a reduction in virus titer. This may be a reflection of the difficulty in doing adsorption work with arboviruses, the viruses most frequently used for testing interferon. Such difficulty was also brought out by Wagner (74). The inhibitor was found to be effective in mouse-cell cultures. It was apparent then that it had no species specificity. Therefore, one of the hallmarks of chick interferon was missing. In addition, the partial sedimentability of the inhibitor suggested that it was not interferon, but probably an inhibitor with a high molecular weight or a smaller molecule adsorbed to some such large sedimentable material. Although I believe that this endogenous inhibitor is most likely virus receptor rather than interferon, in the absence of clean separation it is impossible to rule out the possibility that low titer interferon-like substances may be in this homogenate.

The lesson from these experiments is that viral receptors must be ruled out in any study

TABLE 4. *Summary of findings on an endogenous chick-cell viral inhibitor**

| Procedure | Result |
|---|---|
| Effect of washing treated chick cultures | No wash: 93% plaque reduction<br>1 wash: 85% plaque reduction<br>3 washes: 78% plaque reduction |
| Effect of heating (56 C, 30 min) | No effect |
| Effect of dialysis | No effect |
| Effect of dilution | 50% cell homogenate, effective up to 1:64 |
| Direct inactivation of EEE | 23 to 75% reduction of virus titer |
| Effect on heterologous mouse-embryo cultures | 92% VSV plaque reduction |
| Effect of fractions of inhibitor homogenate | Ribosomal† supernatant fluid, 32% plaque reduction<br>Ribosomal sediment, 76% plaque reduction |

* Inhibitory effect was measured by adsorbing 0.3 ml of inhibitor for 3 hr at 37 C on duplicate chick-cell cultures, washing off inhibitor with 5 ml of Hanks' solution, challenging with 100 to 200 PFU of EEE virus, and calculating reduction of plaque formation as compared with similarly treated control culture.

† Obtained after 22,500 rev/min for 30 min (#40 rotor, model L Spinco centrifuge).

purporting to demonstrate induction of interferon or the presence of interferon. This is especially true if the process of induction involves prolonged incubation and cell injury (40) such that more opportunities exist for the "leaking out" of cell receptors. However, since little appears to be known about the complex properties of different cell receptors for different viruses, the process of distinguishing between these two viral inhibitors may be more difficult than is apparent at first sight.

One final point should be considered: a possible relationship between interferon and cell receptors. Is it possible that receptors are incomplete interferons or vice versa? One might still maintain that interferon is an intracellular inactivator of virus, except for the fact that it inhibits the infectivity of infectious RNA (23, 15, 19). Though the notion that interferon acts by binding infectious nucleic acid is still plausible (78), the idea that it binds whole virus directly either extracellularly or intracellularly is untenable. Therefore, there is as yet no obvious bridge to connect the different mechanisms of action of interferon and cell receptors of virus. Nevertheless, there are other similarities between these two cellular products. It is possible that the production of cell receptors under certain circumstances requires "inducers." It is well established that the amount of cell receptors varies with types of tissue (29) and with age (46), and increases upon in vitro cultivation (29, 58). Further studies on both interferon and cell receptors are required before definitive formulation of the situation is possible.

## SUMMARY

Certain problems regarding the identity and induction of interferon have been discussed and reviewed. Many of these arise because of the large number of heterogeneous substances reported to be inducers of interferon. A few, especially intracellular microorganisms, appear well established.

Interferon formation seems metabolically to be a cellular, as distinguished from a viral, process. It is mediated by cell messenger RNA and requires new protein synthesis. This is well demonstrated by work with antimetabolites. However, relatively little is known about the cellular control of this mechanism, and the role of the "inducer." The resemblance to induced enzyme synthesis is attractive but unproven.

Induction of interferon in experimental ani-

mals by intravenous inoculation of virus is a well-established phenomenon. There is suggestive evidence that there are specialized host cells which make more interferon and release it faster than others.

There are no precise criteria for the recognition of interferon, only general ones. Attention is called to cellular substances which may simulate interferon, especially if inhibitory activity is of low potency. One such substance is cell receptors for viruses.

ACKNOWLEDGMENT

Portions of this work were supported by Public Health Service grant AI-01953 from the National Institutes of Health.

LITERATURE CITED

1. ANDREWS, R. D. 1961. Specificity of interferon. Brit. Med. J. **1**:1728–1730.
2. BALTIMORE, D., AND R. M. FRANKLIN. 1962. The effect of Mengo virus infection on the activity of the DNA-dependent RNA polymerase of L cells. Proc. Natl. Acad. Sci. U.S. **48**:1383–1390.
3. BARON, S. 1963. Mechanism of recovery from viral infection. Advan. Virus Res. **10**:39–64.
4. BARON, S., AND C. E. BUCKLER. 1963. Circulating interferon in mice after intravenous injection of virus. Science **141**:1061–1063.
5. BARON, S., J. S. PORTERFIELD, AND A. ISAACS. 1961. The influence of oxygenation on virus growth. I. Effect on plaque formation of different viruses. Virology **14**:444–449.
6. BURKE, D. C. 1962. Interferon—some aspects of production and action, p. 294–319. *In* L. G. Goodwin and R. H. Nimmo-Smith [ed.], Biological Council Symposium on Drugs. Parasites and Hosts.
7. BURKE, D. C., AND A. ISAACS. 1958. Some factors affecting the production of interferon. Brit. J. Exptl. Pathol. **39**:452–458.
8. CHANY, M. C. 1961. An interferon-like inhibitor of viral multiplication from malignant cells (the viral autoinhibition phenomenon). Virology **13**:485–492.
9. COCITO, C., E. DEMAEYER, AND P. DESOMER. 1962. The action of interferon on the synthesis of RNA in fibroblasts infected with a RNA virus. Life Sci. **12**:753–758.
10. COCITO, C., E. DEMAEYER, AND P. DESOMER. 1962. Synthesis of messenger RNA in neoplastic cells treated *in vitro* with interferon. Life Sci. **12**:759–764.
11. COHN, Z. A., AND K. F. AUSTEN. 1963. Contribution of serum and cellular factors in host defense reactions. New Engl. J. Med. **268**:1056–1064.
12. DEMAEYER, E., AND J. DEMAEYER-GUIGNARD. 1963. Inhibition of 20-methylcholanthrene on interferon production in rat cells. Virology **20**:536–538.
13. DEMAEYER, E., AND P. DESOMER. 1962. Influence of pH on interferon production and activity. Nature **194**:1252–1253.
14. DEMAEYER, E., AND J. F. ENDERS. 1961. An interferon appearing in cell cultures infected with measles virus. Proc. Soc. Exptl. Biol. Med. **107**:573–578.
15. DESOMER, P., P. PRINZIE, P. DENYS, AND E. SCHONNE. 1962. Mechanism of action of interferon. I. Relationship with viral ribonucleic acid. Virology **16**:63–70.
16. ENDERS, J. F. 1960. A consideration of the mechanism of resistance to viral infection based on studies of the agents of measles and poliomyelitis. Trans. Coll. Physicians, Philadelphia **28**:68–79.
17. GLASGOW, L. A., AND K. HABEL. 1962. The role of interferon in vaccinia virus infection of mouse embryo tissue culture. J. Exptl. Med. **115**:503–512.
18. GIFFORD, G. E. 1963. Studies on the specificity of interferon. J. Gen. Microbiol. **33**:437–443.
19. GROSSBERG, S., AND J. J. HOLLAND. 1962. Interferon and viral ribonucleic acid. Effect of virus-susceptible and insusceptible cells. J. Immunol. **88**:708–714.
20. HELLER, E. 1963. Enhancement of Chikungunya virus replication and inhibition of interferon production by actinomycin D. Virology **21**:652–656.
21. HENDERSON, J. R., AND R. M. TAYLOR. 1961. Studies on mechanisms of arthropod-borne virus interference in tissue culture. Virology **13**:477–484.
22. HILLEMAN, M. R. 1963. Interferon in prospect and perspective. J. Cellular Comp. Physiol. **62**:337–353.
23. HO, M. 1961. Inhibition of the infectivity of poliovirus ribonucleic acid by an interferon. Proc. Soc. Exptl. Biol. Med. **107**:639–644.
24. HO, M. 1962. Role of infection in viral interference. I. Inhibition of lethal infections in chick embryos preinfected with influenza viruses. Arch. Internal Med. **110**:653–659.
25. HO, M. 1962. Interferons. New Engl. J. Med. **266**:1258–1264a, 1313–1318b, 1367–1371c.
26. HO, M., AND M. K. BREINIG. 1962. Conditions for the production of an interferon appearing in chick cell cultures infected with Sindbis virus. J. Immunol. **89**:177–186.

27. HO, M., AND J. F. ENDERS. 1959. Further studies on an inhibitor of viral activity appearing in infected cell cultures and its role in chronic viral infections. Virology **9**:446–477.
28. HO, M., AND M. K. BREINIG. 1964. Metabolic conditions of interferon formation. Virology, *in press*.
29. HOLLAND, J. J. 1961. Receptor affinities as major determinants of enterovirus tissue tropism in humans. Virology **15**:312–326.
30. HOLLAND, J. J. 1963. Depression of host-controlled RNA synthesis in human cells during poliovirus infection. Proc. Natl. Acad. Sci. U.S. **49**:23–28.
31. HOLLAND, J. J. 1964. Enterovirus entrance into specific host cells and subsequent alterations of cell protein and nucleic acid synthesis. Bacteriol. Rev. **28**:3–13.
32. HOPPS, H. E., S. KOHNO, M. KOHNO, AND J. E. SMADEL. 1964. Production of interferon in tissue cultures infected with *Rickettsia tsutsugamushi*. Bacteriol. Proc., p. 115.
33. HURWITZ, J. J., J. FURTH, M. MALAMY, AND M. ALEXANDER. 1962. The role of DNA in RNA synthesis. III. The inhibition of the enzymatic synthesis of RNA and DNA by actinomycin D and proflavin. Proc. Natl. Acad. Sci. U.S. **48**:1222.
34. ISAACS, A. 1963. Foreign nucleic acids. Sci. Am. **209**:46–50.
35. ISAACS, A. 1963. Interferon. Advan. Virus Res. **10**:1–38.
36. ISAACS, A., AND J. LINDENMANN. 1957. Virus interference. I. Interferon. Proc. Roy. Soc. (London) Ser. B **147**:258–267.
37. ISAACS, A., AND M. WESTWOOD. 1959. Inhibition by interferon of the growth of vaccinia virus in the rabbit skin. Lancet **2**:324–325.
38. ISAACS, A., J. S. PORTERFIELD, AND S. BARON. 1961. The influence of oxygenation on virus growth. II. Effect on the antiviral action of interferon. Virology **14**:450–455.
39. JACOB, F., AND J. MONOD. 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. **3**:318–356.
40. JENSEN, K. E., A. L. NEAL, R. E. OWENS, AND J. WARREN. 1963. Interferon responses of chick embryo fibroblasts to nucleic acids and related compounds. Nature **200**:433–434.
41. KILBOURNE, E. D., K. M. SMART, AND A. POKORNY. 1961. Inhibition by cortisone of the synthesis and action of interferon. Nature **190**:650–651.
42. KLEINSCHMIDT, W. J., J. C. CLINE, AND E. B. MURPHY. 1964. The production of interferon by statolon. Federation Proc. **23**:507.
43. KONO, Y. 1962. An interferon-like substance produced in cells infected with Newcastle disease virus and its properties. Natl. Inst. Animal Health Quart. **2**:183–188.
44. KONO, Y., AND M. HO. 1964. The role of the reticulo-endothelial system in interferon formation. Virology, *in press*.
45. KREUZ, L. E., AND A. H. LEVY. 1964. Physical properties of chick embryo interferon. Bacteriol. Proc., p. 115.
46. KUNIN, C. M., AND W. S. JORDAN, JR. 1961. *In vitro* adsorption of poliovirus by noncultured tissues. Effect of species, age and malignancy. Am. J. Hyg. **73**:245.
47. LAMPSON, G. P., A. A. TYTELL, M. M. NEMES, AND M. R. HILLEMAN. 1963. Purification and characterization of chick embryo interferon. Proc. Soc. Exptl. Biol. Med. **112**:468–478.
48. LEVINE, S. 1962. Some characteristics of an interferon derived from embryonated eggs infected with Newcastle disease virus. Virology **17**:593–595.
49. LEVY, H. B., L. F. SNELLBAKER, AND S. BARON. 1963. Studies on the mechanism of action of interferon. Virology **21**:48–55.
50. LINDENMANN, J. 1960. Interferon und inverse Interferenz. Z. Hyg. Infektionskrankh. **146**:287–309.
51. LINDENMANN, J., D. C. BURKE, AND A. ISAACS. 1957. Studies on the production, mode of action and properties of interferon. Brit. J. Exptl. Pathol. **38**:551–562.
52. MAHDY, M. S., AND M. HO. 1964. Potentiating effect of fractions of Eastern equine encephalomyelitis virus on interferon production. Proc. Soc. Exptl. Biol. Med. **116**:174–177.
53. MERIGAN, T. C. 1964. Purified interferon: physical properties and species specificity. Science **145**:811–813.
54. MIMS, C. A. 1964. Aspects of the pathogenesis of virus diseases. Bacteriol. Rev. **28**:30–71.
55. PELLEGRINI, A., A. M. PASSAGGIO, AND P. G. PAGANO. 1961. The antivirogenic activity of normal and neoplastic human cells and cell fractions cultured *in vitro*. Panminerva Med. **3**:64–66.
56. POSTIC, B., S. H. SINGER, AND M. HO. 1964. Determinants of pathogenicity of Sindbis virus for adult mice. Federation Proc. **23**:193.
57. PUMPER, R. W., H. M. YAMASHIROYA, AND L. J. ALEDED. 1961. Cytopathic protective factor: its production by normal uninfected serum-free cell cultures. Nature **192**:1101.
58. QUERSIN-THIRY, L. 1961. Interaction between cellular extracts and animal viruses. I.

Kinetic studies and some notes on the specificity of the interaction. Acta Virol. **5**:141–152.

59. QUERSIN-THIRY, L. 1961. Interaction between cellular extracts and animal viruses. II. Evidence for the presence of different inactivators corresponding to different viruses. Acta Virol. **5**:283–293.
60. REICH, E. 1964. Inhibition of RNA biosynthesis by actinomycin D. Federation Proc. **23**:91.
61. REICH, E., R. M. FRANKLIN, A. J. SHATKIN, AND E. L. TATUM. 1962. Action of actinomycin D on animal cells and virus. Proc. Natl. Acad. Sci. U.S. **48**:1238–1245.
62. RITA, G., M. RUSSI, F. BIANCHI BANDINELLI, AND F. DIANZANI. 1963. Fattori biologici della interferenza virale: l'interferone. Estratto da Atti del XII Congresso Naz. di Microbiologia, Perugia, 4–7 Ottobre 1963, **1**:9–90.
63. ROTEM, Z., R. A. COX, AND A. ISAACS. 1963. Inhibition of virus multiplication by foreign nucleic acid. Nature **197**:564–566.
64. RUIZ-GOMEZ, J., AND A. ISAACS. 1963. Optimal temperature for growth and sensitivity to interferon among different viruses. Virology **19**:1–7.
65. RUIZ-GOMEZ, J., AND A. ISAACS. 1963. Interferon production by different viruses. Virology **19**:8–12.
66. SUTTON, R. N. P., AND D. A. J. TYRRELL. 1961. Some observations on interferon prepared in tissue culture. Brit. J. Exptl. Pathol. **42**:99–105.
67. TAKANO, K., J. WARREN, AND K. E. JENSEN. 1964. Resistance conferred by interferons stimulated with non-viral nucleic acid. Federation Proc. **23**:506.
68. TAMM, I. 1959. Chemotherapy and virus infection, p. 156–171. *In* T. M. Rivers and F. L. Horsfall [ed.], Viral and rickettsial infections of man. J. B. Lippincott Co., Philadelphia.
69. TSILINSKY, Y. Y. 1963. Inhibitors of viral activity from uninfected cultures of stable cell lines. I. Inhibition of the cytopathic effect, plaque formation and multiplication of some enteroviruses. Quantitative assay of the inhibitors. Acta Virol. **7**:350–360.
70. TSILINSKY, Y. Y. 1963. Inhibitors of viral activity from uninfected cultures of stable cell lines. II. Properties of inhibitors. Acta Virol. **7**:437–446.
71. TSILINSKY, Y. Y. 1963. Inhibitors of viral activity from uninfected cultures of stable cell lines. III. Interaction of inhibitors with trypsinized monkey kidney cells. Acta Virol. **7**:542–548.
72. TSILINSKY, Y. Y., AND V. S. LEVASHEV. 1963. Inhibitors of viral activity from uninfected cultures of stable cell lines. IV. Study of the relationship between the contamination of cell cultures with PPLO and occurrence of inhibitors. Acta Virol. **7**:549–553.
73. TYRRELL, D. A. J. 1959. Interferon produced by cultures of calf kidney cells. Nature **184**:452–453.
74. WAGNER, R. R. 1961. Biological studies of interferon. I. Suppression of cellular infection with Eastern equine encephalomyelitis virus. Virology **13**:323–337.
75. WAGNER, R. R. 1963. Biological studies of interferon. II. Temporal relationships of virus and interferon production by cells infected with Eastern equine encephalomyelitis and influenza viruses. Virology **19**: 215–224.
76. WAGNER, R. R. 1963. Interferon control of viral infection. Trans. Assoc. Am. Physicians **76**:92–101.
77. WAGNER, R. R. 1963. The interferons—cellular inhibitors of viral infection. Ann. Rev. Microbiol. **17**:285–296.
78. WAGNER, R. R., AND A. H. LEVY. 1960. Interferon as a chemical intermediary in viral interference. Ann. N.Y. Acad. Sci. **88**:1308–1318.
79. YARMOLINSKI, M., AND G. DE LA HABA. 1959. Inhibition of puromycin of amino acid incorporation into protein. Proc. Natl. Acad. Sci. U.S. **45**:1721.
80. YOUNGNER, J. S., AND W. R. STINEBRING. 1964. Interferon production in chickens injected with *Brucella abortus*. Science **144**: 1022–1023.
81. ZAJAC, I., AND R. L. CROWELL. 1964. Enzymatic differentiation between Coxsackie $B_3$ and poliovirus type 1 receptors of living HeLa cells. Federation Proc. **23**:295.