3. Manuelidis, E. E., Pond, A. R., PROC. SOC. EXP. BIOL. AND MED., 1959, v102, 693.

4. Manuelidis, E. E., *Cancer Res.*, in press.

5. Parker, R. C., *Methods of tissue culture,* 2nd ed., 1950, P. B. Hoeber, Inc., N. Y.

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## Inhibition of the Infectivity of Poliovirus Ribonucleic Acid by an Interferon.* (26712)

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The site at which interferon inhibits virus multiplication has been assumed to be an intracellular one, since interferon does not interfere with adsorption or release of virus (1,2). It was thought that further evidence with regard to its site of action could be obtained using infectious ribonucleic acid (RNA). If the infectivity of intact virus but not that of infectious RNA were inhibited in a cell treated with interferon, it could be assumed that interferon acted at the site of penetration of intact virus, or at the site of reactions involved in dissociation or uncoupling of the intact virus into its infectious nucleic acid and protein components. On the other hand, if the infectivity of RNA as well as intact virus were blocked in interferon treated cells, this would be supportive evidence that the site of interference is at some point of the viral reproductive cycle mediated by infectious RNA. If in addition it could be shown that infectious RNA is adequately taken up and is not preferentially inactivated by cells treated with interferon, the thesis that interferon acts at some intracellular site of virus synthesis or assembly would be further supported. These hypotheses were tested in experiments described in the following report.

*Materials and methods. Viruses, cultures and media.* Sindbis virus (Egypt AR 339), vesicular stomatitis virus (VSV, Indiana), and poliovirus type III (Saukett) were employed. Primary cell cultures of chick embryo, monkey kidney and human amnion were prepared in 3 oz. bottles. These were nourished in medium consisting of 0.5% lactalbumin hydrolysate (LAH) in Hanks' balanced salt solution (BSS), 4% calf serum and antibiotics. Plaquing medium included in addition 1.5% bacto agar, and 1.0% or 1.5% of 1:1000 neutral red depending on whether monkey kidney or chick cultures were overlaid. *RNA.* Infectious poliovirus RNA was extracted by the cold phenol method from partially concentrated poliovirus harvests of infected Roux bottle cultures of monkey kidney or human amnion cells by the modified method of Holland *et al.*(3). The infectivity of RNA was also determined by their method(4) as follows: RNA was diluted 1:1 in 2M $MgSO_4$ and 0.2 ml was inoculated in monkey kidney cultures that had been washed sequentially with 5 ml phosphate buffered saline (PBS) and 1 ml 2M $MgSO_4$. After 15′ adsorption at room temperature, the inoculated cultures were decanted, washed with 5 ml medium and overlaid with plaquing medium. Various lots of RNA titered $10^2$-$10^3$ p.f.u./0.1 ml. Its infectivity was destroyed by incubation for 20′ at 25° with $10^{-2}$ $\mu$g/1.0 ml of ribonuclease (Worthington), or for 5′ at 25° with medium. *Interferon and controls.* An interferon, hereafter abbreviated S-VIF, was prepared as follows: Stock Sindbis virus titering $10^6$-$10^8$ p.f.u./0.1 ml was first inactivated at 56°C for 4 hours. Five-tenths ml of the inactivated material was added to a chick culture and incubated at 36° for 18 hours, after which it was decanted and washed with 5 ml of Hanks' BSS before addition of 5 ml of medium. The culture then received 0.1

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ml containing about $10^7$ p.f.u. infectious Sindbis virus. On the day after infection, the fluid was collected, centrifuged at 2,500 r.p.m. and inactivated at 56°C for 4 hours before use. Control fluids (CF) were prepared concurrently with S-VIF by omitting the addition of virus. *Measures of inhibition.* When a culture was "treated" with S-VIF, 0.25 ml of undiluted S-VIF or a dilution of S-VIF made up in medium was overlaid on decanted chick cultures and incubated at 36°C for 3 hours. These cultures were then washed with 5 ml medium before use. To measure the inhibition of intact virus, treated cultures were challenged with 100-200 p.f.u. VSV. Controls that had been treated with CF were challenged concurrently. Plaques were counted on the 2nd and 3rd days after inoculation, and inhibition was expressed as a comparison of plaque counts in treated and control cultures.

The measurements of inhibition of infectivity of polio-RNA were based on a comparison of 2 parameters: 1) virus production, 2) cell infection as expressed by infectious center formations. 1) To compare virus production, S-VIF treated and control chick cultures were inoculated with RNA, replenished with 5 ml of medium and incubated for 18 to 24 hours. To titer extracellular virus, 0.1 ml aliquots of the overlay were taken at various intervals after infection and inoculated in monkey kidney cultures for poliovirus plaque formation. Total virus was similarly measured after release of intracellular virus by 3 cycles of freezing and thawing. 2) To compare infectious center formations, treated and control cultures inoculated with RNA were further washed sequentially with 10 ml LAH and 10 ml PBS at room temperature. The cells were detached from the glass surface by incubation at 36° for 5′ after addition of 1.0 ml of 0.05% trypsin and 0.01% versene to each culture. To stop tryptic activity, 4.0 ml cold medium was added and cells were sedimented by centrifugation at 800 rpm for 5′. These were washed in 5 ml medium and again centrifuged. The resulting supernatant in preliminary experiments had no detectable infective activity presumably because of the inactivating effect of medium on infectious RNA. The final cell sediment was resuspended in 2.0 ml and serial 10-fold dilutions were made in medium. Cell counts varied from $10^6$ to $6 \times 10^6$ per culture. Dilutions of cells in 0.2 ml were then added to duplicate monkey kidney cultures. After incubating for one hour at 36° to allow for cell attachment, 0.5 ml of plaque overlay was first added to the monolayer to minimize cell migration before addition of the usual 7.0 ml. The number of plaques representing infectious centers were counted in 2-3 days.

*Results. Properties of S-VIF.* The multiplication of intact viruses such as VSV is inhibited in chick cultures treated with S-VIF(5). To demonstrate that the effect of S-VIF conforms to that of previously described types of interferon, it was desirable to show that the inhibitory factor is a protein like substance which is not identifiable with Sindbis virus and that it does not act by directly inactivating virus.

Experiments measuring its inhibitory activity after high speed centrifugation, treatment with antiserum to Sindbis virus and dialysis are summarized in Table I. It is apparent that the inhibitory factor in S-VIF is not substantially reduced by any of these procedures.

To test whether S-VIF directly inactivated VSV virus: 0.4 ml of a $10^{-4}$ stock solution of VSV virus was added to each of 2 separate tubes containing 0.4 ml of undiluted S-VIF and 0.4 ml of CF. The mixtures were incubated at room temperature for one hour, and titrated by plaque formation in quadruplicate chick cultures. The means of plaque counts were 51 and 52 respectively. It appears then that S-VIF does not directly inactivate VSV under these circumstances.

To test whether the inhibitory activity of S-VIF could be destroyed by a proteolytic enzyme(1), trypsin was added to S-VIF to a concentration of 0.05% and incubated at 37°. Before testing the digest for inhibitory activity, the tryptic action was stopped by adding soy-bean trypsin inhibitor (SBI) to a concentration of 0.25%. SBI was also added to control fluids (CF) in the same concentrations. Two 0.3 ml aliquots of each

TABLE I. Effect of Centrifugation, Antiserum and Dialysis on Viral Inhibitory Effect of S-VIF against VSV.

| Dilution of inhibitor (0.25 ml) | S-VIF | | S-VIF centrifuged† | | S-VIF | |
|---|---|---|---|---|---|---|
| | Not centrifuged | Centrifuged* | No antiserum | Antiserum | Not dialyzed | Dialyzed‡ |
| 1:1 | 35 | 34 | 52 | 35 | 5 | 9 |
| 1:2 | 44 | 45 | ND | ND | ND | ND |
| 1:4 | 82 | 65 | 86 | 91 | 41 | 37 |
| 1:8 | 112 | 86 | ND | ND | ND | ND |
| 1:16 | 116 | 130 | 118 | 116 | 68 | 77 |
| 1:32 | 121 | 138 | ND | ND | ND | ND |
| 1:64 | 116 | 131 | 125 | 143 | ND | ND |
| Control | 168 | | 165 | | 117 | |

Figures are the means of quadruplicate plaque counts except for those in first 2 columns, which represent duplicates.

ND = Not done.

* Centrifuged twice for a total of 20 hr at 104,000 g. Reduction in virus titer is usually from $10^8$ p.f.u. to about $10^2$ p.f.u./0.1 ml. Heat inactivated before use.

† Each dilution of centrifuged S-VIF contained a 1:4 dilution of either normal rabbit serum or an antiserum against Sindbis virus sufficient to eliminate all infective activity. These mixtures were preincubated for 30′ at 36° before they were added to chick cultures.

‡ Dialyzed in tap water for 18 hr, distilled water for 4 hr, and reconstituted in medium by 2 cycles of dialysis in LAH in Hanks' BSS for 3 and 18 hr at 4°C.

of the following 4 fluids: CF, CF plus SBI, undiluted S-VIF, and a 8′ tryptic digest of S-VIF were added to separate chick cultures and these were incubated for 3 hours at 36°. The cultures were then inoculated with VSV after being washed with 5 ml of medium. The mean plaque counts of the cultures treated with these 4 fluids were: CF alone: 148, CF + SBI: 173, S-VIF alone: 0, S-VIF after 8′ trypsinization: 112. These results indicate that the no inhibitory activity is apparent in S-VIF after tryptic digestion.

The biological and chemical properties mentioned above show that the viral inhibitor in S-VIF is not associated with sedimentable virus or virus antigen and that it is a non-dialyzable, heat stable substance inactivated by a proteolytic enzyme. It does not act by directly inactivating virus. These are the properties of a class of inhibitors called viral inhibitory factor (VIF) by us(1), and interferon by Isaacs and Lindemann(6).

*Effect of S-VIF on infectivity of RNA.* The inhibitory effect of S-VIF on the infectivity of polio-RNA in chick cultures was first tested by comparing virus production in treated and control cultures. Fig. 1 is a summary of 3 separate experiments measuring the growth curves of poliovirus in treated and control cultures inoculated with polio-RNA. Virus is produced in a one step fashion in both types of cultures at 7-11 hours after inoculation with no evidence of further cycles of infection. This is consistent with the findings of Holland *et al.*(3). Judging from the similarity of the extracellular and total virus titers when these were tested simultaneously, virus release into the medium is also complete at this time. The inhibition of virus production in S-VIF treated cultures is indicated by the fact that these produced only 1.7% to 0.8% of the amount of virus produced in control cultures, a degree of in-

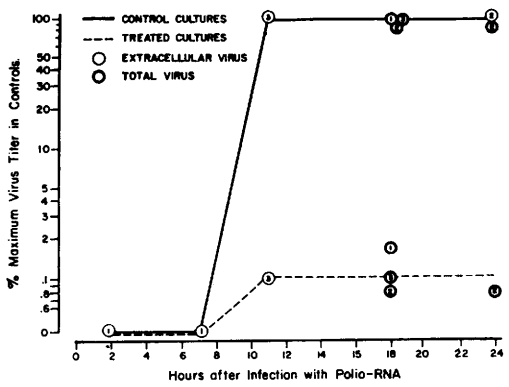


FIG. 1. Inhibition of virus production in chick cultures treated with S-VIF and inoculated with polio-RNA. Cultures were treated with undiluted S-VIF and CF and were inoculated with polio-RNA in 3 separate experiments denoted by numbers within circles. Maximum total virus produced in the control chick cultures in these 3 experiments were $10^{3.8}$, $10^{3.8}$, and $10^{5.7}$ p.f.u. respectively.

TABLE II. Inhibition of Poliovirus Yield and Infectious Center Formation in Chick Cultures Treated with Varying Dilutions of S-VIF.

| | Virus yield, % of control | | | | | No. p.f.u. $\times 10^4$ per | Infectious centers, % of control | | | | | No. I.C. $\times 10^2$ per |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Dilution | 1 (a) | 2 (b) | 3 (c) | 4 (d) | Mean (e) | culture (f) | 1 (g) | 2 (h) | 3 (i) | 4 (j) | Mean (k) | culture (l) |
| C.F. | 100 | 100 | 100 | 100 | 100 | 27.0* | 100 | 100 | 100 | 100 | 100 | 28.4† |
| 1:1 ‡ | 2.2 | 0 | ND | ND | 1.1 | .3 | 1.0 | 2.2 | ND | ND | 1.6 | .5 |
| 1:2 | 3.6 | 4.0 | ND | ND | 3.8 | 1.0 | 2.1 | 1.4 | ND | ND | 1.8 | .5 |
| 1:4 | 6.2 | 9.9 | 1.0 | .8 | 4.5 | 1.2 | 3.9 | 15.0 | 4.6 | 17.0 | 10.1 | 2.9 |
| 1:8 | 8.6 | 11.0 | 4.9 | 3.9 | 7.1 | 1.9 | 15.4 | 10.0 | 20.0 | 9.6 | 13.8 | 3.9 |
| 1:16 | 71.0 | 34.0 | 2.4 | 10.0 | 29.4 | 7.9 | 21.2 | 11.0 | 42.0 | 15.7 | 22.5 | 6.4 |
| 1:32 | ND | ND | 20.2 | 55.5 | 37.9 | 10.2 | ND | ND | 37.0 | 23.0 | 30.0 | 8.5 |

1, 2, 3, 4 represent separate experiments; C.F. = control fluid; p.f.u. = plaque forming units; I.C. = infectious centers; ND = not done.

a, b, c, d: Calculated as follows: virus yield in S-VIF treated cultures ÷ virus yield in controls × 100.

e: Mean of a, b, c, d.

f: e × 27.0; * 27.0 × $10^4$ = mean total virus yield in control cultures.

g, h, i, j: Calculated as follows: No. I.C. in S-VIF treated cultures/cell count of culture ÷ No. I.C. in control culture/cell count in control × 100.

k: Mean of g, h, i, j.

l: k × 28.4; † 28.4 × $10^2$ = mean of total I.C. in control cultures.

‡ Dilution of S-VIF applied to chick cultures.

hibition at least as great as that expressed by reduced plaque formation in cultures treated with S-VIF and challenged with VSV (Table I).

To test whether or not inhibition of virus production in S-VIF treated cultures was accompanied by inhibition of cell infection as well, both virus production and infectious centers were titrated in a series of cultures treated with different concentrations of S-VIF. Duplicate chick cultures treated with CF and serial dilutions of S-VIF were inoculated with undiluted polio-RNA. One set was allowed to incubate for 18 hours for measurement of total virus yield, while the other set was used for titration of infectious centers. Four separate such experiments using one lot of S-VIF and polio-RNA are summarized in Table II. Three observations may be made from these data: 1) both infectious center formations and virus yield are inhibited in S-VIF treated cultures; 2) the magnitudes of inhibition of these 2 parameters at each dilution of S-VIF are comparable. This suggests that the inhibition of virus yield is due to prevention of cell infection, and that inhibition of virus yield is therefore an all or none phenomenon. However there is too great a fluctuation of the amount of inhibition from experiment to experiment to state this with certitude. 3) It appears that if we were to plot either inhibition of virus yield or inhibition of cell infection with varying concentrations of S-VIF, we would not obtain a linear curve. There is much less increase in inhibition at higher concentrations of S-VIF than at lower.

*Direct effect on polio-RNA.* From the foregoing it is apparent that S-VIF prevents both infection of cells and production of virus. This inhibition may be due to a block of a reaction required for virus synthesis within the cell. Alternatively, it may be postulated that either 1) RNA is inactivated at the cell surface or within the cells of cultures treated with S-VIF, or 2) adsorption of infective RNA is impaired in treated cells.

To test these possibilities, 3 types of experiments were undertaken. In the first, an attempt was made to see if inhibition of virus yield was obtained if S-VIF was added *after* inoculation of polio-RNA. Two chick cultures were infected with 0.2 ml polio-RNA suspended 1:1 in 2M $MgSO_4$. After washing, one of these received 0.3 ml undiluted S-VIF, while the other received 0.3 ml CF. The 2 cultures were incubated at 36° for 3 hours, after which the fluids were washed off and 5 ml medium added to each culture. After 18 hours of incubation at 36°, total virus titrations were carried out by making 10-fold serial dilutions of aliquots from both

cultures and inoculating 0.1 ml in monkey kidney tube cultures. Titers for treated and control cultures were $10^{2.5}$ and $10^{3.5}$ $TCD_{50}$/0.1 ml respectively. The fact that inhibition of virus yield is detectable *after* adsorption of polio-RNA in treated cells suggests that at least some of the inhibition cannot be accounted for by inhibition of adsorption of RNA, or by inactivation of RNA at the cell surface of treated cells.

In the second type of experiment, residual RNA was titrated after exposure to S-VIF treated cells. 0.2 ml of a 1:1 dilution of polio-RNA was added to 2 of each of the following types of chick cultures: 1) control cultures with CF, 2) cultures treated with undiluted S-VIF and 3) blank bottles. At various time intervals, residual RNA was titrated by diluting the contents of a bottle 1:10 and titrating on duplicate monkey kidney cultures. At 0 hour, the RNA in a blank bottle titrated 70 p.f.u. representing the initial inocula; at 10′, the residual RNA in control and treated cultures titrated 39 and 41; at 30′, the corresponding values were 21 and 21. The RNA in the blank bottle also measured 21 p.f.u. at this time. Figures of similar magnitude were found in repeat experiments. It is apparent that there is no difference between rate of inactivation of RNA upon exposure to control and treated cells. On the other hand, there is also a fairly rapid rate of degradation of RNA in $MgSO_4$. This precludes accurate measurements of the adsorption of the infectious RNA in either the control or treated cultures by this method. However, in so far as the residual titers reflect adsorption, there is no difference between the control and treated cultures.

In the third type of experiment, cells were scraped from a control and a S-VIF treated culture and suspended separately in 0.5 ml 2M $MgSO_4$. After freezing and thawing the suspension 10× to release intracellular contents that may preferentially inactivate polio-RNA 0.15 ml of undiluted RNA was added to each of the suspensions and these were incubated at 37° for one hour. The initial titer was 80 p.f.u. and the mean of duplicate plaque counts for the control and treated cultures at 10′, 30′ and 60′ were respectively: 49 and 52; 32 and 42; 14 and 24. Such results indicate that under the assumptions and conditions described, there is no release of intracellular RNA inactivating material.

These 3 types of experiments taken together indicate that the inhibition of infectivity of polio-RNA in S-VIF treated cells cannot be entirely accounted for by inhibition of adsorption of polio-RNA, and no evidence for an intracellular RNA inactivating substance in S-VIF treated cells was found.

*Discussion.* The inhibition of virus production in interferon-treated cells inoculated with polio-RNA is presumptive evidence that intreferon acts intracellularly, which fits well with Isaacs' conception of the biochemical site of action of interferon(7). Additional data suggesting that S-VIF interferon acts *after* adsorption of polio-RNA and that treated cells do not preferentially inactivate RNA constitute further evidence favoring the intracellular site of action for interferon. More direct evidence with respect to adsorption of RNA by treated and untreated cells would be desirable. In our hands, however, measurement of adsorption of infectious RNA by the method of titration of residual activity has not proved as clear cut as in the work of Holland *et al.* Adsorption studies using isotopes may not be more informative because infectious RNA is difficult to label specifically(4).

In addition to inhibiting the infectivity of RNA, it is logically possible that interferon acts at a second site with respect to intact virus. This additional site of action could be where intact virus penetrates or where it uncouples into nucleic acid and protein components. This appears unlikely in view of the fact that degree of inhibition of RNA infectivity is as great if not greater than for intact viruses tested such as VSV. Ideally however, one should compare the inhibitory effect of interferon on one species of intact virus and its infectious RNA derivative.

There are advantages to the use of infectious RNA to study the kinetics of inhibition of interferon in a system in which the cells are resistant to the action of the intact virus

progeny of the RNA. One advantage is that in such a system measurement of virus production is limited precisely to one cycle even when a small inoculum is applied. This would not be the case if instead intact virus such as VSV were used. Under these conditions, as virus production increases, the number of infectious cycles multiply and unsuspected factors such as secondary elaboration of viral inhibitors or even potentiators of infection may be added to the experimental conditions.

This study of the kinetics of inhibition of RNA infectivity brings out 2 interesting points. 1) Inhibition of virus production and inhibition of cell infection are of approximately the same magnitude. 2) Degree of inhibition of either virus production or infectious center formation does not increase linearly with concentration of the inhibitor. At high concentration of S-VIF, there is a rapid decline in rate of inhibition such that even with greater concentration of S-VIF, one may not expect complete protection of all cells in the presence of a large enough challenge inoculum. A similar rapid decline in degree of inhibition at higher concentrations of S-VIF using intact vesicular stomatitis virus was noted (Table I)(5). This suggests a limit to the amount of inhibitor that can be made available for cell protection. Such may be a characteristic of interferons in general or only of our particular preparation. Further kinetic data of this type including studies on adsorption of the inhibitor and secondary cell-inhibitor reactions would be desirable.

With respect to the first point, because of the fluctuations of inhibition measurements from one experiment to another, the data presented are inadequate to prove the suggested thesis that inhibition of cell infection alone can account for inhibition of virus production. Such fluctuations are partially due to the methodological difficulties inherent in the many steps necessary for titering infectious centers, and use of separate cell cultures for measuring cell infection and virus production in a particular experiment. Forthcoming improvements in titration methods may help overcome both of these difficulties. In addition, correlation with dose-response data using intact virus(1,5) will also be important.

*Summary.* An inhibitor with the properties of interferon obtained from harvests of Sindbis virus inhibits the infectivity of poliovirus RNA in chick embryo cell cultures. This inhibition is expressed by a reduction of cell infection as well as total poliovirus production. These results are compatible with the concept that interferon inhibits certain intracellular metabolic activities necessary for virus synthesis.

1. Ho, M., Enders, J. F., *Virology,* 1959, v9, 446.
2. Wagner, R. A., Levy, A. H., *Ann. N. Y. Acad. Sci.,* 1960, v88, 1308.
3. Holland, J. J., McLaren, L. C., Syverton, J. T., *J. Exp. Med.,* 1959, v110, 65.
4. Holland, J. J., Hoyer, B. H., McLaren, L. C., Syverton, J. T., *ibid.,* 1960, v112, 821.
5. Ho, M., *Fed. Proc.,* 1961, v20, 442.
6. Isaacs, A., Lindemann, J., *Proc. Roy. Soc., (B),* 1957, v147, 258.
7. Isaacs, A., Klemperer, H. G., Hitchcock, G., *Virology,* 1961, v13, 191.

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