Interferon Production by Nonviral Stimuli of Microbial Origin

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ABSTRACT An increasing number of nonviral materials of microbial origin has been reported to stimulate the production of interferon in cell cultures and (or) in animals. These materials include (a) gram-negative bacteria or the endotoxins prepared from their cell walls, (b) other microorganisms such as Rickettsiae, Bedsoniae, Protozoa, and (c) fungal products such as a mannan from Candida and various antibiotics which act as protein synthesis inhibitors, e.g., glutarimide antibiotics and tenuazonic acid. A summary is presented of the current state of knowledge about interferon production in animals by the most thoroughly studied nonviral substance of microbial origin, bacterial endotoxin. Further evidence is presented which clearly distinguishes the “endotoxin-type” of interferon response in animals from the response seen after the injection of virus. The data suggest that the release of preformed interferon from the tissues occurs in animals injected with endotoxin. On the other hand, interferon produced in response to the injection of virus is newly synthesized protein. While the exact chemical structure of the component of bacterial endotoxin responsible for interferon release has not yet been elucidated, it is clear that the lipid portion of the lipopolysaccharide, rather than the O-specific polysaccharide side chains or the core polysaccharide, is the active moiety.

Since the discovery of interferon by Isaacs and Lindenmann in 1957, it has become apparent that the production of this inhibitor is not solely a response of host cells to infection by viruses. It has been well documented that interferon is produced by animals or cell cultures exposed to a wide variety of nonviral stimuli. These include (a) stimuli of microbial origin, (b) substances from higher plants, and (c) synthetic polymers. I have been asked to discuss interferon stimuli of microbial origin. Other contributors to this symposium will consider stimuli not covered in this presentation.

Table I lists the known nonviral materials of microbial origin which have been reported to be capable of stimulating the production of interferon in cell cultures and (or) in animals. For the purposes of this table, the term “cell cultures” excludes leukocytes obtained from the blood or peritoneal cavity. This exclusion precludes the listing as cell cultures any cells used directly


| Gram-negative bacteria                      | Interferon production in |
|--------------------------------------------|--------------------------|
| **Stimulus** | **Cell cultures** | **Animals** | **References** |
| Brucella       | 0*                  | +*          | 1, 2          |
| Escherichia    | 0                   | +           | 3             |
| Aerobacter     | 0                   | +           | †             |
| Serratia       | 0                   | +           | 2             |
| Salmonella     | 0                   | +           | 2             |
| Vibrio         | n.d.                | +           | †             |
| Bordetella     | 0                   | +           | 4             |
| Francisella    | n.d.                | +           | 5             |

| Endotoxin and cell wall fractions from     | Interferon production in |
|--------------------------------------------|--------------------------|
| **Stimulus** | **Cell cultures** | **Animals** | **References** |
| Escherichia    | 0                   | +           | 2, 3          |
| Aerobacter     | 0                   | +           | †             |
| Salmonella     | 0                   | +           | 2             |
| Brucella       | 0                   | +           | 6             |
| Pseudomonas    | n.d.                | +           | †             |
| Hemophilus     | ?                   | +           | 35            |

| Organism other than bacteria               | Interferon production in |
|--------------------------------------------|--------------------------|
| **Stimulus** | **Cell cultures** | **Animals** | **References** |
| Rickettsia       | +                   | +           | 7, 8          |
| Bedsonia        | +                   | +           | 9             |

| Protozoa        | Interferon production in |
|-----------------|--------------------------|
| **Stimulus** | **Cell cultures** | **Animals** | **References** |
| Plasmodium      | n.d.                  | +           | 10            |
| Toxoplasma      | 0                    | +           | 11, 12        |

| Fungal products | Interferon production in |
|-----------------|--------------------------|
| **Stimulus** | **Cell cultures** | **Animals** | **References** |
| Candida: mannan | 0                   | +           | 13            |
| Streptomyces (glutarimide antibiotics) |  |  |  |
| Cycloheximide   | 0                   | +           | 14            |
| Acetoxy-cycloheximide | 0 | + | 15 |
| Streptovitacin A | 0          | +           | 15            |
| Streptimidone   | 0                   | +           | 15            |
| (Aminoglycosides) kanamycin | n.d. | + | 34 |
| Alternaria: tenuazonic acid | 0 | + | 15 |

* 0 = no interferon production.
+ = interferon production.
n.d. = not done.
† J. S. Youngner, unpublished data.

From the animal without intervening mitosis and cell division. In other words, experiments involving the use of macrophages or other leukocytes are considered ambiguous since these cells are only "maintained" in vitro.

From the information in Table I it is readily seen that extremely diverse organisms and products are capable of stimulating the production of interferon in cell cultures and(or) in animals. In the case of bacteria, only gram-negative genera have been found to be active. In my laboratory, many species
of gram-positive organisms have been injected intravenously into mice in concentrations similar to those used with gram-negative bacteria. In no case have we noted (a) the appearance of circulating interferon or (b) the establishment of a hyporeactive state which reduced the ability of the animals to give an interferon response within 24–48 hr to an injection of gram-negative bacteria or virus (16). Among the gram-positive organisms injected were one or more species of the following genera: *Bacillus, Mycobacterium, Corynebacterium, Staphylococcus, Streptococcus, Cryptococcus, Saccharomyces, Candida, Hansenula.* It should be noted that there has been one report (5) of interferon production in chickens injected intravenously with large numbers of living *Listeria monocytogenes*; heat-killed bacteria failed to produce this result.

The majority of stimuli listed in Table I are active in animals and not in cell cultures, and it is interesting to consider possible explanations for this finding. One must suppose either the failure of cell cultures to take up effective amounts of the stimulus (e.g. bacterial endotoxin) or the large variety of cell types available in the intact animal. This discrepancy in interferon production in cell cultures and in animals has also been observed in a special case involving Newcastle disease virus (NDV) (17). This virus is an excellent stimulus of interferon production in a continuous line of mouse fibroblasts (L cells). When the virus is inactivated by heating at 56°C, its ability to induce interferon production in L cells is lost. In contrast, the intravenous injection of the same heat-inactivated NDV into mice is followed by the appearance of circulating interferon. This observation emphasizes that, although cell cultures fail to respond, there are cells in the animal which can interact with such noninfective, heat-inactivated virus and produce interferon.

It is of interest to note that of the stimuli of microbial origin listed in Table I, Rickettsiae and Bedsoniae are the only agents which have been reported to produce interferon in cell cultures as well as in animals. In contrast to all the other agents tested, these organisms multiple intracellularly, and it is under such conditions of replication that interferon is produced in cell cultures.

In order to bring some order out of the diversity of the organisms and products capable of producing interferon in animals, the remainder of this presentation will be devoted to summarizing what is known about interferon production in animals by the most thoroughly studied nonviral stimulus of microbial origin, bacterial endotoxin.

**Comparison of Interferon Production in Animals by Endotoxin and by Virus**

Previous reports from this laboratory have identified two separate and distinct patterns of interferon production in animals: the "endotoxin type" and

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1 The methods employed in these studies can be briefly summarized. Interferon was produced by the intravenous injection of 0.1-ml volumes of the different stimuli into mice, using at least 10 animals per sample. At appropriate times, blood was obtained by cardiac puncture using heparin-
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the "virus type" (6, 14). In mice, doses of cycloheximide which effectively inhibited protein synthesis did not prevent the appearance of circulating interferon when *Escherichia coli* endotoxin was injected intravenously. This observation suggested the existence of preformed or precursor interferon in the tissues of intact animals and the release of this interferon by endotoxin. In contrast, the appearance of interferon in plasma after the injection of Newcastle disease virus was markedly suppressed in animals pretreated with cycloheximide. This indicated that the interferon produced in response to injection of virus was newly synthesized protein. Similar conclusions have been reached by Ke and his coworkers (18), using inhibitors of protein synthesis in rabbits.

The contrasting effects of blockade of protein synthesis on interferon production in mice inoculated with NDV or endotoxin raised questions which could be answered experimentally. In the case of the virus type of response, is the synthesis of interferon continuous over a 10-12 hr period, or does the synthesis take place shortly after the virus is injected? And does release occur over a period of many hours? Alternatively, is interferon synthesized throughout the 10 hr period of rising plasma titers? What patterns occur when blockade of protein synthesis is imposed at different times after the injection of virus?

The following experiment was carried out in an attempt to answer these questions. Mice were inoculated intraperitoneally with saline or with cycloheximide (244 mg/kg) 1 hr before or 2, 4, or 6 hr after a large dose of NDV was given intravenously. Groups of 10 mice were bled at different times, and the interferon content of the pooled plasmas was determined. It can be seen from the curves in Fig. 1 that when cycloheximide was given 1 hr prior to NDV, the expected 90% inhibition of interferon production was observed. When cycloheximide was given at 2, 4, or 6 hr after NDV, there was a shutoff of further increase of interferon in the plasma. In fact, a marked decrease in titer was seen when the animals were bled 2 or 4 hr after cycloheximide had been injected. The similar slopes of the declining titers after the injection of cycloheximide can be interpreted as representing the clearance of circulating interferon from the bloodstream after inhibition of protein synthesis rapidly curtailed further production. These data indicate that when NDV is used as the stimulus, interferon synthesis occurs throughout the period of rising plasma concentration and rules out the possibility that the rising titer of plasma interferon is due to an early synthesis and a slow release.

Rinsed syringes, and pooled plasmas were tested for interferon by the plaque reduction method. Cultures of a continuous line of mouse fibroblasts (L cells, clone 929) were exposed for 20 hr to dilutions of plasma and then challenged with 40-60 plaque-forming units (p.f.u.) of vesicular stomatitis virus. The inhibitory titers of the plasma were expressed as the reciprocal of the dilution which reduced the plaque count to 50% of that of control cultures. The details of these methods have been published elsewhere (17).
The pattern of interferon release in mice given endotoxin was studied by the same technique. Mice were inoculated with saline or with cycloheximide at different times before or after 100 µg of endotoxin was injected intravenously. The interferon titer of pooled plasma was determined at different times after the injection of endotoxin, and the results are plotted in Fig. 2. As expected, endotoxin produced the usual early peak titer of plasma interferon in control animals pretreated with saline. Cycloheximide injected 1 hr prior to endotoxin produced an enhanced and prolonged interferon response. When the injection of cycloheximide was delayed until 2 hr after endotoxin was given (when plasma interferon titer had reached its peak), no enhancement or prolongation of the interferon response was elicited. These results indicated that the events involved in the potentiation of interferon release by cycloheximide occurred earlier than 2 hr after the injection of endotoxin.

In order to determine more precisely the early events involved in the potentiation of interferon release by cycloheximide in animals given endotoxin, the following experiment was carried out. Mice were given cycloheximide 60
min before, or at the same time, that endotoxin was injected intravenously; other animals were injected with cycloheximide 15, 30, 60, or 120 min after the endotoxin was given. Groups of animals were bled at appropriate intervals, and the interferon titers of the plasma pools were compared to the titers obtained in animals given saline 60 min prior to endotoxin, or in those given only cycloheximide. The data in Table II show that cycloheximide produced an enhanced and prolonged interferon release when given to mice up to 30 min after the endotoxin was injected. By 1 or 2 hr after the injection of endotoxin, no significant enhancement of interferon release was produced by the blockade of protein synthesis. These results indicate that the critical events involved in the release of interferon by endotoxin occur within 30 min after the stimulus is injected.

It is necessary at this point to make an exception to the limits set on the scope of this presentation; essential data dealing with interferon production in mice by a synthetic polymer are included for reasons that will be apparent. We have reported previously that there is a striking similarity between inter-
TABLE II
TIME OF INJECTION OF CYCLOHEXIMIDE: INFLUENCE ON INTERFERON PRODUCTION IN MICE BY ENDOXOIN*

| Mice inoculated with | At 0 time (i.v.) | Interferon titer of plasma at |
|----------------------|-----------------|-----------------------------|
|                      |     | 2 hr | 5 hr | 8 hr |
| Saline† at:          |     |      |      |      |
| -60 min              | 50 µg endotoxin| 400  | 65   | 40   |
| Cycloheximide§ at:   |     |      |      |      |
| -60 min              | 50 µg endotoxin| 280  | 5,200| 7,600|
| 0 min                | "    | 120  | 2,600| 7,500|
| +15 min              | "    | 200  | 1,700| 4,000|
| +30 min              | "    | 700  | 2,000| 4,700|
| +60 min              | "    | 900  | 500  | n.d.||
| +120 min             | "    | 450  | 520  |      |
| Cycloheximide only at: | -60 min | <32  | 110  | 200  |

* From reference 15.
† 1.0 ml intraperitoneally.
§ 244 mg/kg in 1.0 ml saline intraperitoneally.
|| Not done.

The experiments which have been described provide additional information about the two different patterns of interferon production in mice injected with virus or bacterial endotoxin. Blockade of protein synthesis at different times...
after injection of NDV established that the interferon synthesis induced by virus is continuous for at least 10–12 hr. This finding ruled out the possibility of early synthesis and later release following induction of interferon synthesis by the virus. In addition, these results seem to refute the hypothesis that in the virus-induced interferon response protein synthesis was needed for the synthesis of an enzyme (or enzymes) capable of activating a preformed precursor interferon molecule (6). The sharp decrease of circulating interferon which resulted from blockade of protein synthesis at different times after injection of NDV did not support a mechanism involving a hypothetical interferon-activating enzyme. One would expect an enzyme with a reasonably long half-life to function for a significant period of time.

The mechanism by which the inhibition of protein synthesis in the animal enhances and prolongs the interferon response elicited by endotoxin is not clear. The importance of very early events in the interaction of endotoxin and host cells is shown by the need to give the protein synthesis inhibitor within 30 min after the endotoxin is injected. Since endotoxin produces a 2 hr peak of circulating interferon under ordinary circumstances, it is apparent that the release of interferon from the participating host cells is effected rather quickly. Significant amounts of interferon appeared in the circulation between 30 and 60 min after the intravenous injection of endotoxin (2). It is possible that the enhancement of interferon production by endotoxin in animals pretreated with protein synthesis inhibitors may be the result of a failure of detoxifying mechanisms dependent on protein synthesis. The failure of early detoxification of endotoxin could permit the involvement of more cells in the release of interferon over a longer period of time. In addition, the release

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**Table III**

**TIME OF INJECTION OF CYCLOHEXIMIDE: INFLUENCE ON INTERFERON PRODUCTION IN MICE BY POLY I:C**

| Mice inoculated with | At 0 hr (i.v.) | Interferon titer of plasma at |
|----------------------|---------------|-----------------------------|
|                      | hr            | 2 hr | 5 hr | 8 hr |
| Saline§ at:          | -1            | 10 μg poly I:C             | 250  | 200  | 100  |
| Cycloheximide§ at:   | -1            | 10 μg poly I:C             | 100  | 4,200| 10,000|
|                      | +1            | "                      | 300  | 2,500| 6,000 |
|                      | +2            | "                      | 410  | 1,000|       |
|                      | +3            | "                      | 170  | 320  |       |
|                      | +5            | "                      | <128 |      |       |
| Cycloheximide only at: | -1          | ---              | 60   | 200  |       |

* From reference 15.

§ 1.0 ml intraperitoneally.

§ 244 mg/kg in 1.0 ml saline intraperitoneally.
of interferon by glutarimide antibiotics or tenuazonic acid alone is a phenomenon which must be taken into consideration. The enhancing effect of the antibiotics on interferon release by endotoxin may be the result of a synergistic action of the two agents by mechanisms which are poorly understood and difficult to determine in intact animals. It is of interest that Vilček and his coworkers (22; J. Vilček, personal communication) have reported that inhibition of protein synthesis enhances and prolongs interferon production in rabbit kidney cells stimulated by poly rI:rC. The interferon production curves they described were strikingly similar to those we have found in mice given poly rI:rC or endotoxin. The availability of a cell culture system for the study of the phenomena involved in release of preformed precursor interferon may make feasible experiments which cannot be done in animals.

INVESTIGATIONS OF THE CHEMICAL PORTION OF BACTERIAL ENDOTOXIN RESPONSIBLE FOR RELEASE OF INTERFERON IN ANIMALS

Earlier presentations in this symposium have dealt with the mechanism of induction of interferon synthesis in cells infected with viruses. In this section of my presentation, I shall summarize our attempts to determine which chemical structure of complex endotoxin preparations is responsible for the release of interferon in mice (23-25). These experiments were carried out with Salmonella typhimurium mutants with known mutational blocks in the biosynthesis of cell wall lipopolysaccharides (26-29).

Using intravenous injection of suspensions of intact heat-killed Salmonella typhimurium mutants, it was found that the interferon response elicited in mice was not dependent on the presence of a complete cell wall lipopolysaccharide. In fact, a mutant (G30/C21) which has lost all the polysaccharide side chains and sugars of the O-antigen and contains only 2-keto-3-deoxyoctonate and lipid was indistinguishable in its interferon-stimulating ability from the wild type (LT-2) which possesses a complete O-antigen with polysaccharide side chains. This conclusion, based on the use of intact heat-killed bacteria, was confirmed using lipopolysaccharides and glycolipids extracted from the different mutants by methods which have been described elsewhere in detail (24). The data obtained with these materials showed that purified cell wall glycolipid from the heptoseless mutant G30/C21 and lipopolysaccharide from the wild-type organism did not differ significantly in their interferon-releasing activity in mice. These results suggested that it was not the polysaccharide or heptose-phosphate backbone portion of endotoxin which was responsible for the release of interferon. It was essential, however, to eliminate two possibilities: (a) that despite mutational blocks in synthesis of complete O-antigen, certain polysaccharide components could be present but not incorporated into the cell wall of the deficient mutants and (b) that these components were re-
sponsible for the interferon-stimulating effect of whole cells with major deficiencies in cell wall composition.

Interferon-releasing activity of O-specific hapten and core glycolipid were studied in the following manner. Core glycolipid from strain TV-161 (Rb chemotype) was prepared by a phenol extraction technique which has been described elsewhere (24). Pure O-specific hapten from strain TV-161 was isolated by the method of Kent and Osborne (30). Both the core glycolipid and O-specific hapten of strain TV-161 were tested in mice for their interferon-releasing activity. Pretreatment of mice with 244 mg/kg of cycloheximide was used to enhance interferon release (14). In addition to interferon-releasing activity, the lethality of the bacterial preparations was tested. Groups of 10 mice were injected intravenously with twofold dilutions of the materials under test, and deaths were recorded over a 72 hr period. The LD$_{50}$ was calculated by the method of Reed and Muench (31). The lethal effect of the preparations tested was potentiated by pretreatment of the mice with 80 mg/kg of cycloheximide, a dose of antibiotic which causes a transient interruption of protein synthesis but is not in itself lethal (14, 15). From the data in Table IV it can be seen that core glycolipid was lethal only for mice pretreated with cycloheximide, the LD$_{50}$ being about 40 µg in these animals. In contrast, 10-fold greater doses of the pure hapten had no demonstrable lethality for mice. With regard to interferon, the dose response to core glycolipid was similar to what would be seen following injection of complete lipopolysaccharide with O side-chains or with Re chemotype glycolipid. However, no evidence of interferon release by hapten was observed; even in mice pretreated with 244 mg/kg of cycloheximide, a dose which markedly enhanced interferon release by core glycolipid, the level of interferon present in mice treated with hapten did not differ significantly from that released by cyclo-

| Preparation | LD$_{50}$ dose in mice treated with | Interferon titer of plasma of mice treated with |
|-------------|-------------------------------------|-----------------------------------------------|
|             | Saline | Cycloheximide (80 mg/kg) | Dose | Saline | Cycloheximide (244 mg/kg) |
| Core glycolipid | >500 | 30 | 100 | 100 | 7200 |
| Hapten | >500 | >500 | 10 | <32 | 400 |
| Saline | — | — | — | <32 | 100 |

* From reference 25.  
† Mice bled at 2 hr.  
§ Mice bled at 8 hr.
heximide alone. Detailed analytical data obtained spectrometrically and by
gas-liquid chromatography (32) showed that the contamination of hapten
by core glycolipid was negligible.

The essential role of the lipid portion of Salmonella lipopolysaccharide in the
production of interferon in mice was demonstrated in the following manner.
Glycolipid from the heptoseless mutant G30/C21 (Re chemotype) was hy-
drolyzed by acid treatment to produce a series of hydrolysates containing
decreasing amounts of KDO (2-keto-3-deoxyoctonate) (33). The macro-
molecular materials produced by partial hydrolysis were isolated by ultra-
centrifugation, washed with water, and lyophilized. The conditions of hydroly-
sis and the interferon-releasing activity of a number of materials are presented
in Table V. It can be seen that acid treatment of G30/C21 glycolipid hy-

| TABLE V |
| INTERFERON PRODUCTION IN MICE BY S. TYPHIMURIUM LIPOPOLYSACCHARIDE AND GLYCOLIPIDS—EFFECT OF ACID TREATMENT* |

| Glycolipid or lipopolysaccharide (chemotype) | Treatment                  | Residual KDO content‡ | Dose | Saline§ (244 mg/kg) |
|--------------------------------------------|---------------------------|-----------------------|------|--------------------|
| G30/C21 (Re)                               | None                      | 100                   | 100  | 370                |
|                                           | 0.05 N Acetic acid (pH 3.1) | 5 min 100°C           | 100  | 300                |
|                                           |                           |                       | 10   | 250                |
|                                           |                           |                       | 1    | 300                |
|                                           |                           |                       | 1    | 450                |
|                                           | 0.05 N Acetic acid (pH 3.1)| 10 min 100°C          | 100  | 250                |
|                                           |                           |                       | 10   | 220                |
|                                           |                           |                       | 1    | 80                 |
|                                           |                           |                       | 1    | 45<32              |
|                                           | 0.05 N Acetic acid (pH 3.1)| 20 min 100°C          | 100  | 250                |
|                                           |                           |                       | 10   | 250                |
|                                           |                           |                       | 1    | <32                |
|                                           | 0.02 N H₂SO₄ (pH 1.7)     | 20 min 100°C          | 100  | 360                |
|                                           |                           |                       | 10   | 320                |
|                                           |                           |                       | 1    | 45<70              |
| LT-2 (S)                                   | None                      | 100                   | 100  | 270                |
|                                           |                           |                       | 10   | 290                |
|                                           |                           |                       | 1    | 180                |
| Saline only                                | —                         | —                     | —    | <32                |

* From reference 25.
‡ The KDO content of native G30/C21 glycolipid (18%) was taken as 100%.
§ Mice bled at 2 hr.
¶ Mice bled at 8 hr.
¶¶ These conditions of hydrolysis yield Lipid A (25).
drolytically removes KDO from the compound; however, this hydrolysis does not alter the interferon-releasing activity of the material. In fact, hydrolyzed glycolipid from strain G30/C21, from which all detectable KDO had been removed, was identical with LPS (lipopolysaccharide) from the wild-type organism in interferon-releasing activity. Similar results have been obtained with glycolipids from two different Salmonella species (25).

The results which have been presented show that wild-type Salmonella typhimurium lipopolysaccharide and glycolipids from Rb and Re chemotypes are equally active in releasing interferon in mice. Furthermore, materials obtained by graded acid hydrolysis of Re glycolipid, resulting in lipid products with decreasing content of KDO, did not differ from lipopolysaccharide in their interferon-releasing activity. Lipid A, which contains no KDO, was also fully active in releasing interferon. While the exact nature of the chemical structure responsible for interferon release has not yet been elucidated, it is clear that the lipid portion of the lipopolysaccharide, rather than the polysaccharide side chains or the core polysaccharide, is the active moiety.

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REFERENCES

1. Youngner, J. S., and W. R. Stinebring. 1964. Interferon production in chickens injected with Brucella abortus. Science (Washington). 144:1022.
2. Stinebring, W. R., and J. S. Youngner. 1964. Patterns of interferon appearance in mice injected with bacteria or bacterial endotoxin. Nature (London). 204:712.
3. Ho, M. 1964. Interferon-like viral inhibition in rabbits after intravenous administration of endotoxin. Science (Washington). 146:1472.
4. Borecký, L., and V. Lackovíc. 1966. Partial heterologous tolerance to interferon production in mice infected with Br. pertussis. Acta Virol. 10:271.
5. Lukás, B., and J. Hrušková. 1967. A virus inhibitor circulating in the blood of chickens, induced by Francisella tularensis and Listeria monocytogenes. Folia Microbiol. 12:137.
6. Youngner, J. S. 1968. Interferon production in mice injected with viral and non-viral stimuli. In Medical and Applied Virology. M. Sanders and E. H. Lennette, editors. Warren H. Green, Inc., St. Louis, Mo. 210.
7. Hopps, H. E., S. Kohno, M. Kohno, and J. E. Smadel. 1964. Production of interferon in tissue cultures infected with rickettsia tsutsugamushi. Bacterial. Proc. 115.
8. Kázar, J. 1966. Interferon-like inhibitor in mouse sera induced by rickettsiae. Acta Virol. 10:277.
9. McGan, T. C., and L. Hanna. 1966. Characteristics of interferon induced in vitro and in vivo by a TRIC agent. Proc. Soc. Exp. Biol. Med. 122:421.
10. Huang, K. Y., W. W. Schultz, and F. B. Gordon. 1968. Interferon induced by Plasmodium berghei. Science (Washington). 162:123.
11. Rytel, M. W., and T. C. Jones. 1966. Induction of interferon in mice infected with Toxoplasma gondii. Proc. Soc. Exp. Biol. Med. 123:859.
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12. Freshman, M. M., T. C. Merigan, J. S. Remington, and I. E. Brownlee. 1966. In vitro and in vivo antiviral action of an interferon-like substance induced by Toxoplasma gondii. Proc. Soc. Exp. Biol. Med. 123:862.

13. Borečky, L., V. Lackovič, D. Blaškovič, L. Masler, and D. Škrl. 1967. An interferon-like substance induced by mannans. Acta Virol. 11:264.

14. Youngner, J. S., W. R. Stinebring, and S. E. Taube. 1965. Influence of inhibitors of protein synthesis on interferon formation in mice. Virology. 27:541.

15. Youngner, J. S. 1970. Influence of inhibitors of protein synthesis on interferon formation in mice. II. Comparison of effects of glutarimide antibiotics and temuazonic acid. Virology. 40:335.

16. Youngner, J. S., and W. R. Stinebring. 1965. Interferon appearance stimulated by endotoxin, bacteria, or viruses in mice pretreated with E. coli endotoxin or infected with Mycobacterium tuberculosis. Nature (London). 208:456.

17. Youngner, J. S., A. W. Scott, J. V. Hallum, and W. R. Stinebring. 1966. Interferon production by inactivated Newcastle disease virus in cell cultures and in mice. J. Bacteriol. 92:2862.

18. Ke, Y. H., S. H. Singer, B. Poitsch, and M. Ho. 1966. Effect of puromycin on virus and endotoxin induced interferon-like inhibitors in rabbits. Proc. Soc. Exp. Biol. Med. 121:181.

19. Youngner, J. S., and J. V. Hallum. 1968. Interferon production in mice by double-stranded synthetic polynucleotides: induction or release? Virology. 35:177.

20. Absheer, M., and W. R. Stinebring. 1969. Toxic properties of a synthetic double-stranded RNA. Nature (London). 223:715.

21. Lindsay, H. L., P. W. Trown, J. Brandt, and M. Forbes. 1969. Pyrogenicity of poly I-poly C in rabbits. Nature (London). 223:717.

22. Vilček, J., T. G. Rosman, and F. Varacalli. 1969. Differential effects of actinomycin D and puromycin on the release of interferon induced by double stranded RNA. Nature (London). 222:682.

23. Youngner, J. S., and D. S. Feingold. 1967. Interferon production in mice by cell wall mutants of Salmonella typhimurium. J. Virol. 1:1164.

24. Feingold, D. S., J. S. Youngner, and J. Chen. 1968. Interferon production in mice by cell wall mutants of Salmonella typhimurium. II. Effect of purified glycolipid from S and Re chemotypes. Biochem. Biophys. Res. Commun. 32: 554.

25. Feingold, D. S., J. S. Youngner, and J. Chen. 1970. Interferon production in mice by cell wall mutants of Salmonella typhimurium. III. Role of lipid moiety of bacterial lipopolysaccharide in interferon production in animals. Ann. N. Y. Acad. Sci. In press.

26. Lüderitz, O., A. M. Staub, and O. Westphal. 1966. Immunochemistry of O and R antigens of Salmonella and related Enterobacteriaceae. Bacteriol. Rev. 30:192.

27. Nikaido, H., K. Nikaido, T. V. Subbaiah, and B. A. D. Stocker. 1964. Rough mutants of Salmonella typhimurium. III. Enzymatic synthesis of nucleotide-sugar compounds. Nature (London). 201:1301.

28. Osborn, M. J., S. M. Rosen, L. Rothfield, L. D. Zeleznick, and B. L. Horecker. 1964. Lipopolysaccharide of the gram-negative cell wall. Science (Washington). 145:783.

29. Subbaiah, T. V., and B. A. D. Stocker. 1964. Rough mutants of Salmonella typhimurium. I. Genetics. Nature (London). 201:1298.

30. Kent, J. L., and M. J. Osborn. 1968. Properties of the O-specific hapten formed in vivo by mutant strains of Salmonella typhimurium. Biochemistry. 7:4396.

31. Reed, L., and H. Muench. 1938. A simple method for estimating fifty per cent endpoints. Amer. J. Hyg. 27:493.

32. Davies, C., S. Freedman, H. Douglas, and A. Braude. 1969. Analysis of sugars in bacterial endotoxins by gas-liquid chromatography. Anal. Biochem. 28:243.

33. Westphal, O., A. Nowotny, O. Lüderitz, H. Hwong, and E. Egger-Derger. 1958. Die Bedeutung der Lipoid-Komponente (Lipoid A) für die biologischen Wirkungen bakterieller Endotoxine (Lipopolysaccharide). Pharm. Acta Helv. 33:401.

34. Lukáš, B., and J. Hrstíčková. 1968. In vitro induction of an interferon-like inhibitor by kanamycin. Acta Virol. 12:263.
Discussion from the Floor

Dr. B. L. Wasilauskas (Columbia-Presbyterian Medical Center, New York): I'd like to ask Dr. Youngner if he has ever used any other acid hydrolysis to obtain his KDO. I noticed he used 0.2 N sulfuric. I was just wondering if he ever used hydrochloric or formic acid. We've used these procedures and found that we were able to isolate KDO in significant amounts, using 1 N and higher normalities of these acids for periods up to 30 min.

Dr. Youngner: The only other acid treatment that we've used is, I believe, 0.05 N acetic acid for longer periods of time, and we've obtained graded hydrolysis. We've never used the acids which you mentioned.

Dr. Wasilauskas: What procedures did you use to determine your KDO? The TBA method?

Dr. Youngner: That's right, the method of Osborne.

Dr. Wasilauskas: And you were not able to isolate KDO from sulfuric acid?

Dr. Youngner: We were not. We hydrolyzed the KDO and separated the material by ultracentrifugation after treatment. There was no KDO present in this material. We could account for it all in the hydrolysate.

Dr. Wasilauskas: Also, I'd like to ask if you've had any problem with this glucan that Nikaido has indicated is present in many lipopolysaccharide preparations?

Dr. Youngner: From the analytical material, which I did not present for purposes of brevity, it is not likely that we have contamination with this material.

Dr. Levy: Let me presume upon my authority as the Chairman to ask a more generalized question. The nature of preformed interferon has been a troublesome concept for many people, including myself. As everybody has observed, you can stimulate an animal to release the preformed material. Then you can't stimulate it again immediately, but after a reasonably short period of time the animal can be restimulated. This implies, I think, a replenishment of the supplies of the stored material. Does this mean that interferon is being continually produced and stored up somehow, or is the preformed material present in the animal as a result of prior infections? How do you envision this?

Dr. Youngner: This is a rather difficult question to get at experimentally, but what we've assumed is that the depletion effect by the first inoculation is due to the toxicity of the materials. This depletion may result from release, and then the interferon has to be resynthesized. Or there may be appreciable destruction of the cells involved, and then these cell populations would have to be repopulated in order for the animal to become reactive again. The interesting thing is that there is what we refer to as cross-hyporeactivity, so that an injection of lipopolysaccharide produces hyporeactivity to virus, and vice versa. This must mean that the same cells are affected by the first stimulus in terms of its toxicity or depletion effect, and then the second stimulus cannot act. Two different mechanisms are envisaged here, release or synthesis. It's possible that cells are capable of more than one type of interferon response, and we have to take that into consideration, too.
From the Floor: Has the same experiment been done with germfree mice, because it could perhaps exclude the possibility that previous infection produces the interferon which is released?

Dr. Youngner: We have not done any experiments with germfree mice, but others have reported that germfree mice give good interferon responses with endotoxin.

Dr. Lockart: I would like to add a comment to your previous question, Dr. Levy, concerning whether some interferons might not be being made continuously in animals. I have here six references that are in the literature which show that lymphoid cells, of various sorts, kept in culture can make interferon spontaneously.

We reject the idea of spontaneous interferon production by saying that because most of these cells are from leukemia patients they are really infected cells and there's a latent inducer there. But I think that when you start accumulating this much data that some cells make interferon over long periods of time continuously, we may be at the stage where we should consider that not all interferon has to be induced.

Dr. Youngner: I would like to make one additional comment. I recall to you my stricture in what I classified as cell cultures in the first four slides I showed. It was because of this stricture and the ambiguity of the use of lymphoid cells, peritoneal exudate cells, anduffy-coat cells from animals that I did not call them cell cultures but “maintained” cells because there have been so many references to these primary cells producing interferon without any stimulus added.

Dr. Levy: Just as further reference to Dr. Lockart's comments to my question—With Dr. Haase we have shown similar effects with lymphocytes and have pretty well excluded the likelihood that infecting virus is responsible for the continuous interferon production by lymphocytes in culture.

Dr. Robert M. Friedman: You can produce interferon in rabbit cells in the presence of cycloheximide and antimycin. We have done extensive work in human cells in culture and shown the same thing. Although we were very skeptical when we started this work, I think we have come around to the feeling that there is such a thing as preformed interferon in constant production. In my lab we have tried to sum this up in a form that's easy to remember and maybe familiar to all of you—"You can take interferon out of the animal, but you can't take the animal out of interferon."

Dr. James G. Gallagher (University of Vermont, Burlington, Vt.): Dr. Youngner, would you care to comment on the mechanism of release of interferon elicited by the active lipid fraction? What do you envisage actually occurs at the molecular level? Is this a membrane-mediated function?

Dr. Youngner: It took us 3 yr to get Bob Friedman to admit that there may be such a thing as preformed interferon, and I'm afraid it will take me twice as long to be able to answer your question. This is very difficult for us to even get at, although efforts with these purified materials are going to be made. I have absolutely no idea.

Dr. Gallagher: Is it possible that a physical interaction between the lipid and the cell membrane results in a lesion, rendering the cells in a sense "leaky," the end result being a release of preformed interferon? This hypothesis appears to be consistent with an interpretation of the enhancing effect of cycloheximide on release of preformed interferon as possibly being due to inhibition of repair of membrane defects.

Dr. Youngner: This I find as general as your original question. That there is some damage to the membrane which releases interferon still doesn't tell us in what state the interferon is in the cell, or what that particular lesion at the membrane is. I
think these are very difficult and thorny questions for which to design the proper experiments.

From the Floor: I have a question about endotoxin which has been earlier described as an immunologic adjuvant. And now here, years later, we’re describing endotoxin lipid as an interferon inducer. I wonder if you care to speculate on any relationship existing at all between endotoxin as viral immunologic adjuvant, and endotoxin as interferon inducer.

Dr. Youngner: Well, I certainly won’t make a single comment on endotoxin as an inducer, but I will make a comment on interferon release by endotoxin and immunologic adjuvants.

These effects we observe are rather quick. The interaction of endotoxin with cells showed that interferon release in mice occurred within a half hour. I think the stimulating effects on antibody production, under certain conditions, that endotoxin has been shown to have by many different workers, are effects which go on for a period of days or weeks. I haven’t got the vaguest idea whether they are even interacting with the same cells. I think it is very difficult to sort these out.

What we’re trying to do is to dissect the chemical portions of the endotoxin molecule which are responsible for the adjuvant effects of the endotoxin, and the part of this complex molecule that is responsible for the interferon release. And this may give us some clues as to how to approach the questions that you raise very accurately.

Dr. Richard H. Adamson (National Institutes of Health, Bethesda, Md.): You seem to feel it is the lipid portion of your molecule that’s important. Have any simple lipids or naturally-occurring lipids been tested to see if they release interferon?

Dr. Youngner: We haven’t done any. I’m just trying to think whether anybody else has. But we are very much involved now in creating models. Lipid A is a difficult material to work with. We’re doing the things that can be done with the chemistry of lipid A, but we’re also trying to create models of lipid A structure with which we’ll be able to work. There are some advantages to lipid A, as far as one may bring in the practical here. It is a substance we all carry around with us and it would offer few complications in using such material as an interferon stimulus. In fact, one could even go wild and speculate that the autolysis of intestinal organisms and the release of lipid A, or products like it, could be the source of some of the innate resistance to virus infection, that is, by constant, low level stimulation of interferon production; but that’s pretty wild. But we are looking at models.