Nonviral Interferon Inducers

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ABSTRACT Interferon production can be stimulated by a great variety of microbial and nonmicrobial agents other than viruses. The nonmicrobial inducers can be divided into polyanions, mitogens, and a miscellaneous category including the various endotoxins and antibiotics. The polyanions appear to require a stable, high molecular weight backbone and a high density of free anionic groups whether they are polynucleotides, plastics, or polysaccharides. Mitogen-induced interferon appears to be but one of a constellation of substances produced following lymphocyte transformation. The process of transformation can be stimulated either by specific immune recognition or nonspecifically by phytohemagglutinin. Synthetic polynucleotide inducers are active; the thermostable, double-stranded RNA's are much more active than the double-stranded DNA's or 1-, 3-, or 4-stranded RNA's. Some success has been obtained with potentiation of nucleotide inducers through the use of polycationic substances, complexing with a polysaccharide, concurrent administration of a metabolic antagonist, or substitution of phosphate by thiophosphate in the polynucleotide backbone. The stages in the interaction of interferon stimulating RNA and cells can be divided into three steps: first, binding to cell surface, next, a temperature dependent “recognition” step, and finally, degradation and utilization of monomers in cellular RNA synthesis; the critical recognition site has not yet been determined. The vast majority of cell-associated polynucleotide remains at the surface of the cell. Information from animal models resembling human diseases suggests that certain of these nucleotide inducers may have clinical usefulness in therapy or prophylaxis.

INTRODUCTION

In 1964 interferon was observed in the serum of chickens that had been injected intravenously with Brucella abortus by Youngner and Stinebring (1). This finding indicated that substances other than viruses could lead to interferon stimulation. Youngner has discussed the observations with nonviral interferon inducers of microbial origin earlier in this Symposium.

This presentation will focus on nonmicrobial inducers, in particular on synthetic inducers from which one can draw conclusions as to the critical structural requirements for this process. The RNA inducers have received a
great deal of attention recently as they are the most potent on a weight basis and are among the least toxic presently available as well as perhaps most resembling the natural stimuli to interferon production during virus infection.

**Classes of Inducers**

A classification of nonviral agents stimulating interferon production is presented in Table I. The microorganisms which are capable of interferon induction are intracellular parasites in the main, whereas another large group of interferon inducers are the polyanions (the multi-stranded RNA polynucleotides, the plastics, and the polysaccharides). So far, wherever it has been examined, the critical features for the activity of the polyanions appear to be a stable, high molecular weight backbone and a high density of free anionic groups which may be phosphates, thiophosphates, carboxylates, or sulfates (2–4). There are, of course, other structural features required for interferon-inducing capacity, as not all polyanions with a high molecular weight, high charge density, and structural stability are active, e.g., polygalacturonic acid (4) and polyaspartic and polyglutamic acids (2) are not active.

A interesting group of polysaccharide derivatives recently described by Claes and his associates (5, 6) are the polyacetal carboxylic acids. These polycarboxylates are formed by a two step oxidation of polysaccharides with chlorite. The backbone of these polyanions contains acetal (\(-\text{C}--\text{C}--\text{O}--\text{C}--\text{O}--\)) instead of the ethylene (\(-\text{C}--\text{C}--\text{C}--\text{C}--\)) linkages of the plastics. They are markedly less toxic than the plastic polycarboxylates and
Nonviral Interferon Inducers

have a therapeutic ratio in the range of 300-500, and they had a more transient protective effect in various animal models against virus infection than did the plastic polycarboxylates. This suggests that the acetal-containing backbone is more easily biodegraded than the plastics. The requirement of free anionic charges for antiviral activity was established for this series of compounds (as it had been shown for pyran copolymer [2] and polyacrylic acid [3]), in that the partially oxidized polysaccharides were of lesser activity, and the nonoxidized parent compounds were inactive.

Another interesting polysaccharide shown to be active by De Somer et al. (3) was a sulfated polysaccharide extracted from seaweed. The activity may be related to the recently observed double-stranded helical configuration of a seaweed-derived sulfated polysaccharide (carrageenan) (7).

In addition to the microorganisms and the polyanions, another group of interferon inducers are the mitogens. The mitogens have been most well studied in vitro, and they include both specific antigens and nonspecific agents such as phytohemagglutinin. The interferon is produced by lymphocytes prior to their transformation (8).

The fourth class of interferon inducers is grouped somewhat arbitrarily. Here belong the various endotoxins (9, 10), together with the two antibiotics, cycloheximide (11) and kanamycin (12), which have been shown to induce interferon. These inducers were active in the whole animal with a rather narrow therapeutic ratio. Endotoxin has also been found to be active in peritoneal macrophages (13, 14) and in leukocytes (14, 15) in releasing small amounts of interferon, but it has been quite inactive in all other cells. As these macrophages and leukocytes only represent explants and are not purified cell preparations, it is possible that here, as in the whole animal, there may be multiple cell types involved in this activity.

Effector substances are released during lymphocyte transformation and possibly are involved in delayed hypersensitivity, homograft reaction, and other forms of cell-mediated immunity. The effector or mediator substances which are produced during lymphocyte transformation (16) are as follows: interferon, lymphotoxin, migration inhibitory factor, chemotaxic factor, skin-reactivity factor, and mitogenic factor. The biological significance of Wheadock's finding of phytohemagglutinin stimulation of interferon production from lymphocytes (8) was not clear initially. With the discovery of these other activities produced during lymphocyte transformation, one can propose a more specific role for interferon. It seems reasonable to suggest that it acts at the site of virus infection to block further local extension of the virus working with the other effector or mediator substances. These components of the delayed hypersensitivity or cell-mediated immunity system appear to be triggered during transformation, each having a useful role in the inflammatory response. Lymphotoxin acts by destroying certain cells; the migration in-
hibitory factor brings to the site of transformation macrophages whose phago-
cytic capacity is useful in dealing with the foreign materials; the chemotaxic
factor brings polymorphonuclear leukocytes to the infiltrate, and the mito-
genic factor recruits other lymphocytes into the inflammatory filtrate through
their transformation. The skin-reactivity factor is capable of producing many
of the cellular changes of delayed hypersensitivity in the skin within a few
hours. Only interferon, by virtue of its low molecular weight (18,000), is
clearly differentiatable from the other five activities whose molecular weights
are all in the range of 70-80,000. The specificity to this inflammatory response
is conferred by the specific immune recognition inherent in lymphocyte
transformation. This area will be discussed further by Dr. Lowell Glasgow
later in this Symposium.

Green and his associates (17) have been able to show that tuberculo-
protein, diphtheria toxin, and tetanus toxin will cause the production of
interferon from lymphocytes if they are obtained from specific sensitized in-
dividuals. In addition, Stinebring and Absher (18) have shown that, in BCG-
infected mice, injection of tuberculo-protein causes a circulating interferon
response within 6 hr. In collaboration with Dr. Lois Epstein we have also
observed transformation-induced interferon produced in vivo in the mouse.
Low levels of serum interferon were observed for several days following
administration of high doses of phytohemagglutinin. However, these studies
may only be examining “the top of the iceberg” as to the extent or role of
this antiviral effect. It is possible that the critical amount of interferon or
other mediator substances being released at the site of virus infection, either
at the port of entry or at the end organ, may be extremely low and yet quite
useful to the host as a rapidly mobilizable antiviral function.

The requirements for activity of synthetic polynucleotide inducers are
reviewed in Table II. Initial studies by Isaacs and his coworkers in England
suggested that foreign polynucleotides were active in interferon production
(19, 20), but there was some difficulty in reproducing these studies in his

| Inducer                | Activity                                                                 |
|------------------------|--------------------------------------------------------------------------|
| Double-stranded RNA    | Very active if strands are complementary                                  |
|                        | *Thermostability* is important as reflected in Tm > 60°C necessary for full activity |
|                        | Salt, pH, and divalent cation content all may alter Tm and activity        |
| 3- or 4-stranded RNA   | Other *unknown* factors very important                                    |
| Double-stranded DNA    | Of two different types or a single type                                     |
|                        | *Less active* than double-stranded RNA's                                  |
|                        | *Much less active* than double-stranded RNA's                            |
laboratory (21). However, the studies of Hilleman and his associates (as reviewed in reference 22) sharply focused interest on this area by clearly demonstrating the importance of the double strand in polyribonucleotides in their in vivo and in vitro interferon-inducing capacity. These polyribonucleotides were obtained from a wide variety of sources, including natural sources such as double-stranded mammalian viruses (23), bacterial viruses (24), plant viruses (25), and fungal viruses (26), as well as synthetic RNA's enzymatically polymerized in the laboratory by a variety of techniques (27). Activity has been shown with both alternating copolymers (which form a base-paired, double-stranded single molecule) as well as with homopolymer pairs (composed of two complementary strands, each of repeating sequences of the same bases). This work will be discussed in more detailed by Dr. A. K. Field later in this Symposium.

We have been interested in the structural requirements for the activity of these synthetic polymers, and our conclusions to date are reviewed in Table II. With the active double-stranded complexes, it appears as though a threshold degree of thermostability is important in that complexes with a low degree of stability are inactive, and that increasing this stability of the double-stranded pair, either by changes in salt, pH, or divalent cation content, appears to alter the stability and biologic activity in parallel (28). Interestingly enough, certain chemical substitutions, such as halogenation in the C5 position which do not effect the stability (29), do not alter the activity (30), whereas methylation of the N7 lowers the stability (31) and decreases the activity (30). As we survey the polymers studied in our laboratory (28) and elsewhere (22, 30, 32), the thermostability requirement is not adequate to wholly explain the high activity of certain double-stranded RNA complexes. A similar conclusion was reached by Colby and Chamberlin (32). It is clear that other factors in the three-dimensional structure of these polynucleotide complexes must be highly critical in the interaction with the cell. These factors might be acting through a variety of means. Either they act to promote the binding or penetration of the cell by the polymer, or they may alternatively act to promote the affinity of the polymer for the interferon-triggering site on or within the cell. A third alternative explanation (which will be discussed below) is that the three-dimensional structural specificity is acting through the specific recognition of nucleases which can destroy, and thereby compete for, the polynucleotide before its interaction with the final receptor site. Therefore, this specificity can be either a positive one in that those molecules which are recognized act as inducers, or a negative system in that recognition by nuclease prevents certain molecules from acting as inducers. At present, there are no convincing data to decide among these possibilities.

One of the unknown factors may be degree of packing of negative charges in the polyanion. It is known that the homopolymer pair formed of poly-
inosinic acid-polycytidylic acid (I:C) is one of the most densely packed of the homopolymer pairs in that, when it is annealed, the negative charges are closer together in this structure than in the less active polyadenylic-polyuridylic acid homopolymer pair (A:U) (33). A number of triple- or four-stranded RNA complexes, formed from either two different homopolymer types or from a single self-aggregating homopolymer type were less active than the double-stranded RNA’s (28). In addition, as has been stressed by Hilleman, single-stranded RNA’s appear to be inactive (22). Polyxanthylic, polynosinic, and polyguanylic acids each tended to form multi-stranded complexes under the physiologic conditions usually employed in tissue cultures and had a borderline activity in this form (28). By altering the pH or divalent cation concentrations one can cause polyadenylic and cytidylic acids also to shift from single-stranded to multi-stranded complexes (28) and to become active. Double-stranded DNA complexes of a composition similar to that of active double-stranded RNA complexes are much less active (4). Exactly how this marked difference in activity is achieved is unclear.

POTENTIATION OF RNA INDUCERS

Mechanisms for potentiating the activity of polyribonucleotide inducers have been recently evolved (Table III). Almost 2 yr ago, Dianzani and his associates (34), taking a lead from studies with infectious viral RNA’s and DNA’s, observed that DEAE-dextran promoted interferon-inducing activity of I:C in tissue culture. They observed that L cells would only produce interferon if they were treated with DEAE-dextran in addition to I:C (34). Subsequently, others have confirmed this finding (35-37), and Colby and Chamberlin studied a large series of helical, double-stranded RNA in chick embryo fibroblasts by using DEAE-dextran to potentiate their antiviral activity (32). DEAE-dextran increased interferon production in vivo with I:C in one study (38) but not in others.1

It is of interest that the kinetics of interferon production are altered in cells treated with DEAE-dextran when they are studied in vitro. Dianzani and his associates (34) noticed the peak of interferon production in tissue culture at 24 hr in cells treated with DEAE-dextran, in contrast to observation of earlier interferon production in other cell types (35-37, 39) responding in the absence of DEAE-dextran. Billiau and his associates (40) have studied a variety of basic substances, including methylated albumin, protamine, neomycin, histone, streptomycin, etc., all of which appeared similar to DEAE-dextran in potentiating interferon induction in vitro. Lampson and his colleagues (41) found polyamines (such as neomycin) active in potentiating the antiviral

1De Clercq, E., M. R. Nuwer, and T. C. Merigan. 1970. The role of interferon in the protective effect of a synthetic double-stranded polyribonucleotide against intranasal vesicular stomatitis virus challenge in mice. J. Clin. Invest. In press.
activity of I:C in primary rabbit kidney cells, but neomycin failed to demonstrate an enhanced antiviral activity in other cell lines or an enhanced interferon production or resistance to virus infection in the whole animal. Considering the complexity of the whole animal and the possible multiple interactions between polyanionic and polycationic molecules, it is not surprising that in vivo results are not reproducible. DEAE-dextran has been observed to protect I:C from nuclease degradation (42) and has increased the binding of radiolabeled I:C by cells and has also increased the amount of labeled material seen within cells (37). Lampson et al. (41) showed that neomycin increased the stability of double-stranded RNA, but they were unable to demonstrate increased rates of cell binding for double-stranded RNA in the presence of neomycin.

Fig. 1 presents another observation which we have made in regard to binding of radiolabeled I:C in the presence of DEAE-dextran. We have noted microscopically visible aggregates of radioactive material on cells when they are treated with tritium-labeled I:C in the presence of DEAE-dextran. It appears also that there is more uptake or binding of the polyanion to glass under these circumstances. These aggregates can be seen in areas where there are no cells; therefore, this effect is nonspecific. On the other hand, it is possible that these aggregates are more resistant to nuclease degradation and that their presence on the surface or within the cell is a critical mechanism for the potentiation of interferon production. It is important to remember that in cells that respond to I:C in the absence of DEAE-dextran, no more interferon is made with DEAE-dextran despite the fact that more polynucleotide is bound (37). Perhaps in certain cells the presence of these aggregates is useful. In others, the threshold for the response is exceeded under the normal conditions of binding, and therefore the effects of DEAE-dextran are not necessary.

Our laboratory has also been interested in the possibility of potentiating the biologic activity of the synthetic polyribonucleotides and, in fact, has

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### Table III

| Approach                                      | Mechanism                                      |
|----------------------------------------------|------------------------------------------------|
| DEAE-dextran and related compounds           | Complex protected from nuclease degradation or increased cell binding or penetration |
| (methylated albumin, neomycin, etc.)         | Protected from nuclease degradation             |
| Thiophosphate analogues                      | Protected from nuclease degradation             |
| RNA-polysaccharide complex                   | Possibly interference with synthesis of interferon repressor (?) |
| Cycloheximide and actinomycin                |                                                 |

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made some progress in that direction. It appears difficult to obtain both a uniform material by complexing polyanions with polycations and a uniform in vivo delivery of polycations and polyanions separately to cells. Therefore, we looked for a change in the intrinsic structure of the polyanion (RNA) which would potentiate the activity. Certain changes in the RNA structure increase or decrease the stability of the double-stranded complex (see above), but there were no remarkable changes in activity. Our analysis of the stability studies indicated that any chemical change in the RNA backbone to potentiate their activity should not decrease the affinity of one of the two strands for the other. However, it would seem useful to heighten the resistance of the inducer to break down either outside of or within the cell, the latter having been demonstrated with radiolabeled I:C (as will be described below). Therefore, we aimed toward blocking nuclease degradation with the smallest possible change in polynucleotide structure.

It came to our attention that Dr. Fritz Eckstein (Max-Planck-Institute, Göttingen, Germany) was synthesizing thiophosphate analogues of double-stranded RNA (43, 44). The general structure can be seen in Fig. 2. In the series of compounds that Eckstein was then working with, only one of the oxygens on the phosphate (bridging the sugar moieties in the RNA backbone) was substituted with sulfur, but this was carried out throughout the polynucleotide backbone. This minimal change in structure was not associated with any significant change in stability as is demonstrated by the fact that the thio-
phosphate analogues, poly r(A-U)s and poly r(As-U)s, and their parent compound, poly r(A-U) (alternating copolymer of riboadenylic and ribouridylic acid), showed an identical Tm (thermal transition midpoint) (43, 44). The thermal transition midpoint assesses the point at which the polynucleotides change from helical to random coil structure and can be measured with a high degree of accuracy under any given salt conditions. However, this modest change (substitution of sulfur for oxygen in the phosphate linkages) produced a major change in sensitivity to several different nucleases (44). All of these nucleases were markedly less active on the RNA analogue than on the parent compound because of this change which essentially involved only

the addition of a single shell of electrons on one atom out of each of the subunits of this macromolecule.

When the thiophosphate-substituted polynucleotides were studied for their ability to produce resistance to virus infection in tissue culture, they were potentiated by several orders of magnitude (45). We also found them more potent on a weight basis in inducing interferon both in tissue culture and in vivo (45). The potentiation of the interferon-inducing capacity is most likely due to increased resistance to nucleases within cells with the specificity of pancreatic ribonuclease. Levy and his associates pointed out that the nuclease activity of the serum may be involved in the interferon inducing activity of polynucleotides. The thiophosphates showed an increased resistance to serum

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2 Norlund, J. J., S. M. Wolff, and H. B. Levy. 1970. Inhibition of biological activity of poly I: poly C by human plasma. Proc. Soc. Exp. Biol. Med. 133:439.
inactivation when compared to their parent compounds as measured by their ability to confer cellular resistance to virus infection in vitro. The increased resistance to serum closely paralleled the decreased sensitivity to degradation by pancreatic ribonuclease and the increased capacity to induce interferon and resistance to virus infection (45). Therefore, we feel it is of great importance to explore the results with thiophosphates in animal models in regard to potential usefulness in clinical medicine. We are currently looking for the most active congener in this series. We then plan to produce larger quantities of this material in order to determine whether it has a more favorable therapeutic ratio in animal models against virus infection when compared to their parent compounds. As we have observed a somewhat longer period of interferon production with these analogues in tissue culture (3), it is possible that they will give a more prolonged period of antiviral protection in animal models as well as being more active on a weight basis.

To return to Table III, Goore and his associates (46) have recently discovered an RNA-polysaccharide complex which they obtained from a fungus of the Cunninghamella species. It appears active in producing resistance to virus infection in cells in tissue culture and animals on the basis of nuclease resistance conferred on the RNA by complexing with a polysaccharide. They were able to demonstrate interferon production with a polysaccharide RNA complex in rabbit kidney cells, and they found that the complex was much more active in producing both interferon and antiviral protection than either of its components. When they studied the melting curve of the RNA in the complex in a variety of salt concentrations, they found that there was no evidence of a sharp thermal transition, and they interpreted this to indicate that this RNA was of a single-stranded variety and active in interferon induction in tissue culture, because the polysaccharide protected it from nuclease degradation. This complex is of fundamental interest as it has not been reported in any other context and as yet has no role in the metabolism of the organism from which it is obtained. Alternatively, it may be an artifact of isolation of RNA from these particular cells since the complex may form through nonspecific association of RNA with a cellular polysaccharide component.

A fourth mechanism which has been demonstrated for potentiating interferon production by polynucleotides both in vitro and in vivo is the administration of proper doses and at a proper timing of certain metabolic inhibitors such as actinomycin (47) or cycloheximide (48, 49). These studies, which will be reviewed by Vilcek in this Symposium, have demonstrated that either in the mouse or in tissue culture, these metabolic antagonists increase the pro-

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3 De Clercq, E., F. Eckstein, H. Sternbach, T. C. Merigan. The antiviral activity of thiophosphate substituted polyribonucleotides in vitro and in vivo. In preparation.
duction of interferon when given together with I:C in vitro (47, 49) or in vivo (48). It has been interpreted that these agents are acting by interfering with the synthesis of an endogenous inhibitor of interferon formation (47, 49). The main return from these studies of the effect of antibiotics and lead acetate (50) on the interferon induction by polynucleotides is insight into the mechanism of interferon induction rather than a practically useful augmented response.

For example, the studies outlined in Table IV suggest that the postulated inhibitor or repressor of interferon induction by double-stranded synthetic RNA has no effect on an RNA virus induction of interferon. Full yields of the early interferon stimulated by I:C and the late interferon induced by NDV were produced in cell cultures undergoing dual stimulation. Hence the presence of the postulated repressor in cells did not block the NDV stimulation while it functioned to turn off synthetic RNA production of interferon. Verification that the first peak was entirely due to I:C and that the second was due to NDV was achieved through the use of actinomycin D and puromycin, respectively. Actinomycin failed to block the first round of interferon, whereas puromycin blocked the second. The dose of both drugs employed was selected on the basis of previous studies which showed them to inhibit NDV production without effecting yields from I:C (14).

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**Table IV**

NONVIRAL AND VIRAL INTERFERON RESPONSE IN DULLY INDUCED CELL CULTURES*

| Interval after dual stimulation | Interferon | IU/ml |
|-------------------------------|------------|-------|
|                              | I:C alone  | NDV alone | NDV and I:C |
|-----------------------------|------------|-----------|-------------|
| hr                           |            |           |             |
| 0-8                          | 154        | <5        | 240         |
| 8-23                         | 8          | 606       | 765         |
| 0-8                          | 135        | <5        | 280         |
| (Actinomycin D pretreated)   |            |           |             |
| 8-24                         | 30         | 34        | 34          |
| (Puromycin present)         |            |           |             |

* Cell monolayers were incubated for 1 hr at 37°C with either control media or NDV as indicated. After this time, the cell supernatants were replaced with either I:C (50 μg/ml) or control media. The fluids were then collected after the indicated intervals and assayed for interferon.

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4 Bausek, G. H., and T. C. Merigan. Simultaneous viral and non-viral interferon induction in human cell cultures. Proc. Soc. Exp. Biol. Med. 135:982.
CELL INTERACTION WITH SYNTHETIC RIBONUCLEOTIDES

Our conception as to the stages in the interaction of interferon-inducing RNA and cells is as follows: (a) binding to cell surface, (b) temperature dependent recognition step, and (c) degradation and utilization in cell RNA synthesis. By exposing cells to RNA in the cold, thereby arresting events at the binding step which appears to be an electrostatic nonenergy-requiring step, we are able to separate this step from a second step. If the cells are exposed to the enzyme ribonuclease after binding the RNA, this RNA will be destroyed while it is still at the cell surface. Thereby the biological effect of the RNA on the cells will be blocked. However, if the cells are warmed up to 38°C for 1–2 hr before this enzyme treatment, the commitment to interferon production has then been initiated and the enzyme has no effect as compared to controls.

Our studies (37) indicate that only a very small percentage of the RNA over cells is bound to the cell surface, that is 1% or less. In addition, the majority of this RNA stays on the surface of the cell during the interferon induction process, and only a very small amount actually penetrates the cell. However, as the third step (c) demonstrates, if cells are left in contact with RNA for longer periods of time, there clearly is degradation of this RNA to monomeric units which would then be utilized by the cell in its own RNA synthesis. This process of utilization is unessential for the interferon production process. On the other hand, it is not clear where the critical step for triggering interferon is. That is, whether it is related to the large amount of polymer on the surface of the cell or a small fraction of polymer taken within the cell.

Table V presents studies which followed cellular binding of radioactivity

| Interferon | Cell radioactivity | Calculated approx. number of I:C-3H |
|------------|-------------------|-------------------------------------|
|            | Time of 1 hr treatment | Time of 1 hr treatment | Time of 1 hr treatment |
| Treatment at 37°C | 0 hr | 1.5 hr | 0 hr | 1.5 hr | 0 hr | 1.5 hr |
| RNase | U/μl | cpm/2 × 10⁶ cells | molecules/cell |
| 2 | 68 | 10 | 11 | 5000 | 5000 |
| None | 132 | 137 | 420 | 412 | 150,000 | 150,000 |

* Cells were exposed to I:C-3H for 1 hr at 4°C, then washed 6 times with minimal essential medium (MEM) and treated at 37°C as indicated. Interferon production was measured after 11 hr total incubation at 37°C; cell radioactivity was determined immediately after 37°C treatment.
and interferon production in parallel, after the exposure of human skin cells to tritium-labeled I:C. In this system the cells were exposed to the polymer in the cold for 1 hr, all unbound material then was washed off, and the cells incubated at 37°C. The cell supernatants were collected for interferon assay after 10 hr. In the absence of ribonuclease approximately 130 units of interferon were made, whereas no interferon was made with ribonuclease treatment immediately after the binding step. However, if one delays ribonuclease treatment 1.5 hr, 68 units of interferon were made. Hence, during that 1.5 hr, a temperature-dependent recognition step occurred. One can see the fate of cell bound radioactivity during that same period in the middle section of Table V. There is 30-40-fold more radioactivity on the surface of the cell than within the cell—that is, 150,000 molecular equivalents per cell on the cell and no more than 5000 molecular equivalents within the cell. During this 1.5 hr period no increase in intracellular tritium-labeled I:C molecules can be seen. Hence, either some very small number of molecules enters the cell to interact with cytoplasmic or nuclear constituents in the initiation of interferon production, or the presence of the large amount of RNA on the surface of the cell at 37°C is a critical factor in triggering the induction of interferon. To make the system more sensitive, a higher specific activity material could be used, and perhaps then one could sense a critical increase in the intracellular fraction of the RNA molecule associated with interferon production during the 1.5 hr at 37°C.

To demonstrate the third stage of interaction of RNA with interferon-producing cells, that is the stage of degradation and incorporation into host RNA, we adopted a fundamentally different experimental design. All studies on the inducer-cell interaction described up to now were based on treatment of the cells with a short pulse of I:C in the cold and removal of extraneous polymer before further treatment. To study the degradation and incorporation, the cells were continuously incubated with I:C for several hours at 37°C. Here it was found that progressively more I:C was taken up by the fibroblasts and became resistant to degradation by ribonuclease.

In order to study the intracellular fate of the cell associated I:C, we performed radioautography of cells incubated with tritium-labeled I:C for various periods of time. These studies indicated that this ribonucleotide homopolymer pair was degraded in cells and incorporated into cellular RNA. The radioactivity was largely localized over nucleoli after 3 hr of incubation of the cells with the tritium-labeled I:C as shown in Fig. 3. Radioautographs at later times showed more grains distributed over the nucleoplasm and later over the cytoplasm as well. To verify that the isotope in cells was due to breakdown and reincorporation into newly synthesized host RNA, actinomycin and unlabeled cytidine were added. It was observed that this nucleolar labeling could be completely blocked. Here we believe the I:C was still
broken down but that the fragments were washed out during the preparation for radioautography, as they could not be incorporated into polymeric form. Since interferon was produced under these conditions, it is clear the incorporation of degraded I:C into cellular RNA is not required for interferon production, although it does occur as a major event in the cell interaction with I:C.

ANIMAL PROTECTION EXPERIMENTS

Table VI presents a summary of the animal virus challenge models from various laboratories which have been studied so far with I:C and which are most readily related to human disease. Although challenge doses may be much greater than in man, the route of infection and pathogenesis of disease would seem to resemble the human model. In all cases systemically administered I:C is active against the virus challenge if given prophylactically and in at least two models I:C is effective when given after the virus challenge at the time the virus is actively multiplying in the target organ (51, 53).

Obviously these findings are encouraging to those who would like to study polynucleotide inducers in human virus disease. However, another set of findings must be balanced against these positive findings. Systemically administered I:C, as any other pharmacologically active agent, has been shown to have a number of side effects in the whole animal. These include tumor-necrotizing effects (59), provocation (but not sensitization) of the local Schwartzman reaction (60), pyrogenicity in rabbits (61), and embryo-
toxicity (62), all of which resemble effects reported with endotoxin. Recently, a picture resembling "runt disease" has been produced in mice with several types of double-stranded RNA, including I:C. Also, synthetic double-stranded RNA has been shown to potentiate adjuvants and have an adjuvant effect in vivo (63), and to increase cell-mediated immunity (64) and RE cell activity (65). Whether any of these other in vivo effects will be limiting for clinical studies or give evidence regarding the fundamental mechanism of interferon stimulation by this class of agents is only speculative.

### Table VI

**STUDIES WITH POLYANIONIC INTERFERON INDUCERS IN ANIMAL MODELS RELATING TO HUMAN VIRAL DISEASE**

| Route          | Virus                  | Animal | Human parallel                  |
|----------------|------------------------|--------|--------------------------------|
| Intracorneal   | Herpes simplex         | Rabbit (51, 52)* | Herpes simplex keratitis       |
| Intramuscular  | Rabies                 | Rabbit‡ | Rabies Encephalomyelitis       |
| Intranasal     | Vesicular stomatitis   | Mouse (53)§  | Herpes or enterovirus encephalitis |
| Intranasal     | Influenza              | Mouse (6, 54–56) | Influenza                      |
| Intranasal     | Sendai                 | Mouse (55, 56) | Influenza and other respiratory infections |
| Intranasal     | Vaccinia               | Mouse (55)  | Smallpox                       |
| Intravenous    | Vaccinia               | Mouse (2, 5, 6, 56-58) | Smallpox (Viremic phase)        |
| Intranasal     | Columbia SK            | Mouse (55, 56) | Polio, Coxsackie, and ECHO infections |

* Kaufman, H. E., E. D. Ellison, and S. R. Waltman. 1969. Double stranded RNA, an interferon inducer, in herpes simplex keratitis. Personal communication. 
‡ Postic, B., and P. Fenje. 1970. Prophylaxis of rabies in rabbits by poly I:C. *Bacteriol. Proc.* 155; and Janis, B., and K. Habel. 1970. Polyribioncinic and polyribocytidylic acid polymers (poly I:C) in rabies prophylaxis. *Fed. Proc.* 29:636. § De Clercq, E., M. R. Nuwer, and T. C. Merigan. 1970. The role of interferon in the protective effect of a synthetic double-stranded polyribonucleotide against intranasal vesicular stomatitis virus challenge in mice. *J. Clin. Invest.* In press.

As preparations of I:C can vary one from another in terms of annealing, and the studies demonstrating side effects have not, in the main, been carried out with studies of concomitant interferon induction or antiviral protection, we can not directly relate results of antiviral activity and toxicity in different laboratories. Accurate therapeutic ratios must be established in animal models before we can make predictions regarding human efficacy. Another complication in such predictions is that toxicity of these materials varies widely from...
one animal species to another (56), and hence, therapeutic ratios must ultimately be established in man. Local rather than systemic approaches to therapy, such as can be utilized in the skin and eye and perhaps in the respiratory tract, are obviously less complicated, but with macromolecules like I:C there are likely to be problems in delivery or distribution to the diseased area. However, by combining knowledge of the best routes of delivery and of the timing of various effects of the inducers in animal models with knowledge of viral infectious processes in any given human disease, one can set up the most favorable approach to clinical prophylaxis or therapy. It appears that successful manipulation of host resistance mechanisms in the face of disease is likely to require greater knowledge of details of the disease process than chemotherapy with agents which act directly against parasites.

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