Mechanisms of Interferon Induced Transfer of Viral Resistance Between Animal Cells

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SUMMARY
The sequence of events initiated by interferon and leading to the antiviral state were studied as possible sites for the cell-to-cell transfer of interferon induced viral resistance. The possible role of interferon produced by recipient cells was negated by the demonstration of transfer of resistance in the presence of anti-human interferon antibody and under conditions of a single cycle of VSV growth. Transfer of sensitivity of WISH cells to mouse interferon, possibly through transfer of a membrane receptor, seems unlikely since resistance was transferred in the absence of mouse interferon. From kinetic data and the fact that actinomycin D blocked resistance in human cells for 3 h longer than in mouse cells, it seems unlikely that the mouse antiviral protein itself or its mRNA alone is a likely candidate for the transfer of resistance. Thus, by a process of elimination, we suggest that secondary messenger molecules which transmit the interferon signal from the membrane to the nucleus are the effector substance(s) for the transfer process.

INTRODUCTION
The action of polypeptide hormones on transcriptional and translational processes are mediated by secondary molecules which are produced in response to a hormone–cell membrane interaction (Cuatrecasas, 1975). Interferon is thought to act in a fashion similar to polypeptide hormones (Baron, 1966), and as such is not consumed (Buckler et al. 1966) and does not penetrate the cell membrane (Ankel et al. 1973; Chany et al. 1974). The interferon–membrane interaction in turn leads to the derepression and production of the antiviral protein (Taylor, 1964, 1965). An obvious question arises as to whether the induction of the antiviral protein is mediated by secondary molecules. A stage between the interferon–cell membrane reaction and the derepression has been identified (Dianzani & Baron, 1975; Dianzani et al. 1976) but little is known about the events which occur during this stage.

We have recently studied a system which may involve these events. Briefly, we found that while human (WISH) amnion or baby hamster kidney (BHK) cells alone are not sensitive to the action of mouse interferon, co-cultivation of either of these cells with sensitive mouse L cells in the presence of mouse interferon resulted in a marked inhibition of virus yield from the insensitive cells (Blalock & Baron, 1977). The efficient transfer of viral resistance requires cell to cell contact which suggests that intercellular communication is involved.

The sequence of events initiated by interferon is thought to be: interaction of interferon with a membrane receptor (Besancon & Ankel, 1974a, b; Besancon et al. 1976; Kohn et al. 1976); production of a putative secondary messenger molecule(s) which transmits the signal...
from the membrane to the nucleus (Friedman & Pastan, 1969; Dianzani & Baron, 1975; Dianzani et al. 1976; Blalock & Baron, 1977); induction by this secondary molecule of the messenger (m)RNA for the antiviral protein (Taylor, 1964, 1965); and translation of the messenger RNA into the antiviral protein (Friedman & Sonnabend, 1964, 1965; Levine, 1964; Lockart, 1964; Dianzani, et al. 1969). Theoretically, any one of these molecules [the membrane receptor, the putative secondary messenger molecule(s), the mRNA for the antiviral protein or the antiviral protein] could be the effector for the transferred resistance. This study was done to help distinguish between these possible mechanisms of transfer.

METHODS

Materials. Mouse interferon was obtained from Bionetics Laboratory Products, Kensington, Md. and human fibroblast interferon from Dr Jan Vilček. Rabbit anti-human fibroblast interferon serum was supplied by the Antiviral Substances Program of the National Institute of Allergy and Infectious Diseases, Bethesda, Md. Actinomycin D was purchased from Sigma Chemical Company, St Louis, Mo. Interferon titres are expressed in terms of the NIH research reference standard (G-023-901-527) for human leucocyte interferon.

Methods. Mouse L and human WISH cells were cultured in Eagle's medium (supplemented with 10% foetal calf serum) alone or in a 1:1 ratio in Micro Test II tissue culture plates (Falcon Plastics, Oxnard, Calif.). The total number of cells in each well (about 28 mm²) was 2-25 x 10⁶. Control experiments showed that on the following day the same numbers of cells were recorded. Thereafter the cells were in Eagle’s medium supplemented with 2% foetal calf serum, which allows only minimal cell proliferation, and thus the ratio of cells was constant during the experiments. Controls consisted of an equivalent total number of each cell type alone. Interferon or an equal volume of medium was added and cultures were incubated overnight at 37 °C in a 4% CO₂ atmosphere. Supernatant fluids were decanted and each well was infected with vesicular stomatitis virus (VSV) at 3 p.f.u./cell. After 1-5 h at 37 °C, the inoculum was decanted and the cell sheets were washed and replenished with fresh medium. Virus yields from pooled triplicate cultures were determined approx. 24 h later by a slightly modified microplaque assay in which methylcellulose was substituted for carboxymethylcellulose (Campbell et al. 1975). Except where specified this procedure was used throughout this study. Differences in virus yields of 0.5 log₁₀ are significant at P < 0.05 by Student’s t test.

Since cells treated with homospecific interferon produced a negligible amount of virus, and since either cell type alone in the absence of interferon produced an equivalent amount of virus, the expected virus yield in a mixed culture was calculated in terms of the 50% of cells not treated with the homospecific interferon. The % inhibition was determined by the following formula:

\[
\text{% inhibition} = \left( \frac{\text{Virus yield from interferon treated mixed cells}}{\text{Half virus yield from non-treated mixed cells}} \right) \times 100
\]

A control was used in every experiment to show that mouse interferon had no activity on human WISH cells.

RESULTS

Kinetics of mouse interferon action on mixed cultures of L and WISH cells

Experiments were conducted to determine the time of treatment with interferon required for mixed cultures to manifest transferred resistance. Fig. 1 shows that after only 1 h of treatment of L cells with 150 units/ml of mouse interferon, the replication of VSV was
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Is interferon production by the recipient cells involved in transfer of viral resistance?

One explanation for the observed transfer of viral resistance is that it results from the production of human interferon by human WISH cells in the presence of mouse L cells and mouse interferon. However, we could detect no human interferon by a very sensitive assay and the fact that VERO cells, which produce essentially no interferon (Desmyter et al. 1969), were capable of receiving transferred resistance from L cells argues against this possibility, (Hughes et al. 1978). The following experiments tend to support the suggestion that interferon production in the recipient cells is not involved.

Interferon is thought to be externalized from a cell before acting and therefore its action
**Table 1. Effect of antiserum to human fibroblast interferon on the transfer of resistance induced by mouse interferon from mouse L cells to human WISH cells***

<table>
<thead>
<tr>
<th>Cells</th>
<th>Interferon</th>
<th>Rabbit anti-human fibroblast interferon</th>
<th>Normal rabbit serum</th>
<th>Inhibition of expected virus yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mouse (150 units/ml)</td>
<td>Human (300 units/ml)</td>
<td>(1:40)</td>
<td>(1:40)</td>
</tr>
<tr>
<td>1L:1 WISH†</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>L</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>WISH</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

* Mouse L cells and human WISH cells were cultured as described in Methods. Supernatant fluids were removed and replaced with mouse or human interferon and rabbit antiserum to human fibroblast interferon or normal rabbit serum in the combinations shown. Following overnight incubation cells were washed and challenged with VSV.

† 1·25 x 10⁶ L cells were co-cultivated with an equal number of WISH cells.

‡ Statistically insignificant inhibition (P > 0·5 by Student’s t test).

is susceptible to inhibition by antisera to interferon (Pitha *et al.* 1976). However, in a mixture of equal numbers of L cells and WISH cells treated with mouse interferon, transfer of viral resistance occurred to the same extent in the presence of sufficient antiserum to human fibroblast interferon to neutralize 300 units/ml of any human interferon which might be produced (Table 1). These data show that if human interferon is produced in this system it plays no role in the transfer process.

Another test for a possible mediating role of interferon is to challenge the mixed culture with a high input multiplicity of infection (m.o.i.) of virus to allow virus replication to occur before the virus can induce interferon (Wagner *et al.* 1963; Lefkowitz & Baron, 1974). Our initial experiments (Blalock & Baron, 1977) had been performed with a low m.o.i. and the multiple cycles of VSV growth could have allowed time for endogenous interferon production and action. Such an experiment showed that transfer of viral resistance occurred during conditions of a single cycle of growth of VSV (Table 2) during which there is little time for interferon production and action. There is a diminution in the amount of transferred resistance with increasing input m.o.i., but this also occurred with mouse interferon treatment of the control mouse L cells alone. Similar results were obtained when VSV was replaced with vaccinia virus as the challenge virus (data not shown). These data, in addition to further ruling out a role for interferon production by the recipient cells, indicate that the resistance transferred has the characteristics of interferon-induced resistance: the level of transferred resistance showed an m.o.i. dependency similar to the interferon-induced antiviral resistance in the homologous L cells.

**Lack of effect of removal of mouse interferon on transfer of viral resistance**

Another possible explanation for the transfer process is that human WISH cells are made sensitive to mouse interferon by co-cultivation with mouse L-cells. One could imagine that such a process might occur through the transfer to WISH cells of membrane receptors for mouse interferon. If this were the case then to demonstrate transferred resistance should require the presence of mouse interferon with the human WISH cells. Contrary to this, we
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Table 2. Effect of input multiplicity of infection with VSV on interferon-induced transfer of viral resistance

<table>
<thead>
<tr>
<th>Input multiplicity of infection*</th>
<th>1L:1 WISH Cells (2·25 × 10^6 cells/well)</th>
<th>L</th>
<th>WISH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse interferon units (7500 units/ml)</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Log_{10} virus yield</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expected</td>
<td>6·65</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>% inhibition</td>
<td>66</td>
<td>-</td>
<td>99·2</td>
</tr>
<tr>
<td>Log_{10} virus yield</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Observed</td>
<td>6·19</td>
<td>6·95</td>
<td>4·95</td>
</tr>
<tr>
<td>Expected</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>% inhibition</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

2:1

| Log_{10} virus yield | | | | | | |
| Observed | 5·93 | 6·90 | < 4·00 | 6·95 | 7·16 | 6·93 |
| Expected | 6·60 | - | - | - | - | - |
| % inhibition | 80 | - | > 99·9 | - | 0 | - |

0:21

| Log_{10} virus yield | | | | | | |
| Observed | 4·40 | 6·54 | < 4·00 | 6·78 | 6·00 | 6·00 |
| Expected | 6·24 | - | - | - | - | - |
| % inhibition | 98·6 | - | > 99·8 | - | 0 | - |

* After overnight incubation cells were washed and challenged with VSV at the m.o.i. shown. Virus yields from pooled triplicate cultures were determined 24 h later.

Table 3. Lack of effect of removal of mouse interferon on transfer of viral resistance from L cells to WISH cells*

<table>
<thead>
<tr>
<th>Hours of treatment of L cells with mouse interferon (150 units/ml)</th>
<th>% inhibition of expected virus yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L cells</td>
</tr>
<tr>
<td>0·5</td>
<td>88</td>
</tr>
<tr>
<td>1·0</td>
<td>95</td>
</tr>
<tr>
<td>4·0</td>
<td>91</td>
</tr>
</tbody>
</table>

* L cells were treated for the indicated times with mouse interferon and then the supernatant fluids were decanted, the cells were washed and an equal number of WISH cells was added. Following overnight incubation the cells were washed and challenged with VSV.

found that L cells treated with mouse interferon and washed extensively to reduce the interferon to less than 2 units/ml (too little to cause the transfer) could still transfer resistance (Table 3). It follows that the transfer of viral resistance does not require the continued presence of interferon, which is inconsistent with the possibility that co-cultivation makes the WISH cells sensitive to mouse interferon.

Kinetics of actinomycin D inhibition of interferon-induced transfer of viral resistance

Another possibility is that the mouse mRNA for the antiviral protein is transferred and causes the WISH cells to become resistant to virus infection. To test this, the kinetics of inhibition by actinomycin D of interferon-induced viral resistance in L cells alone and in L: WISH cell mixtures were studied.

It was found that by 1 h after interferon treatment of L cells, actinomycin D could no longer block development of resistance to VSV, indicating that almost complete transcription of large amounts of mRNA for the mouse antiviral protein had occurred (Fig. 2). In contrast,
Mouse L cells and human WISH cells were cultured as described in Methods and treated with 150 units/ml of mouse interferon; at the indicated times all cell cultures were treated with actinomycin D (2 µg/ml) for 2 h. Ten hours after interferon addition cell cultures were challenged with VSV (3 p.f.u./cell). ●——●, L cell cultures; ○——○, WISH cell cultures; ●——●, cultures with equal numbers of L cells and WISH cells. Not added: control WISH cells not treated with actinomycin.

Transferred resistance could be totally blocked by actinomycin D for 4 h after addition of mouse interferon, indicating that the transcription required for the development of transferred resistance in WISH cells occurred between 4 and 6 h. Thus the transferred resistance in the WISH cells cannot be simply attributed to the mouse mRNA since transfer failed to occur for 3 h after the production, complete by 1 h, of the mRNA in the donor L cells. This finding does not exclude the more complex possibility that actinomycin D inhibits the transfer process for the mRNA as well as inhibiting mRNA synthesis, and that both may be required. Actinomycin D, however, has been shown not to interfere with the transfer of small molecules between cells, and RNA molecules are not thought to be transferred (Pitts & Simms, 1977).

Since actinomycin D is thought to inhibit transcription specifically, these data imply that a transcriptional process in the WISH cells may be required by them to develop the antiviral state. They also support the hypothesis that mouse interferon causes the production of
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Fig. 3. Effect on the transfer of viral resistance of adding mouse interferon or WISH cells at different times: •—•, 150 units of mouse interferon added at the times shown to mixtures of equal numbers of L cells and WISH cells; ○—○, L cells were treated with 150 units of mouse interferon and WISH cells were added at the times shown. Following overnight incubation the cultures were washed and challenged with VSV.

Effects of varying the time of addition of mouse interferon or WISH cells on the transfer of viral resistance

If interferon initiates an antiviral process in the L cells which is subsequently transferred to the WISH cells, one can determine the length of time during which this occurs, WISH cells were therefore added to L cells at varying times following the addition of mouse interferon. Fig. 3, lower curve, shows that there was a linear decrease in the transfer of viral resistance as WISH cells were added successively later, relative to interferon addition. Since viral resistance is not transferred following 8 h treatment of L cells with mouse interferon, these data indicate that an essential interferon-induced material or process which is responsible for the transfer is either unstable or otherwise unavailable for transfer at this time.

Also shown in Fig. 3 is that when L cells and WISH cells are seeded simultaneously,
interferon can be added at any time up to 8 h and a maximum transfer of resistance still occurs. This indicates that the potential to transfer resistance is stable in the co-cultivated cells in the absence of interferon.

DISCUSSION

The present study was undertaken to examine the possible mechanism(s) governing the transfer of interferon-induced viral resistance between heterologous cells (Blalock & Baron, 1977). The possible mechanisms include: (a) interferon production by the recipient cells; (b) transfer to the recipient cells of sensitivity to heterologous interferon possibly through transfer of a membrane receptor; (c) transfer of a putative secondary messenger molecule(s) which transmits a depression signal between the cell membrane and the nucleus; (d) transfer of the mRNA for the antiviral protein; and (e) transfer of the antiviral protein.

The available evidence indicates that transfer of viral resistance from interferon-treated mouse L cells to human WISH cells does not result from the production of human interferon by human WISH cells. This idea is supported by the finding that VERO cells, which produce essentially no interferon (Desmyter et al. 1969) receive transferred resistance from L cells (Hughes et al. 1978). Additional evidence arguing against human interferon production is the finding that transfer of virus resistance occurred to the same extent in the presence of antisera to human fibroblast interferon. Also consistent with this finding is the fact that transfer occurs during conditions of a single cycle of VSV growth which allows little time for interferon production and action. Although, there was a diminution in the amount of transferred resistance with increasing input m.o.i. of VSV, this was also seen with L cells alone and indicates that the resistance, once transferred, has the characteristics of an interferon type antiviral state. Taken together these data seem to negate the production of interferon by the recipient cells as the basis for transfer of resistance.

Human WISH cells might be made sensitive to mouse interferon when co-cultivated with mouse L cells by transfer of membrane receptors for interferon. However, this seems unlikely since this mechanism would require the presence of mouse interferon with the recipient WISH cells and we found that after a brief interaction of L cells with mouse interferon, followed by removal of the interferon, resistance was transferred to subsequently added WISH cells. Thus resistance transfer did not require the presence of mouse interferon with the human WISH cells.

Data are presented which show that the development of resistance in the donor L cells precedes the development of resistance in the WISH cells (Fig. 1). This suggests that the mouse interferon initiates an antiviral process in the L cells which is subsequently transferred to the human WISH cells. Theoretically, any one of the following molecules [putative secondary messenger(s), the mRNA for the antiviral protein or the antiviral protein] could be the effector molecule for transferred resistance.

If the transfer process occurs through gap junctions which transfer only small molecules (Pitts & Simms, 1977), then it seems unlikely that mRNA or the antiviral protein is responsible. Data indicate that the interferon-induced material which is responsible for the transfer of resistance is unstable or it becomes unavailable for transfer. Since the mouse antiviral protein is stable for more than 8 h (Baron et al. 1967) and its production continues in the presence of interferon, it seems unlikely that it alone is responsible for resistance in the WISH cells. Thus either a molecule other than the antiviral protein, or the antiviral protein plus another factor (which is no longer available by 8 h) is responsible for the transfer.
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The mRNA for the antiviral protein also seems an unlikely candidate for the effector of transferred resistance. If the mRNA alone caused the viral resistance in the WISH cells, then actinomycin D should not have blocked development of resistance in the WISH cells beyond the 1 h required for substantial transcription of the mRNA in the L cells. Furthermore, since actinomycin D blocked resistance in the WISH cells for 3 h past its effect on L cells, these data imply that a transcription event in the WISH cells is necessary for the development of the antiviral state. These findings also argue against the transfer of the antiviral protein, since its possible action in WISH cells should not require transcription. Again, the more complex possibility that actinomycin D blocks the production of a factor needed to transfer the mRNA cannot be excluded.

The sequence of events, initiated by interferon, leading to the antiviral state were studied as possible sites for the transfer of viral resistance. By a process of elimination, secondary messenger molecules which transmit the interferon signal from the membrane to the nucleus are favoured as the effector substance(s) for the transfer process leading to derepression of the gene for the human antiviral protein. Studies to isolate these molecules are being undertaken.

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REFERENCES


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