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Interferon Induced Transfer of Viral Resistance

Annual Report

J. Edwin Blalock, Ph.D.
Samuel Baron, M.D.

February 1980

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University of Texas Medical Branch
Galveston, Texas 77550

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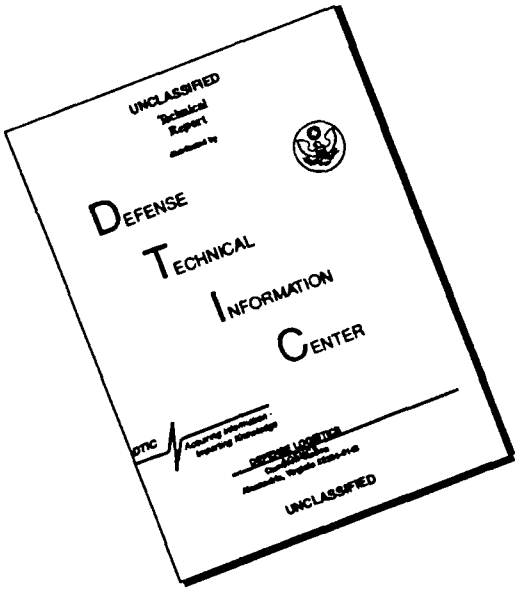

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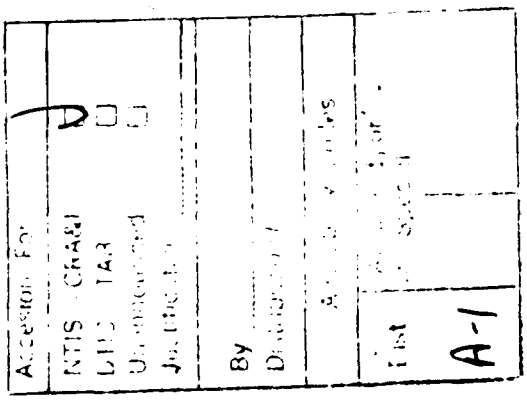
recipient cell and causes derepression of it's gene for the antiviral protein.

During this granting period we have found that: (a) the transfer of viral resistance is operational within cell lines and as few as 10% of the cells determine the response of the population; (b) only a fraction of a heterologous donor cell population acts as donors and this characteristic is unstable and associated with a dense colonial morphology; (c) the transferred signal probably passes through more than one cell; (d) leukocytes can transfer viral resistance to fibroblastic and epithelioid cells; (e) leukocyte transfer of viral resistance is preceded by leukocyte interferon production in response probably to a membrane component of the co-cultured heterologous cells; (f) the inducing component can be obtained in a soluble form; (g) a B lymphocyte is responsible for interferon production in the leukocyte transfer system; (h) interferon-induced immunosuppressive activity is transferable between heterologous as well as homologous lymphoid cells; (i) transfer of immunosuppressive activity is mediated by a soluble suppressor factor produced by interferon-induced suppressor cells (j) the suppressor factor is dissociable from interferon, devoid of antiviral activity and has a molecular weight greater than 10,000 daltons; (k) interferon has hormonal activity and a hormone has interferon-like antiviral activity; (l) the reciprocal activities of interferon and hormones are transmissible between cells and probably involve the same or similar secondary messenger molecules; (m) the viral resistance transfer process is probably mediated by a soluble substance which is highly unstable and found intracellularly within minutes after interferon treatment of cells; (n) there is an antiviral material (probably fibronectin) which is dissociable from the transfer substance and released from non-interferon treated cells.

These findings have shown that: (a) the transfer process is an important major amplification system for interferon's antiviral and immunosuppressive actions; (b) the transfer process is a means to study cell communication, genetic derepression and their molecular control; (c) there is a new process for cells of the immune system to disseminate antiviral and immunoregulatory activity; (d) interferon and hormones are very similar in their activities; (e) interferon may be a hormone; (f) hormones may have functions, such as protection of tissues against viruses and maintenance of differentiation, which are not classically associated with them; (g) a transformation sensitive protein (fibronectin) has antiviral activity.

The understanding of the molecules involved in the aforementioned processes may lead to a new series of antiviral and immunosuppressive substances as well as a possible new strategy of tissue targeted antiviral and antitumor therapy.



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RESEARCH PLAN

A. Introduction

I. Objective:

The overall objective of this research proposal was the continued study of the cells and mechanism(s) involved in the transfer of interferon induced viral resistance. Up to the present time we have studied the following specific problems.

1) Is the transfer process operational within cell lines?
2) What are the characteristics of donor and recipient cells in the transfer system?
3) What is the number of cells through which transferred resistance passes?
4) What are the characteristics of transfer of interferon-induced viral resistance by lymphoid cells?
5) Do lymphoid cells transfer interferon-induced immunoregulatory activity?
6) Do interferon and polypeptide hormones act through the same or similar secondary messenger molecules?
7) What are the optimal conditions for the production of interferon-induced molecule(s) which transfer resistance?

II. Background:

Interferon shares a number of similarities with polypeptide hormones (1). For instance, penetration of the cell membrane is not required for its action (2,3). The interferon-membrane interaction in turn leads to derepression and production of the antiviral protein (4,5). Prior to the work herein reported, essentially nothing was known about the events between interferon action at the cell membrane and derepression of the gene for the antiviral protein. We recently designed a system for the study of these events (6).

This system was based on two observations. First, that many animal cell types exhibit the ability to communicate between themselves in vivo and in vitro (7). This communication is thought to occur through gap junctions which allow cells to share their metabolites and small control molecules (8-12). Second, 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 (13). We hypothesized that if, as the case with polypeptide hormones, the induction of the antiviral protein is mediated via secondary molecules, these might influence adjacent cells. The many instances of the species specificity of interferon action (14) made this a testable hypothesis. Briefly, we found that cells made resistant to virus infection by treatment with their homologous interferon can transfer viral resistance to cells of a heterologous species insensitive to that interferon (6). For instance, while human WISH or baby hamster kidney cells (BHK) alone were not sensitive to the action of mouse interferon, cocultivation of these cells in the presence of mouse interferon and sensitive mouse L cells resulted in a marked inhibition of the expected yield of virus from the interferon insensitive cells. Control cell mixtures, in the absence of interferon, yield

at least the expected amount of virus as compared with the yield from either cell type alone. The controls showed that inhibition of virus yield does not result from cocultivation of different cell species but resulted from the presence of the mouse interferon preparation with the cocultivated cells. Using poliovirus (which infects only human cells) (15), we have directly shown that the human WISH cell cocultured with mouse L cells in the presence of mouse interferon is protected and that this protection is dependent on the interferon dose. These data seem to confirm the hypothesis that the presence of interferon with its homologous cell can induce antiviral activity in heterologous cells and suggest that cell-to-cell communication can be demonstrated with interferon action.

Initially, we determined that the observed transfer of viral resistance from L cells to heterologous cell species was initiated by mouse interferon and that the transferred resistance had the characteristics of interferon action. Taken together, the results strongly suggest that initiation of the transfer process was due to mouse interferon since it occurred with crude as well as highly purified (10^7 units/mg protein) interferon preparations; its action was on the cell rather than the virus since resistance was manifested in the absence of interferon but in the presence of cells which had been acted on by interferon; and the transfer process was initiated by mouse interferon produced in two different cell lines (L and C243). These two interferon preparations have been shown to have the classic characteristics of interferon (i.e., species specificity, broad virus specificity, requirement of RNA and protein synthesis for action, inhibition by antimouse interferon antisera, nondialysability, trypsin sensitivity, pH 2 resistance etc.) (14). The results also show that transferred resistance has the characteristics of the interferon system in that it was blocked by actinomycin D (5 μg/ml) and was effective against both a RNA (VSV) and a DNA (vaccinia virus) virus.

We found that the transfer of resistance is dependent on cell proximity since the degree of transfer was controlled by both the donor (L) to receptor (WISH or BHK) cell ratio as well as the absolute cell density at a given ratio. Interferon-induced transfer of viral resistance was not observed until the majority of cells were in close contact with neighboring cells.

We also found that while transfer of viral resistance to heterologous cells required more interferon than development of resistance in homologous cells, the process was none-the-less efficient in terms of the concentrations of interferon required. The concentrations of interferon necessary (5-15 units/ml) for transfer are well within physiologic limits.

We have examined the possible mechanism(s) governing the transfer of interferon- induced viral resistance between heterologous cells (16). 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 derepression 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 findings that VERO cells, which produce essentially no interferon (17) receive transferred resistance from L cells (18). 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 cocultivated 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 was presented which showed that the development of resistance in the donor L cells precedes the development of resistance in the WISH cells (16). 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 (19), 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 (16). Since the mouse antiviral protein is stable for more than 8 h (20) 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.

The mRNA for the antiviral protein also seemed an unlikely candidate for the effector of transferred resistance. If the mouse 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 (16). 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.

By a process of elimination and in light of the data with actinomycin D, secondary messenger molecules which transmit the interferon signal from the membrane to the nucleus are favored as the effector substance(s) for the transfer process leading to derepression of the gene for the human antiviral protein. This model for the transfer phenomenon is strengthened by our preliminary findings of a soluble interferon induced material from L cells which confers viral resistance on human WISH cells.

To be certain that the transfer phenomenon was not limited to a few cell types, we examined other cell species and their ability to exhibit transfer. We have shown that this phenomenon also occurs when rabbit kidney and human WISH cells, with their corresponding interferons, are cocultivated with human WISH and baby hamster kidney cells, respectively. This finding increases the number of donor cell types to three. The related finding that monkey VERO and chick embryo cells can be recipients of transferred resistance expands the number of heterologous recipient cell species to five (18).

With a fairly large number of cell species demonstrating the transfer of resistance, this could possibly be a general phenomenon among adjacent cells within the body and thus play an important role in the pathogenesis of viral infections. Information relevant to this idea came from the finding that the rate of development of interferon-induced virus resistance in a mixture of two human cell types (U and WISH) is determined by the cell type (WISH) in the mixture which responds first (21).

The transfer of virus resistance from one human cell (WISH) to another (U) (homologous transfer) is much more efficient than the transfer from mouse L cells to WISH cells (heterologous transfer), as was shown by a much lower ratio of donor to recipient cells required for maximum transfer as well as a more rapid transfer. Thus, virus protection afforded by the interferon system is amplified more efficiently in mixtures of different human cells than in mixtures of mouse and human cells (21). An explanation for this difference in efficiency might be found in the mechanism of transfer between cells. For example, if transfer of virus resistance occurs through gap junctions which allow cells to communicate between themselves, then the efficiency of the transfer should be a reflection of the relative ability of the cells to communicate. Recently, specificity of junctional communication was shown and appeared to occur more frequently between homologous than heterologous cells (11, 12). Hence, the demonstration that a lower percentage of donor cells is required in a homologous cell mixture than in a heterologous cell mixture for maximum transfer of virus resistance may be explained in terms of the relative ability of these cells to communicate.

Based on the above findings we predicted that cellular interactions which depend on cell proximity determine the rate and degree of interferon action. Consistent with this prediction are the findings that the response of mouse L, human WISH and secondary ME cells to mouse or human interferon is determined by the cell density. More specifically, it was shown that as the cell density was lowered both the rate of development and degree of viral resistance in response to interferon decreased and reached a minimum when most cells were not in contact with one another (22).

The observed requirement of cell contact or at least close proximity for a maximum response to interferon strongly points to a cell-to-cell transfer

of resistance among these cells and implies this process is operational in vivo where there is three dimensional cell contact. This requirement was shown by the fact that the response to interferon increased as the number of cells touching increased and that even at a concentration of cells equivalent to a confluent monolayer, suspended cells responded slower than attached cells (22). It is, then, not merely the cell concentration which determines the response to interferon, but the spacial relationship of the cells. Interestingly, ME cells and human diploid fibroblasts, even well below confluency, sent out cellular processes and at relatively low cell densities formed a network of cells and showed maximum sensitivity to interferon. With ME cells, a marked decrease in interferon sensitivity only occurred when the majority of these cellular extensions did not touch.

This cell proximity effect might be envisioned to result from variations in interferon sensitivity between subpopulations of cells in vivo and in vitro. The most sensitive and first responding cells to interferon could transfer their resistance to less sensitive, slower responding cells, possibly through gap junctions whose formation requires cell contact. In fact, our preliminary findings indicated sufficient variation in isolated clones of our L cells to account for at least part of the observed effect. Alternatively there could be a solubilization of a transfer material which is conserved by close cell proximity since the local concentration around cells would be higher at higher cell densities. Such a transfer process and substance would, in effect, increase the speed and sensitivity of the interferon system. In our studies, then, when the cells are not in close proximity this amplification does not occur. One intriguing implication of these findings is that in a mixed population of cells in vivo, the more slowly responding cells may be influenced by cells which respond more rapidly to interferon. Thus, the efficiency of the virus protection afforded by the interferon system could be amplified. Since transfer of virus resistance between different human cells occurred almost immediately and with low percentages of donor cells (21), this process probably represents an important component of the interferon system in its defense against virus infections.

This transfer process and the finding of large variations in sensitivity of cloned cells to interferon has the appealing aspect that all cells do not have to expend their cell machinery to a maximum extent for maximum sensitivity to interferon. This process would tend to be conservative for such things as cellular receptors for interferon.

We have also shown that immune-type interferon, a lymphokine, can cause the transfer of viral resistance from mouse to human cells (23). This is similar to findings for virus-type interferon (6), except that immune-type interferon caused the transfer more efficiently. The immune-type interferon molecule(s) was found to be the most likely substance in the interferon preparation to be responsible for the transfer. The transferred resistance had the characteristics of an interferon-induced antiviral state. The kinetics of development of transferred viral resistance in response to mouse immune-type interferon suggest that a antiviral process is initiated in the mouse cells and is subsequently transferred to the human cells. Interestingly, although the kinetics of the response of L cells to virus-type and immune-type interferon are different, there is a similar delay in the development of transferred resistance in the human WISH cells. This indicates that the transfer process and its expression in WISH cells may be similar with both interferon types whereas, the initial events by which the two interferons activate L cells may

be different. These data also indicate indicate that within L cells, immune-type and virus-type interferons probably share some common pathways to the antiviral state. These findings further suggest that, like virus-type, some component(s) of the immune-type interferon system is (are) not species specific because immune-type interferon-treated L cells can transfer viral resistance to human WISH cells.

We have proposed that the natural mechanism of interferon protection may include action on cells near the interferon-responding cell by means of this transfer mechanism (6). The finding that immune-type is better able than virus-type interferon at eliciting the transfer mechanism points to a new and efficient means of disseminating the interferon response of lymphocytes.

In addition to the role of a lymphocyte product in the transfer of viral resistance, preliminary findings indicated that non-sensitized lymphocytes can transfer resistance to heterologous cells (see below). One unexpected result of these studies was the demonstration that foreign cells stimulate non-sensitized lymphocytes to produce a type of interferon which has the properties of leukocyte interferon (24). Further this induction of lymphocytes does not necessarily require transformed cells since normal heterologous cells also induce. Additionally, intimate and or brief contact between the lymphocytes and the foreign cells, but not mycoplasmas or endogenous viruses, appears to be required for induction. The kinetics of production of leukocyte interferon by non-sensitized lymphocytes in response to foreign cells is similar to that induced by viruses. Apparently, in this system leukocyte interferon must be produced prior to transfer of resistance from non-sensitized leukocytes to other cell types.

B. Methods of Procedure and Results

1. Is the transfer process operational within cell lines?

a. The interferon response of individual cells within a cell line: Cell cloning was employed to establish whether there is variation in the interferon response of individual cells within a population (25). Mouse L cells were suspended in MEM supplemented with 10% fetal calf serum, nonessential amino acids, and 25 mM Hepes buffer. Cells (20/ml) were distributed at 5 ml/flask into a series of Falcon tissue culture flasks (area, 25 cm^2). They were incubated undisturbed at 37° for 10 days at which time the medium was replaced with mouse interferon or fresh medium. Following interferon treatment, 10^5 plaque-forming units (PFU) of vesicular stomatitis virus (VSV) was added to each flask (approximate input multiplicity of infection was 20 PFU/cell). After overnight incubation at 37°, surviving clones were stained with crystal violet and counted. The cloning efficiency of L cells was 70-100%. Figure 1 shows that when a low density of L cells is plated such that each cell forms a clone, the number of clones protected against virus challenge increased with the length of interferon treatment. Likewise, at a given time, there is an increase in the number of protected clones with increasing concentrations of interferon. These data show that individual cells differ in the amount of interferon to which they respond as well as the time required for the response. These findings strongly suggest marked differences in the interferon response of individual L cells.

To determine the degree of variation, cell lines were established from individual L cells. For the establishment of cloned L-cell lines, 0.1 ml/well of a cell suspension (30 cells/ml) was added to each well of Falcon Micro Test II tissue culture plates. After 10 days incubation in 4% CO_2 at 37°, wells containing a single clone were visually identified. About 4 days later, the cells from these wells were removed with trypsin and placed in Falcon tissue culture flasks (25 cm^2) for 2 weeks then into 75-cm^2 flasks.

Three days later they were assayed for interferon sensitivity either by a micro-yield reduction assay (1×10^3 cells/well) (6) or by the method of microplaque reduction using methylcellulose overlay instead of carbomethylcellulose (7.5×10^4 cells/well) (26). The VSV challenge dose for these two assays were 3 PFU/cell and 25-30 PFU/well, respectively. Representative dose responses for three of these cloned L cell lines is shown in Fig. 2. At the extremes, clones differed by 10-fold in their sensitivity to interferon and almost 100-fold in the maximum degree of protection against virus. Table 1 summarizes the results of the interferon responses of 18 L cell clones. Clearly, there is a large heterogeneity among individual L cells in both the sensitivity to and maximal protection afforded by interferon.

b. Transfer of interferon-induced viral resistance between cloned L cells: If the transfer of viral resistance occurs between individual cells of a cell line, then this should be demonstrable with cloned cells derived from the parent population.

When cloned L cells of "high" (clone 2) and "low" (clone 1) sensitivity to interferon were cocultivated in a 1-to-1 ratio the response of the mixed population approached that of the "high" responding clone (Fig. 3). The virus yields from non-interferon-treated cells of "high" and "low"

responding clones (alone or mixed) were the same and the yield from interferon-treated "high" responding cells was insignificant compared to equivalently treated "low" responding cells. Thus if transfer had not occurred, in the presence of interferon the yield should have been 50% of the yield from interferon-treated "low" responding cells. In terms of the maximum amount of protection confered by interferon, there was no significant difference between the mixed cells and the "high" responding cells. This observation has been repeated with three different clones. The increase in sensitivity ranged from 3- to 30-fold and the increased maximum degree of protection ranged from 5- to 30-fold. Based on these data, it seems that individual cells within a heterogeneous population can transfer interferon-induced viral resistance amongst themselves.

c. The fraction of cells which determines the interferon response of the population: Since the preceding experiments demonstrated that transfer of viral resistance can occur within a population of cells, it is important to determine what fraction of cells controls the response of the population. This was investigated by comparison of the interferon response of the parent L-cell population, 40 L-cell clones, and a reconstituted L-cell population. In a plaque reduction assay, the response of the reconstituted population (equal numbers of each cloned cells) was not significantly different from the parental population, while both were four to five times more sensitive than the average of the 40 individual clones (Fig. 4). This indicates that transfer occurs within L cells and can be demonstrated with a plaque reduction assay. It also appears that the reconstituted population closely approximates the parental population.

A compilation and analysis of this data (Table 2) shows that with 1 and 3.3 U/ml of interferon the response of the population is determined at most by 10 and 30% of the cells, respectively, whether all of these clones transfer is not known at present. At 10 and 33 U/ml almost all the cells respond equally (100% inhibition). Thus the transfer of viral resistance can play an important role in the action of interferon.

2. What are the characteristics of donor and recipient cells in the heterologous transfer system? While work has not begun on the recipient cells, the following has been completed with donor cells.

a. The percentage of the donor population which transfers viral resistance: Since L cells were shown to be heterogeneous in their sensitivity to interferon and ability to transfer resistance to other L cells we tested whether a similar heterogeneity was observed in transfer to human WISH cells. Cloned L cells derived as above were assayed for their ability to serve as donor cells in the transfer of resistance to WISH cells. We assayed for transfer by the standard technique: Cloned L cells and human WISH cells in Eagle's medium supplemented with 10% fetal calf serum were cultured in a 1-to-1 ratio in Micro Test II tissue-culture plates (Falcon Plastics, Oxnard, California). The total number of cells in each well (about 28 mm^2) was 2.25×10^5. Controls consisted of an equivalent total number of either cell species alone. Interferon or an equal volume of medium was added; cultures were incubated overnight at 37°C in a 4% CO_2 atmosphere. Supernatant fluids were decanted and each well was infected with 5×10^4 pfu of VSV. After 1.5 h at 37°, the inoculum was decanted, cell sheets are washed once and replenished

with fresh medium. Virus yields from pooled triplicate cultures were determined approximately 24 h later by a slightly modified microplaque assay (26). Since interferon-treated L cells produce a negligible amount of virus, the expected yield is calculated from the percentage of the WISH cells in a cell mix without interferon or from the WISH cell yield with interferon.

Table 3 shows that only about 30% of L cell clones are able to transfer viral resistance to human WISH cells. Thus, it appears that at any point in time only a fraction of a donor cell population actually transfer resistance. It is interesting that only a percentage of donors are involved in heterologous and homologous (see Table 2, 1 U of interferon) transfer. Whether the same cell is involved in both systems is presently unknown.

b. Stability of the donor cell "phenotype": In preliminary experiments, it appears that the ability of individual L cells to act as donors in the transfer of viral resistance to WISH cells is an unstable characteristic. While the percentage of clones that donate remains constant the same clones do not always transfer resistance. Hence, it appears that epigenetic factors may be involved in the donor cell "phenotype". It might be pointed out that the parent population of L cells always transfers resistance. This shows that there is always a subpopulation of cells within the parental population which can transfer resistance.

c. Markers of donor cell activity:

i. Degree of interferon sensitivity: Table 3 shows, as expected, that a donor L cell (clone) must be sensitive to its homologous interferon in order to transfer resistance to WISH cells. However, sensitivity to interferon does not assure ability to transfer resistance. Thus the donor cell population falls into three subpopulations: 1) cells insensitive to interferon and unable to transfer; 2) cells sensitive to interferon and unable to transfer; 3) cells sensitive to interferon and able to transfer. Of the cells which transfer resistance there is not a direct correlation between degree of sensitivity and level of transferred resistance. It appears then that with interferon sensitive cells the ability to transfer resistance is independent of the ability to respond to interferon.

ii. Colonial morphology: Our clones of L cells fall into three broad catagories based on morphology. Those which form: dense piled colonies of cells on a plastic surface; contact inhibited colonies which are only one cell deep; and loose colonies where cells in a colony have two or three cell diameters between them and their neighbors. As is seen in Table 3 while only 20% (1/5) of the contact inhibited or loose clones transfer resistance 50% (5/10) of the dense clones are able to transfer. It appears then that colonial morphology may be related to ability to transfer resistance. These studies need to be done with more clones but may be a method for selection of donor cells. The reason behind this phenomenon is unknown and requires further study.

3. What is the number of cells through which transferred resistance passes?

While this has not yet been directly determined, data from the preceding experiments indirectly suggests that resistance passes at least from the donor cell through one recipient then to another. The argument supporting

this is as follows. It is known that only 1 in 10 cells can transfer in the homologous system. Since in a two dimensional culture system such as ours one cell can only be surrounded by about 6 and all cells in the culture appear protected, then protection probably extend at least one cell beyond the donor. More direct and quantitative evidence should come after previously proposed experiments are completed (see Research Proposal).

4. What are the characteristics of transfer of interferon-induced viral resistance by lymphoid cells?

a. The stimulus for leukocyte interferon production: Trinchieri et al, (27) reported and we have confirmed (24) that non-sensitized human leukocytes produce interferon when co-cultured with human tumor but not normal human cells. We also found that both normal and transformed heterologous cells induce this interferon response by non-sensitized human leukocytes (24). We have shown that this induction is for a specific type of interferon, leukocyte interferon (24) and that this interferon must be produced before leukocytes transfer viral resistance to the heterologous cells (see below). Hence the induction of this interferon is intimately involved in the transfer of resistance from leukocytes to heterologous cells. De novo RNA and protein synthesis are not required in the inducing cell since interferon is produced when actinomycin D. treated L cells are co-cultured with nonsensitized human lymphocytes (Table 4). Since interferon production requires new RNA and protein synthesis it also confirms that the interferon produced is a product of the human leukocyte. This data implies that if the inducer of the interferon response is a protein it preexists on the inducer cell.

We have found that Dounce homogenized L cells will induce interferon production by non-sensitized human leukocytes. The inducing component remains in the supernatant fluid during low speed (2,000xG) centrifugation. About 50% of the inducing activity also remains in the supernatant fluid and 50% in the pellet after high speed centrifugation (10,000 xG) (Table 5 & 6). Since this force is sufficient to pellet most intact membranes (28) the inducer may be membrane associated. Additionally, inducer apparently has been solublized by homogenization, possibly as a membrane fragment. We are presently characterizing the inducer by its possible succeptibility to specific enzymes (protease, glycolytic enzymes, RNAse etc) and are about to begin purification and molecular weight determinations (see Research Proposal).

Non-sensitized mouse spleen cells have also been shown to produce a mouse type I interferon when co-cultured with human WISH cells (Table 6). Thus the mouse system appears much like the human and could possibly serve as another assay procedure for the inducer.

b) Cell type that produces heterologous cell-induced leukocyte interferon: Human leukocytes from ficoll-hypaque gradients (29) have been characterized as to which subpopulation produces leukocyte interferon in response to heterologous (L) cells. The producer cells are apparently not T cells since they are not removed by rosetting with sheep erythrocytes (30). They are non-adherent which indicates they are not macrophages (31). Treatment with anti-human immunoglobulin antibody and complement removes B cells (32) as well as the leukocyte interferon producer cell (Table 7). These data strongly suggest that B cells produce leukocyte interferon in response to heterologous cells.

c. Leukocyte transfer of interferon-induced viral resistance: We now have ample evidence that non-sensitized human leukocytes can transfer viral resistance to heterologous cells.

Figure 5 shows that when human leukocytes were co-cultured with mouse L cells there was rapid production of human interferon but not mouse interferon. Shortly thereafter, the mixed cultures became resistant to challenge by Sindbis virus. Since human leukocytes alone failed to yield significant amounts of virus ($<10^2$ PFU), these data showed that the mouse L cells must have acquired resistance to virus replication. The close temporal relationship between the production of human interferon and the development of resistance suggest resistance was initiated by human interferon although not directly since the mouse cells were not sensitive to human interferon at levels present in the culture fluids.

In addition to mouse L cells, mouse embryo fibroblasts and primary chick embryo cells induced human interferon production by non-sensitized human leukocytes and subsequently became resistant to virus infection. Allogeneic human diploid cells failed to induce interferon and were not protected against virus challenge (Table 8). This is in agreement with the previous demonstration that human tumor cells but not normal human cells induced interferon production by non-sensitized human leukocytes (24,27). The results showed that the leukocyte transfer process was not limited to L cells as recipients and that the viral protection was most likely mediated by interferon since human diploid cells failed to induce interferon and were not protected against the virus.

We previously have shown that the transfer of mouse interferon induced viral resistance from mouse L cells to human cells was dependent on the ratio of mouse to human cells (6). Likewise, when a constant number of mouse L cells were co-cultured with increasing number of human leukocytes there was an increase in the degree of viral protection of the mouse cells and the amount of human interferon produced by the human leukocytes. The data (Fig. 6) indicated as few as one leukocyte per one mouse L cell and as little as 100 units of human interferon could initiate protection of the mouse cells, thus indicating that the transfer process between human leukocytes and mouse L cells was very efficient. The viral resistance transferred from non-sensitized leukocytes to mouse L cells was also found to be effective against a wide range of viruses (Table 9).

One possible explanation for the reduced virus yield from mouse L cells co-cultured with non-sensitized human leukocytes could be cytotoxicity mediated by natural killer (NK) cells, especially since NK activity is augmented by interferon (33). This possibility was investigated by studying ^{51}Cr release from labeled mouse L cells co-cultured with human leukocytes. Table 10 shows that significant resistance to virus developed in L cells 4 hours after addition of human leukocytes while significant ^{51}Cr release was not observed until 24 hours. Even considering the 8 to 10 hour replication cycle for Sindbis virus, cell mediated cytotoxicity (CMC) as measured by ^{51}Cr release occurred too late to significantly affect the virus yield. These data strongly suggest that CMC was not responsible for most of the observed reduction in virus yield from these mouse L cells. Further evidence supporting this conclusion are: a) Transfer of viral resistance occurs at a lower ratio of leukocytes to L cells than is commonly used to show CMC by ^{51}Cr release assays;

b) there is no microscopically observable toxicity to mouse L cells co-cultured with non-sensitized human leukocytes for 24 hours; c) 24 hours after co-culture of the two cell types 95% of the L cells exclude trypan blue dye and take up neutral red dye. Thus, the reduction in virus yield does not appear to result from toxicity of non-sensitized human leukocytes for co-cultured mouse L cells over the times observed.

Studies on the cell type responsible for leukocyte transfer of viral resistance are just beginning. However, it appears that both human B and T lymphocytes can transfer resistance.

5. Do lymphoid cells transfer interferon-induced immunoregulatory activity?

a. Heterologous lymphoid cells: Interferon has been shown to exert profound effects on the immune system. A few of the *in vivo* effects include inhibition of both the mixed lymphocyte reaction (34) and the primary *in vitro* antibody response (35,36), and increased phagocytosis by macrophages (31). Our data showing transfer of viral resistance from leukocytes to heterologous cells suggested that the immunoregulatory properties of interferon might be transmissible. We employed inhibition of the mouse primary *in vitro* antibody response to sheep red blood cells (SRBC) as an assay for mouse interferon activity (37). To test for possible transfer of interferon-induced immunosuppressive activity, we co-cultured human leukocytes and mouse (C57Bl/6) spleen cells in the presence of human interferon. Table 11 shows that while human interferon or human leukocytes alone suppressed the mouse antibody response, the combination of the two caused more than an additive suppression. This suggests that human leukocytes interacting with human interferon may transfer immunosuppressive activity to mouse spleen cells.

b. Homologous lymphoid cells: To test interferon-induced transfer of immunosuppressive activity in a homologous system, mouse (C57Bl/6) spleen cells were incubated with mouse fibroblast interferon and washed to remove residual interferon. These cells were added to non-interferon treated spleen cells and the anti-SRBC response determined. We found that interferon treated spleen cells inhibited the anti-SRBC antibody production by the untreated cells (Table 12). Significant suppression of the *in vitro* direct plaque-forming cell (PFC) response after 5 days incubation was observed with cells treated with two concentrations (500 and 5000 units/ml) of interferon and with two concentrations of interferon-treated cells (1×10^7 and 3×10^6/ml) (Table 12). The PFC responses of the cultures to which the interferon-treated cells were added were inhibited by approximately 90% whether expressed as PFC/culture of PFC/10^6 viable cells. Over 90% of the interferon used to treat the spleen cells was recovered. Under conditions where 1×10^8 cells were treated with 500 units of interferon, the addition of 3×10^6 of these washed cells to the PFC cultures would have resulted in a maximum carryover of only 2 units of cell-bound interferon. We have previously determined that at least 50 units of interferon are required in the cultures to suppress the PFC response by 90%. Thus interferon appears to act indirectly through induction of suppressor cell activity. Suppressor cell activity can be induced with as little as 100 units of interferon/ml and with treatment for as short as 2 hr. Cell viabilities for both interferon-treated cells and untreated controls were essentially the same, 75-85%. Interferons of specific activities of 10^5 and $10^{8.5}$ units/mg protein

were both capable of inducing suppressor cell activity. Depletion of macrophages from the suppressor cell preparation by glass beads-glass wool columns did not affect the suppressor cell activity, which suggests that the suppressor cell is a lymphoid cell.

Further characterization of the interferon-induced suppressor cell activity was done by treatment of interferon-induced suppressor cells with antibody to mouse L cell fibroblast interferon to remove residual interferon. Results are presented in Table 13. Specific antibody did not block or reduce the suppression, which was essentially 100%, and was comparable to normal rabbit serum and medium controls. Spleen cells not treated with interferon, as expected, were not suppressive under the same conditions. Further, the immunosuppressive effects of the interferon used to induce the suppressor cells was significantly blocked by preincubation of this antibody with interferon, which is consistent with previous observations. Thus the suppressor cell activity was induced by interferon, did not require the countinued presence of interferon, and could be attributed to either a direct or indirect action of suppressor cells.

c. Interferon-induced suppressor factor: Dose-response studies indicated that 1 to 3 x 10^3 suppressor cells/1.5 x 10^7 untreated spleen cells resulted in significant suppression of the PFC response (data not shown). The high ratio (approximately 100:1) of untreated cells versus suppressor cells that resulted in suppression of the PFC response suggested that direct cell-cell contact of the effector (suppressor) and responder (untreated) cell was not required. Further, this suggested that a mediator derived from the suppressor cell population was probably responsible for the suppression. Direct evidence for such a mediator was obtained by incubating high concentrations of interferon-treated cells for 2 hr at 37°C and adding the supernatants to untreated cultures (Table 13). The PFC response was suppressed over 95% and the suppression was not affected by prior incubation of suppressor supernatants with antibody to interferon. The suppressor factor may be a macromolecule, since it did not pass through an Amicon filter with a molecular weight cut-off of 10,000 daltons, and in fact was concentrated under these conditions. Thus interferon induced suppressor cells, which in turn produced a suppressor factor that was capable of suppressing the *in vitro* PFC response.

It was of interest to ascertain whether the suppressor factor possessed antiviral activity. Undiluted suppressor factor preparation contained antiviral activity equivalent to 10 to 30 units of interferon/ml which could be residual after washing. All of this antiviral activity was neutralized by antibody to interferon which failed to neutralize the suppressor activity. Interferon-induced suppressor factor, then, was active in suppression of the PFC response, but lacked antiviral properties.

Titration of the factor showed a linear relationship between inhibition of PFC response versus suppressor factor dilution (Figure 7). Induction of suppressor factor by interferon was completely blocked if the interferon was first neutralized by specific antibody prior to addition to spleen cell cultures. This is evidence that interferon was the inducer of the suppressor factor.

These data show that interferon's immunosuppressive activity, like it's antiviral activity, is transmissible between cells. The mechanisms of

action however appear different since the suppressor factor lacked antiviral activity. Additionally efficient transfer of suppressor activity did not require cell to cell contact. Whether there is a similar secondary messenger molecule for induction of antiviral activity and suppressor factor is unknown at present.

6. Do interferon and polypeptide hormones act through the same or similar secondary messenger molecules?

Shortly after our demonstration of the transfer of interferon-induced viral resistance, similar transmission of hormonal stimulation by cell-to-cell communication was reported (38). Since interferon is thought to act through secondary messenger molecules there is the possibility that they are the same or similar to those for polypeptide hormones. If this hypothesis is correct then certain predictions can be made. First, interferon should cause a hormonal response which is species specific. Second, a hormone should induce antiviral activity which is tissue specific. Third, if both of these responses are mediated by similar secondary messengers they should be transmissible and cross activate cells.

a. Hormonal activity of interferon: We decided that rather than first studying induction of tyrosinase in melanoma cells or plasminogen activator in ovarian granulosa cells as originally proposed we would initially study changes in the beat frequency of cultured mouse myocardial cells. This system seemed simpler for a pilot study and is sensitive to a polypeptide like hormone, noradrenaline.

It is known that noradrenaline increases the spontaneous beat frequency of cultured mouse myocardial cells. The present studies confirm these results as well as show a noradrenaline dose response which is in good agreement with that previously published (38) (Fig. 8a). Increases in beat frequency also resulted from treatment of the cultures with mouse but not human interferon (Fig. 8a) there by demonstrating species specificity of action. The interferon concentrations required to cause a change in beat frequency are equivalent to those which induce antiviral activity and are well within the concentrations produced in vivo. The similarity of the slopes of the noradrenaline and interferon dose responses suggest that they increase the beat frequency by a similar mechanism. These results show that interferon can elicit a hormonal response in a species specific manner.

b. Antiviral activity of noradrenaline: Figure 8b shows that overnight treatment with noradrenaline caused a dose dependent development of antiviral activity in myocardial cells. Interestingly, the maximal antiviral activity was observed at the same concentration (10^{-6}M) of noradrenaline as the maximal stimulation of beat frequency. The noradrenaline induced antiviral state was not as strong as that induced by interferon (0.7 to 1.0 $\log_{10}$ reduction in virus yield vs 2.0 $\log_{10}$ reduction). As will be shown later, under identical conditions, noradrenaline did not cause antiviral activity in human ammion (WISH) cells thereby demonstrating its tissue specificity. These findings demonstrate that in addition to its classical effect on beat frequency, noradrenaline treatment of its target (myocardial) cells causes the development of an antiviral state.

c. Characterization of the hormonal activity of interferon and the antiviral activity of noradrenline: Experiments were done to characterize the hormonal activity of interferon and the antiviral activity of noradrenaline.

Table 14 shows that purified mouse fibroblast interferon ($10^{8.5}$ units/mg protein) caused an increase in beat frequency. Additionally, the stimulatory effect of a crude interferon preparation was neutralized by specific anti-interferon antisera. In other studies, interferon preparations of varying purity increased the beat frequency in direct proportion to their antiviral titer (data not shown). These results demonstrate that the increase in beat frequency is initiated by the interferon molecule itself. We also found (Table 14) that once myocardial cells are maximally stimulated with mouse interferon, addition of noradrenaline (10^{-5} M) caused no further significant increase. Also, interferon and noradrenaline increased the beat frequency with similar kinetics. Maximal stimulation with noradrenaline required about 1 minute while interferon required 2 minutes (Table 14). This represents the most rapid reported final action of interferon. Taken together these results strongly suggest that the effects of noradrenaline and interferon on the beat frequency of mouse myocardial cells occurs through a common mechanism.

When the antiviral activity of noradrenaline was characterized we found that the continued presence of noradrenaline and interferon was not required since the antiviral state remained after overnight treatment and washing the cells to remove these substances. This shows that the effect of both interferon and noradrenaline are on the cell rather than on the virus. One hallmark of the interferon induced antiviral state is the inhibition of its induction by actinomycin D (4,5). Similarly, the induction of antiviral activity by noradrenaline was blocked by actinomycin D (Table 14). Like interferon, noradrenaline did not show virus specific antiviral activity (see below). Under conditions of high multiplicity of infection, vaccinia virus is not sensitive to interferon (6) and was not inhibited by noradrenaline (data not shown). These data show that the antiviral state that is induced in myocardial cells by noradrenaline bears a marked resemblance to that induced by interferon.

d. Transmission of interferon's hormonal activity: Since our data indicate that interferon and noradrenaline share common pathways that may involve similar secondary messenger molecules, then the hormonal activity of interferon and the antiviral activity of noradrenaline should be transmissible between cells. This hypothesis was tested with co-cultures of mouse myocardial cells and human amnion WISH cells. As demonstrated above (Figure 8a) the stimulatory effect of interferon on beat frequency is species specific. Human interferon did not increase the beat frequency of mouse myocardial cells (Figure 8a and Figure 9). However, when human interferon was added to co-cultures of mouse myocardial cells and human WISH cells (which are sensitive to human interferon) there was a dose dependent increase in the beat frequency of the myocardial cells. This effect was only observed when the myocardial cells were in contact with human cells. When noradrenaline (10^{-5} M) was added to co-cultures which had been maximally stimulated with human interferon there was no further increase in beat frequency, while noradrenaline addition to mouse myocardial cells treated with human interferon increased their beat frequency to that of the co-cultures (Fig. 9). The dose dependence of this response resembled that observed with mouse interferon on mouse myocardial cells (Compare Fig. 8a and 9). Both cell types in the co-cultures in the presence of human interferon also developed antiviral resistance while human interferon treated mouse myocardial cells alone did not. Ten units of human interferon caused a 90% reduction in VSV yield from mouse myocardial cells co-cultured with human WISH cells. Kinetics studies showed that the increased beat frequency due to mouse interferon treatment of mouse myocardial cells precedes by 1 to 2 minutes

that observed in co-cultures treated with human interferon (data not shown). The observed sequence is consistent with the initiation of events in the human cell by human interferon and their subsequent transfer to the myocardial cell. These data show that under conditions which can lead to transfer of interferon induced viral resistance there is transfer of the hormonal activity (increased beat frequency) of interferon.

e. Transmission of noradrenaline's antiviral activity: The possible transmissible nature of noradrenaline's antiviral activity was explored by infection of co-cultures with poliovirus which only infects human cells (15). Figure 10 shows that noradrenaline did not induce antiviral activity in human WISH cells alone. However, noradrenaline treatment caused a dose dependent decrease in poliovirus yield from the WISH cells in the co-cultures. Poliovirus did not replicate in mouse myocardial cells in the presence or absence of noradrenaline and co-culturing myocardial cells and WISH cells did not affect the yield of poliovirus from the WISH cells. Similarly, mouse interferon (50 units/ml) treatment of co-cultures (1:1 ratio) of mouse myocardial cells and WISH cells resulted in a 85% reduction in poliovirus yield from the WISH cells (data not shown). When VSV was substituted for poliovirus the yield of virus from either cell type alone or in combination was similar. Noradrenaline caused a dose dependent decrease in VSV yield from myocardial cells alone (Fig. 8b), while it had no effect on the virus yield from WISH cells along (Fig. 10). Since the cells were mixed in a 1 to 1 ratio, if there had been no development of viral resistance in the co-cultured WISH cells one would have expected about 50% of the yield of a co-culture without noradrenaline. This is because the VSV yield from co-cultured myocardial cells in the presence of noradrenaline is inhibited 80-90% (Fig. 8b) and therefore only 50% of the cells (WISH) would yield the full amount of VSV. Figure 10 shows that noradrenaline caused a dose dependent decrease in the expected VSV yield, indicating antiviral activity in the co-cultured WISH cells. Thus, noradrenaline (like interferon) causes the transfer of non-specific virus resistance between its target cell and other cells.

7. What are the optimal conditions for the production of interferon-induced molecule(s) which transfer resistance?

Previously reported experiments have indicated that cell-free supernates as well as cell extracts from interferon treated L cells can transfer viral resistance to human WISH cells (Fig. 11). We have hypothesized that this substance(s) is responsible for the direct cell-to-cell transfer of viral resistance and that it is an intermediate molecule(s) produced by the interferon-cell membrane reaction and that it is responsible for induction of the antiviral state.

We are following our initial protocol for the production and assay of this material. This is, L cells (5×10^6/ml) are treated with mouse interferon, the cell-free extracts (obtained by sonication) are harvested and placed on human WISH cells. Following overnight incubation at 37°, the recipient (WISH) cells are challenged with virus and observed for protection (by a reduction in virus yield). We are continuing to concentrate our efforts on the detection of the transfer material in the cell extracts (Table 15) since they seem to have more activity than supernatant fluids and are more reproducible.

A number of difficulties have been encountered in our studies of the soluble transfer material. Among the most prominent are the following. The

peak activity of the transfer material appears at different times after addition of interferon to L cells (5-30 min). Since in many instances the activity appears and declines rapidly, this necessitates doing a kinetic experiment each time we work with the transfer material to assure an active preparation. This requirement increases the number of samples in any experiment by a factor of about five (time points). The transfer material is very unstable and activity declines rapidly even at -70°C (Table 16). At present this inherent instability precludes making a stock preparation from which to work. Instead we must generate the material fresh in a kinetic experiment, use it immediately and only once. These qualities while expected of a secondary messenger molecule, (produced and declines rapidly and instability) make it a formidable task to work with the transfer material. Our immediate goals are to find ways to control the time of production of the material and once produced to stabilize it. (see Research Proposal).

Difficulty was also encountered in production when, in some instances, cells not treated with interferon but receiving sonication released a factor which also proved to be antiviral (Table 17). Study and differentiation of this material from the interferon induced substance has provided several interesting observations and implications.

Upon, sonication, the control material (CM) is released concommitantly (though in lower titer and activity) with the interferon induced material. Release does not require major disruption of the cells as determined by microscopic evaluation. This may indicate that CM is released from the cell membrane. Even though CM is released concommitant with the transfer material it appears to be a separate substance (see below). However, evaluation of the transfer material is hindered by CM's presence.

The mode of production of CM has been partially ascertained. Production occurs best in medium supplemented with fetal calf serum (FSC) and there is an indication that 10% FCS is better than 2%. Serumless medium results in inconsistent production. Kinetics of production experiments lasting up to 6 hours seem to indicate that CM's production may cycle. That is, samples taken every 30 minutes for 6 hours from a donor cell population yield quantities of CM which appear to increase and decrease in regular intervals. Further work is needed to confirm this observation.

CM's mode of action has also been partially characterized. It seems to act on recipient cells and not on the virus as washing of recipient cultures does not remove activity. It has been differentiated from interferon in that it acts on heterologous cells and has also been shown not to be an interferon inducer because VERO cells, which do not produce interferon, are sensitive to CM's action (Table 18).

Some preliminary molecular and chemical characteristics of CM have also been determined. The substance is proteinaceous as shown by extreme trypsin sensitivity. It appears to be $\geq$ 300,000 MW as shown by ultrafiltration and its non dialyzable nature. CM is stable for 24 hours at 4°C while the transfer material is unstable.

The above characteristics - release from the cell membrane, extreme trypsin sensitivity, and $\geq$ 300,000 MW - plus an additional observation, that of partial neutralization by anti-cold insoluble globulin antibody indicated that CM may be fibronectin or fibronectin associated (for review see 39).

Fibronectin has been shown to bind specifically to collagen (gelatin) (39). Using this observation, we applied CM to a gelatin/sepharose column. In almost all cells tested, fibronectin was removed from the sonicate (Figures 12). Correspondingly, CM activity was removed from the sonicate by passing it through the column, though this observation at present needs confirmation. It is important to note at this point that the transfer material and CM in one experiment have been separated in this manner. Namely, CM was removed in control preparations whereas the transfer material was not.

These preliminary studies strongly suggest that the CM is fibronectin. They also offer ways of dissociating CM activity from that of the interferon induced transfer material. We feel that a complete understanding of the CM is a prerequesite to meaningful studies on the transfer material (see Research Proposal).

C. Discussion and Conclusions

We have proposed that the natural action of interferon does not require a direct effect of the molecule on each cell. This proposition stemmed from our previous demonstrations of the transfer of interferon-induced viral resistance between cells (6). Supportive of this was the finding that interferon action was determined by the cell density (22). At a cell density where the majority of cells in a population could not contact one another there was a precipitous drop in interferon activity. This cell-proximity effect was proposed to result from variation in interferon sensitivity between individual cells in the population and an inability of the most sensitive, first-responding cells to transfer their viral resistance to less sensitive, slower-responding cells when they were not in contact.

We have shown by the cloning of individual L cells that there is a very marked heterogeneity among individual cells in both their sensitivity to and maximum degree of protection afforded by interferon. Further, cloned L cells of "high" interferon sensitivity can transfer their viral resistance to clones of "low" sensitivity. By studying the interferon response of individually reacting clones and a reconstituted parental population of cells, it was found that as few as 10% of these cells can determine the response of the population (25). These findings strongly support our interpretation of the cell proximity effect and our contention that the action of interferon does not require a direct effect of the molecule on each responding cell.

Our earlier demonstrations of the transfer of interferon-induced viral resistance employed cells of different animal species (6,18). The observation of transfer among cells within a population from a single species suggests that the transfer process is operational *in vivo*. This is all the more likely since the cell proximity effect occurs with primary mouse embryo cells as well as diploid human fibroblasts (which are similar to normal cells *in vivo*) (22). This means that the natural mechanism of interferon protection probably includes action on cells near the interferon-responding cell. This process would amplify the interferon response since sensitive, fast-responding cells would transfer resistance to less sensitive, slower-responding cells which constitute the bulk of the population. The observation that a small fraction of cells (10%) determines the response of the population shows that the transfer process plays a highly important role in the action of interferon. It also implies that the transfer probably proceeds through more than one recipient cell since one cell can only be surrounded by 6 cells in this system.

There are many similarities between interferon and polypeptide hormones (1). For instance, interferon acts at the cell membrane (2,3). It has been proposed that as with polypeptide hormones, the interferon-cell membrane interaction produces a secondary messenber molecule(s) which induces the antiviral state (6). Based on our previous data a likely mechanism for the transfer of interferon-induced viral resistance may be gap junctional transfer of the putative secondary messenger(s). Shortly after our demonstration of cell-to-cell communication of interferon activity, a similar finding with polypeptide hormones was reported (38). This leads to the intriguing possibility that as with interferon there may be marked heterogeneity among "sensitive" cells in their response to polypeptide hormones and only a small number of cells determine the response of the population (or *in vivo* the tissue). If this is correct then direct cell-to-cell communication represents a novel mechanism for the amplification of hormone or hormone-like activity.

We have also found that in a heterologous transfer system only a fraction (30%) of L cell clones can transfer resistance to human WISH cells. Whether the same clones are responsible for homologous and heterologous transfer of viral resistance is not know. The donor cell phenotype seems to be an unstable characteristic and is therefore probably under epigenetic control. It is of interest to understand what controls the ability of cells to communicate since this is such an important system for coordination of functions within tissues. Phenotypically, we have observed a correlation between L cell colonial morphology and the ability to transfer resistance. The processes underlying the dense colony phenotype and its involvement in transfer may be helpful in understanding the control of cell-to-cell communication. Interferon sensitivity, of course, is a prerequisite to ability to transfer viral resistance. However, interferon sensitivity does not appear to control the ability of cells to communicate.

Lymphoid cells have also been found to transfer interferon induced resistance to other cell types. A prerequesite to this transfer is the production of leukocyte interferon by lymphoid cells in response to co-culture with heterologous cells (24). The present findings are the first demonstration that foreign cells stimulate non-sensitized lymphocytes to produce a type of interferon which has the properties of leukocyte interferon. Further this induction of lymphocytes does not necessarily require transformed cells since normal heterologous cells also induce. The inducing component of the foreign cell does not require ongoing RNA and protein synthesis since they induce after treatment with actinomycin D. Additionally, initimate and or brief contact between the lymphocytes and the foreign cells, but not mycoplasmas or endogenous viruses, appears to be required for induction. The kinetics of production of leukocyte interferon by nonsensitized lymphocytes in response to foreign cells is similar to that induced by viruses.

Preliminary data indicate that a component probably of the foreign cell membrane, can be solubilized and in this state cause the induction of interferon by non-sensitized human leukocytes. The nature of the interferon inducer is important for several reasons. First, it initiates interferon production and thereby sets into motion leukocyte transfer of viral resistance. Second, it may prove to be a valuable in vivo interferon inducer. Third it is possible that many of the in vivo interferon inducers (e.g., bacteria, protozoa, and intreacellular parasites including viruses which alter cellular antigens) induce by this mechanism. This is readily testable by characterization of the interferon type stimulated by the various inducers. Finally, a new cellular system for the recognition of foreigness is suggested by the ability of nonsensitized lymphocytes to recognize a foreign cell component and respond with a particular type of interferon. This may be of value in the diagnosis of certain tumors and infections. Further more, it may be a potential system for the easy production of virus-free, high-titered, human, leukocyte-type interferon.

It appears that a B cell is responsible for interferon production in our system. This is a new finding which may be of broad interest in terms of recognition of foreigness and cell-to-cell interactions in the immune system. After leukocyte interferon is produced we have shown that human leukocytes can transfer interferon induced viral resistance to xenogenic cells of fibroblast and epithelial origin. The possible induction of an antiviral state in the recipient cells by endogenous interferon seems unlikely because no interferon to

recipient cells was detected in supernates of any of the systems used and it is unlikely that mycoplasma or viruses were present in the primary chick embryo or secondary mouse embryo cultures that were used in this system. The transfer was rapid, efficient (1:1 cell ratios), and occurred only in the presence of leukocyte interferon. Transfer did not occur when the recipient cell did not induce interferon in the leukocytes. The antiviral state in the xenogenic recipient cell had a characteristic of the antiviral state directly induced by the interferon in syngeneic or allogeneic cells. Specifically it was broadly active against viruses. The reduction of virus yields observed in recipient cells was not due to natural killer cell activity based on the amount of specific ^{51}Cr release, trypan blue dye exclusion, and uptake of neutral red dye observed during the critical parts of the studies and the small ratio of leukocyte to recipient cells required to induce the antiviral activity.

Taken together the studies strongly suggest a new and efficient host defense mechanism against virus infections that may be operating in vivo. The system would be activated by interferon. Once activated, migrating leukocytes could transfer resistance to other tissues, including lymph nodes, spleen, liver, nerves, and other target tissues. Unlike cytotoxic cells, cells capable of transferring resistance could rapidly help protect uninfected and recently infected cells without destroying them.

Lymphoid cells were also shown to transfer interferon's immunosuppressive activity. The indirect immunosuppressive action of interferon was found to be through induction of suppressor cells which in turn produce a suppressor factor that inhibits antibody production. We have the following working model of the production and function of the suppressor factor. Its induction is blocked by treatment of interferon with specific antibody, but the immunosuppression by induced suppressor factor is unaffected by antibody to interferon. The factor is devoid of antiviral activity, which suggests that interferon regulates the immune response by a mechanism(s) that is different from its antiviral property. This differentiates the cell interactions that are involved in immunosuppression by interferon from the cell-to-cell interactions that are associated with the transfer of viral resistance (6). Additionally, efficient transfer of viral resistance requires cell-to-cell contact, which is not required in immunosuppression. This suppressor factor may play a natural role in both normal immune mechanisms and in the host response to viral infections. It may also be a desirable means of suppressing the immune response under certain conditions.

The induction of a suppressor factor by interferon, which lacks antiviral activity, is consistent with two previous observations which suggest dissociation of the antiviral and immunoregulatory actions of interferon. One is the observation that the immunosuppressive effects of fibroblast interferon are blocked by 2-mercaptoethanol, while the antiviral property is unaffected (40). The other is that a ribosome-associated factor(s) obtained from interferon-treated cells is immunosuppressive, but lacks antiviral properties (41). One of the biochemical effects of interferon on cells has recently been shown to be a block of protein synthesis via blockage of formation of initiation complex through ribosome-associated protein kinase activity (41,42). To date the only biological function that this mechanism has been shown to possibly affect is suppression of the immune response (41). It is quite possible, then, that interferon-induced molecular events such as inhibition of initiation complex formation and suppressor factor induction may be related to the non-

antiviral properties of interferon. Therefore, elucidation of the nature and action of the interferon induced suppressor factor should be of much benefit in our understanding of the non-antiviral properties of interferon, such as immunosuppression.

We have shown that like polypeptide hormones, the action of interferon is probably mediated through secondary messenger molecules, and that these influence adjacent cells. Subsequently, the cell-to-cell transmission of hormonal stimulation was reported (38). Both of these cell communications are thought to occur by gap junctional transfer of secondary messenger molecules. These observations, together with other similarities between interferon and polypeptide hormones, have led us to propose that there is a common cellular pathway of interferon and hormonal action.

We have demonstrated that interferon can have hormonal activity and that hormonal stimulation can result in interferon type antiviral activity. These findings have led us to conclude that interferon and hormonal action are probably mediated by common pathway(s). The transmission of the reciprocal actions of interferon and noradrenaline not only gives further credence to a common pathway of their actions but also suggests that common transfered molecule(s) are generated after interaction of either substance with the appropriate cell membrane. Superficially, cyclic AMP seems a candidate for the interferon induced increase in beat frequency since cyclic AMP can cause this response (38) and interferon under certain conditions can elevate cyclic AMP levels (43). However, cyclic AMP alone cannot account for the antiviral effects since it is not antiviral (44) and interferon does not stimulate adenyl cyclase in all cells (43). A more likely situation is that cyclic AMP and/or another small molecule(s) is responsible. This putative molecule may represent a new class of secondary messengers and thereby lead to elucidation of a new cellular control system.

Evidence for this new putative secondary messenger molecule continues to accumulate. We suspect that the soluble substance extracted from interferon treated L cells may represent this molecule and be responsible for transfer of viral resistance and induction of the antiviral state. The transfer material has been found to be rapidly produced intracellularly after interaction of cells with interferon. It is highly unstable and dissappears rapidly from cells. These are qualities we might expect of a secondary messenger molecule.

Detection of the transfer material has been complicated by a control material, probably associated with the cell membrane, which is not induced by interferon, is released from sonicated cells and is antiviral. This material appears to be fibronection. If this could be proven it would be interesting in and of itself. Fibronectin is a molecule of much current interest since it is in much higher levels in normal than transformed cells. Antiviral activity may provide a new function for this molecule and may be related to the transformed phenotype.

Two fundamental questions result from the hormonal studies. First, is interferon a hormone? The numerous similarities between interferon and polypeptide hormones indicate that interferon should be classified as such. These similarities coupled with our inability to distinguish interferon action from a hormonal response would seem to answer this question affimatively. As such, the natural role of interferon may be regulatory with its effects on virus

infections being secondary. The instances of low levels of interferon in normal individuals may not result from inapparent virus infections but may be reflective of this more general interferon regulatory mechanism. Additionally, this could be related to the side effects observed during clinical trials using high levels of interferon (45) as well as some aspects of viral pathogenesis.

If interferon can cross activate for other hormonal activities this might explain the many diverse actions of interferon, (for review see 46). Most attempts to explain these diverse responses have been in terms of a single unique mode of action of interferon. The puzzling aspect has been how these many actions are mediated through one unique pathway. Our data offer a plausible explanation. The mode of action of interferon is not unique, it is shared with other hormones. While hormones are generally tissue targeted, all nucleated cells appear sensitive to interferon. Since interferon and hormones, as well as hormones themselves (38), share common pathways, interferon could cause many responses which are dictated by the particular cell type or tissue. Such a situation would make questionable the present view that the varied biochemical changes in interferon treated cells are interferon specific.

A second important question is; What are the limits of responses to hormones? Classically, the actions of polypeptide hormones are well understood in terms of specific activation of their target tissues. The present findings suggest that there may also be patterns of different, hormonal responses induced by any one hormone and the pattern will vary with the cell type affected. For instance, hormones may not only have their known major action but may also protect tissues against viruses or maintain differentiation through interferon or other hormonal mechanisms. If this could be documented in vivo a new strategy of tissue targeted antiviral and antitumor therapy might evolve.

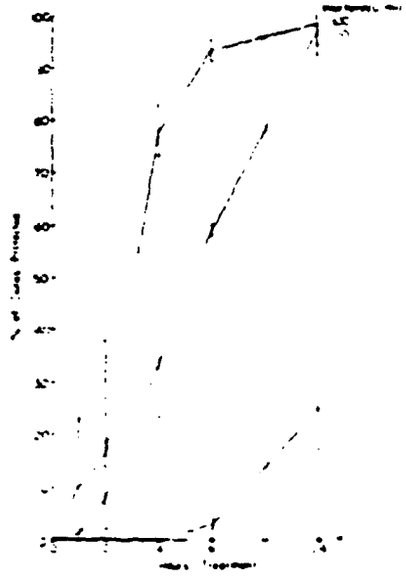


FIG. 1. Variation in interferon protection of L-cell clones against VSV

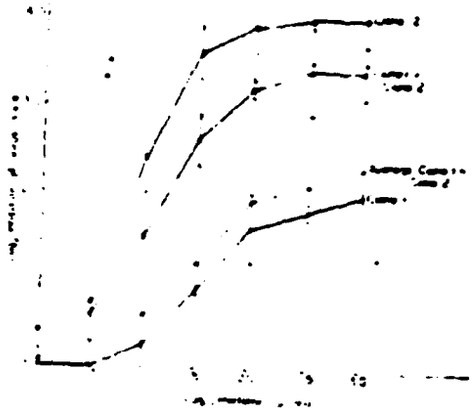


FIG. 3. Transfer of interferon-induced viral resistance between cloned L cells. Data are presented as the mean (three replicates) and 95% confidence limits.

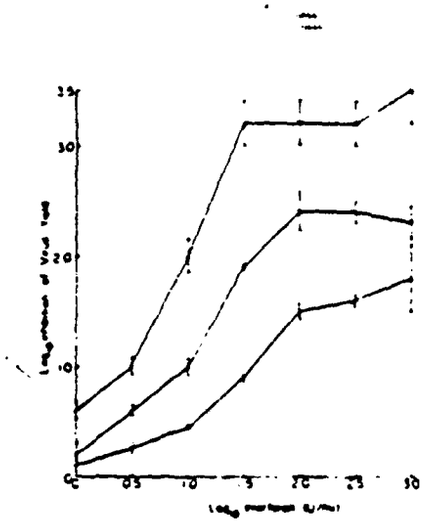


FIG. 2. Representative interferon dose-responses of three cloned L-cell lines. Data are presented as the mean (three replicates) and 95% confidence limits.

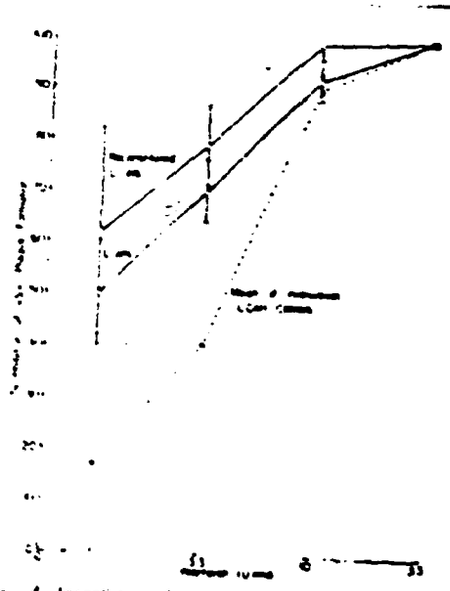


FIG. 4. Interferon dose-responses of "parental" L cells, a reconstituted L-cell population, and the mean response of all L-cell clones. Data for the "parental" L cells and the reconstituted L-cell population are presented as the mean (three replicates) and 95% confidence limits.

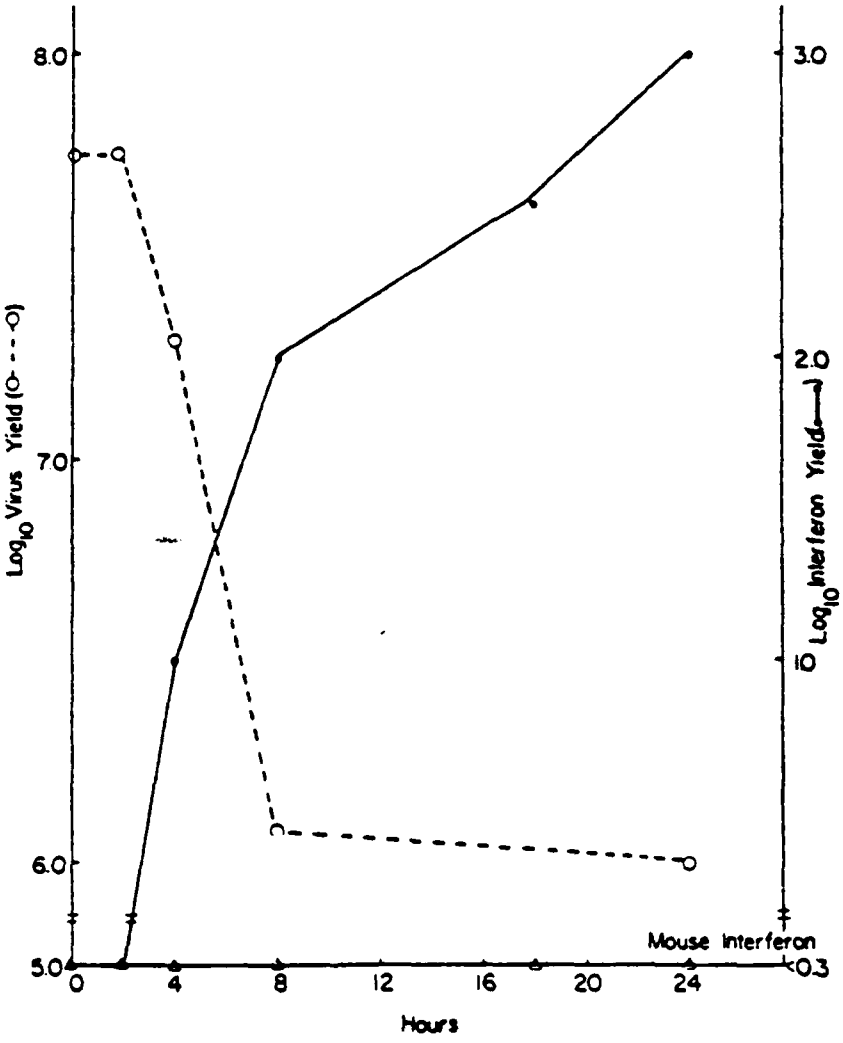


Fig. 5. Leukocyte transfer of interferon-induced viral resistance.

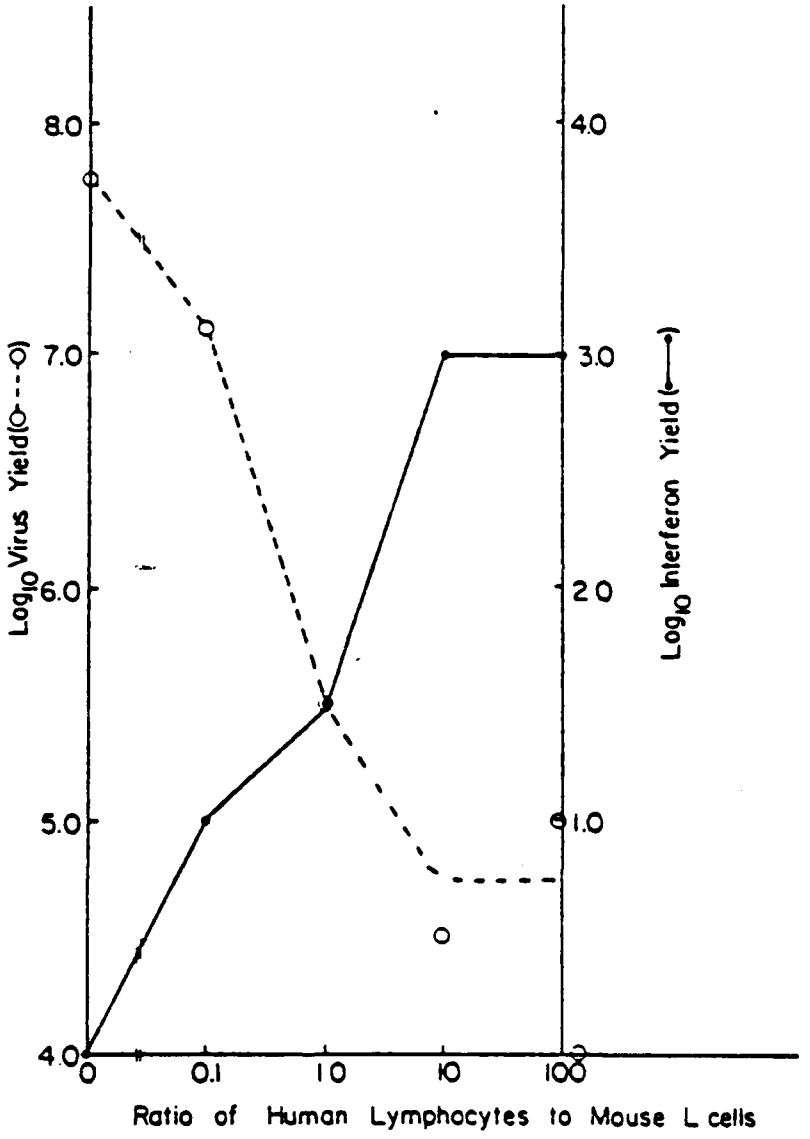


Fig. 6. Effect of ratio of human leukocytes to mouse L cells on interferon production and transfer of viral resistance.

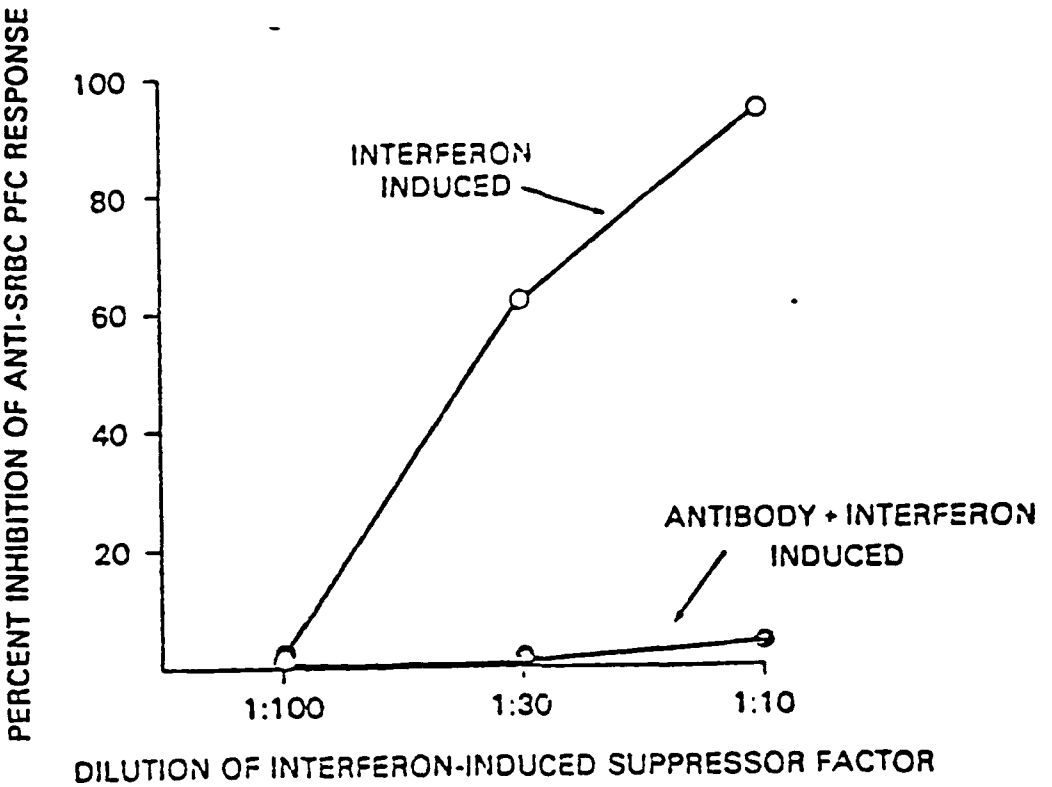


Figure 7. Titration of interferon-induced suppressor factor. Suppressor factor was induced by treatment of 5 x 10^7 cells/ml with 1000 units of interferon/ml for 24 hr at 37°C. The washed cells, 1 x 10^8/ml, were incubated in culture media for 2 hr at 37°C. The resultant supernatant was titrated against syngeneic cells and SRBC for its ability to inhibit the PFC response. O----O, supernatant from cells treated with 1000 units of interferon/ml; ●——●, supernatant from cells treated with 1000 units of interferon that had been neutralized by anti-interferon serum prior to addition to the cultures.

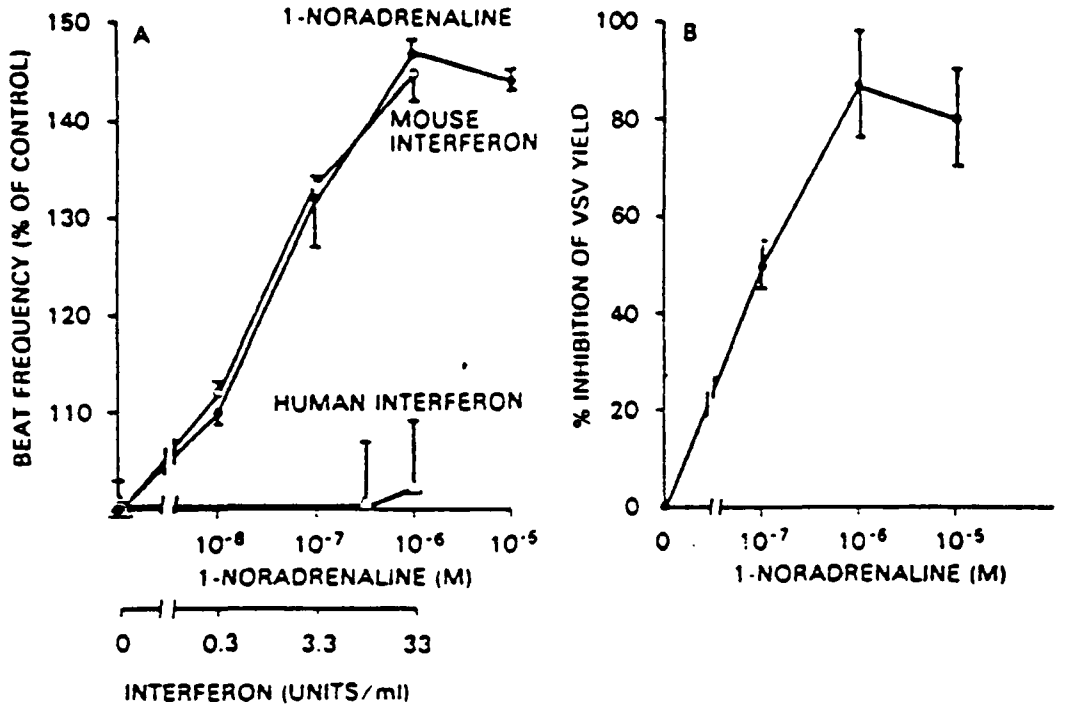


Figure 8 Effect of l-noradrenaline and interferon on the beat frequency (a) and virus replication (b) in cultured mouse myocardial cells. With the exception of omission of filtering through nylon mesh, cells were prepared as previously described (17). For beat frequency experiments about 2.5×10^5 cells were plated into each well of Falcon multiwell tissue culture plates in 1 ml of Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum, penicillin (100 U ml^{-1}) and streptomycin (100 µg ml^{-1}). Twenty-four hours later cultures were placed at room temperature for 1 hr to equilibrate and experiments were performed at this temperature. During the experiments the medium remained near pH 7.4. Each value is the mean ± S.D. (n = 4 cells in parallel culture). Beat frequency was determined visually with the aid of a microscope (100X) and stop watch (Scientific

counts were 60 to 80 beats per minute. Beat frequencies were determined 5 minutes after the addition of 0.1 ml of the indicated concentrations of each compound at room temperature. Mouse fibroblast interferon (2×10^6 units/mg protein was kindly provided by Dr. J. Georgiades, University of Texas Medical Branch, Galveston, Texas). For virus replication studies 2.5×10^4 cells were plated into each well of Falcon microtiter tissue culture plates in 0.1 ml of culture medium. Twenty-four hours later medium was replaced with the indicated concentrations of noradrenaline in 0.1 ml volume in duplicate cultures and incubated overnight at 37°C in 4% CO_2 atmosphere.

Supernatant fluids were removed and cultures were infected with vesicular stomatitis virus (300 plaque forming units, PFU/culture). Virus yields were determined 24 hr later by a previously described microplaque assay (1). Control virus yields were about 1×10^6 PFU/ml. Each point represents the mean % inhibition of the control virus yield $\pm$ S.D. (n = 4).

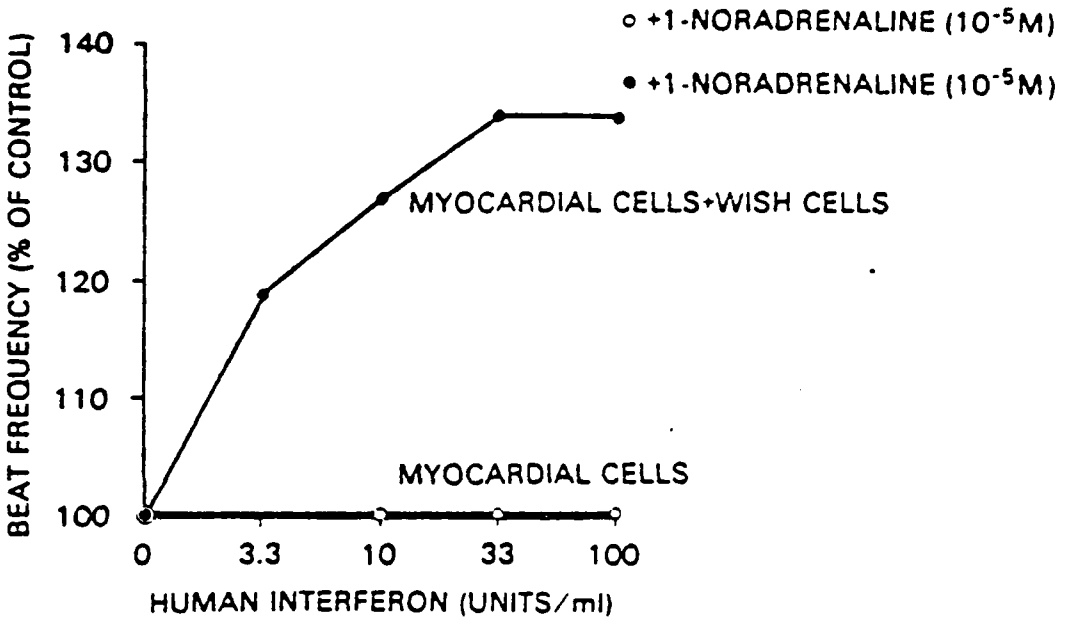
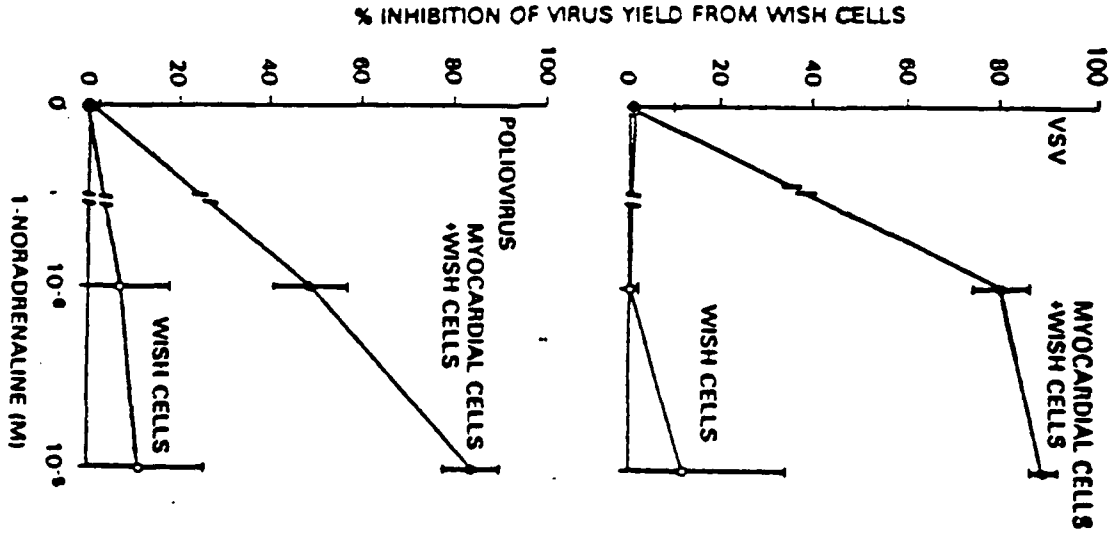


Figure 9. Effect of human interferon on the beat frequency of mouse myocardial cells co-cultured with human amnion (WISH) cells. Mouse myocardial cells (2.5×10^5/well) were plated into adjacent wells of a Falcon multiwell plate. Half of the wells then received 2.5×10^5 WISH cells. After overnight incubation, cultures were equilibrated to room temperature, human fibroblast interferon (1.8×10^6 units/mg protein, kindly provided by HEM Research, Inc., Rockville, Md.) was added to achieve the indicated concentrations and the beat frequency (n = 3) determined as described in Fig. 1. After beat frequencies were determined, l-noradrenaline (10^{-5}M) was added to cultures which had been previously treated with human interferon (100 units/ml) and the beats were again counted.

Figure 10 Effect of l-noradrenaline on virus replication in co-cultures of mouse myocardial cells and human amnion (WISH) cells. Mouse myocardial cells (3.0×10^4/well) and human WISH cells (3.0×10^4/well) were cultured alone or in combination (1:1 ratio) in Falcon microtiter tissue culture plates. Twenty-four hrs later medium was replaced with the indicated concentrations of noradrenaline and incubated overnight. Supernatant fluids were removed and cultures were infected with 300 PFU of either VSV (upper panel) or poliovirus (lower panel). Virus yields were determined 24 hrs later by a microplaque assay (1). Each point represents the mean % inhibition of the control virus yield ± S.D. (n = 6). VSV yield from myocardial cells alone was inhibited (Fig. 1b). Poliovirus did not replicate in mouse myocardial cells.



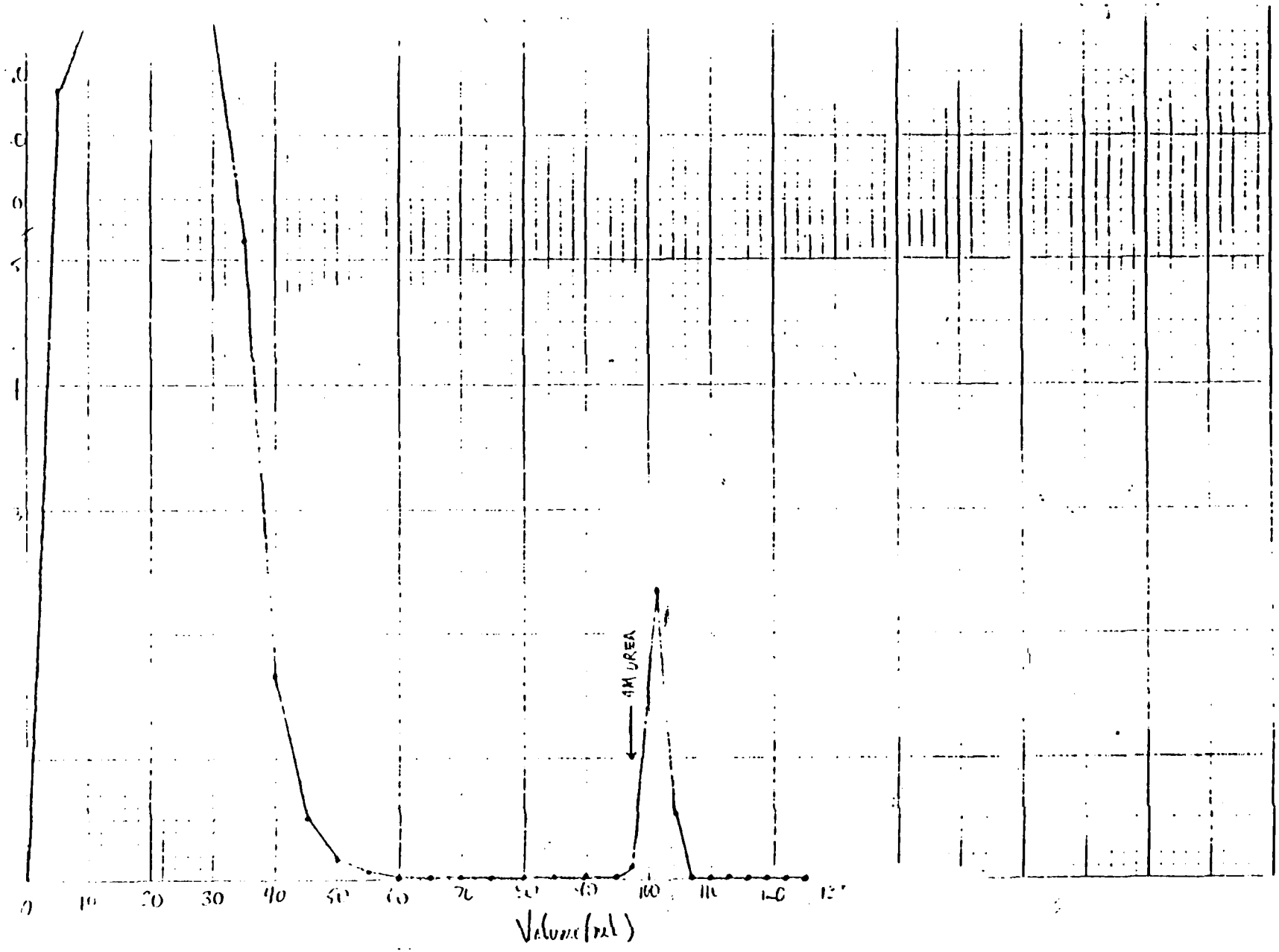


FIG. 12. Gelatin-sepharose affinity chromatography of L-cell sonicate containing

Figure 11. Kinetics of production of interferon-induced cell-free mediator of transf of resistance to virus. Cell-free transfer from mouse L cells to human WISH cells.

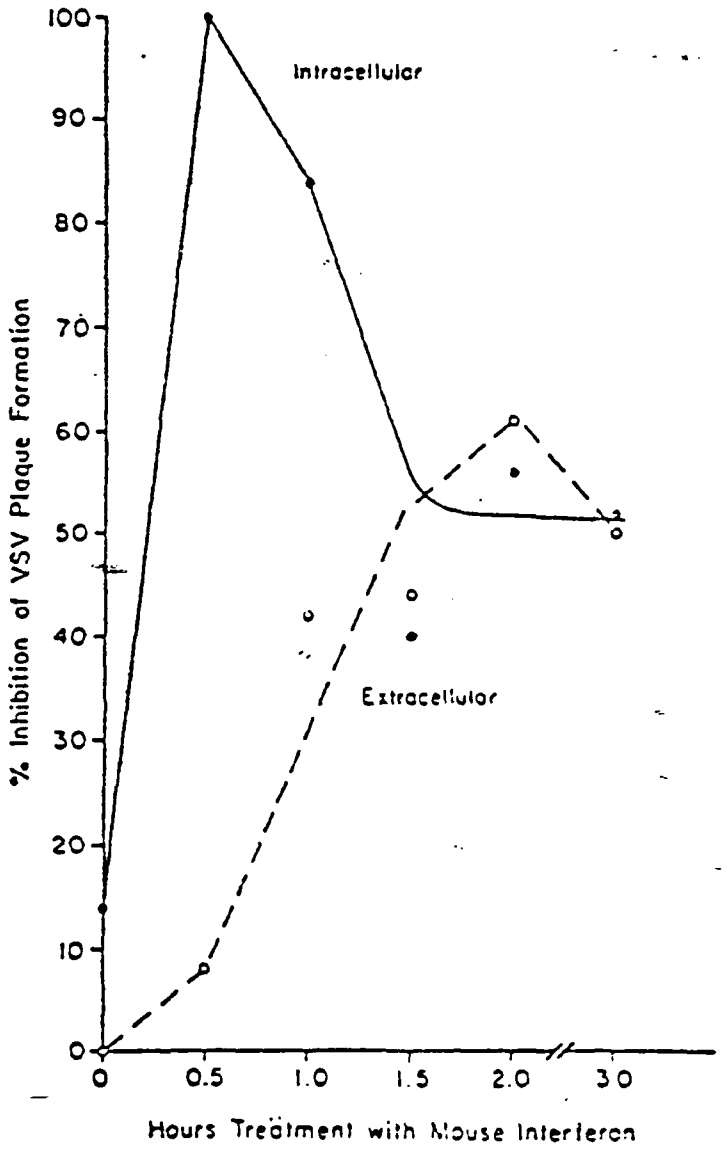


TABLE 1 VARIATION OF THE INTERFERON DOSE-RESPONSES OF CLONED L CELLS

Clone	Interferon dose (U/ml) log$_{10}$ inhibition of VSV yield*					
	1	3	10	30	100	300
A	0.03	0.19	0.37	0.92	1.5	1.6
B	0.20	0.26	0.76	1.5	1.7	1.9
C	0.08	0.88	1.5	2.6	2.7	2.9
D	0.03	0.74	1.8	2.3	2.5	2.8
E	0	0.70	1.8	2.5	2.5	2.8
F	0.09	1.2	1.7	2.6	2.8	3.3
G	0.20	0.45	1.5	1.8	2.1	1.9
H	0.07	0.68	1.7	2.8	2.8	2.6
I	0.10	0.28	0.92	1.5	2.3	2.2
J	0.10	0.85	2.0	3.2	3.2	3.3
K	0	0.44	1.5	2.2	2.6	2.4
L	0	0.43	1.2	1.9	2.2	2.2
M	0.42	0.88	2.0	2.7	2.8	73.5
N	0	0.17	1.2	1.8	2.[illegible]	2.5
O	0	0.4	1.0	1.9	2.4	2.4
P	0.36	0.78	1.8	2.3	2.3	2.5
Q	0.15	1.2	1.9	2.4	2.6	2.5
R	0	0.47	1.1	1.9	2.5	2.5

* Differences of 0.5 log$_{10}$ are significant at $P < 0.05$

TABLE 2 THE FRACTION OF CELLS WHICH DETERMINES THE INTERFERON RESPONSE OF THE POPULATION

Interferon (U/ml)	Percentage inhibition of VSV plaque number, number of clones			Percentage inhibition of VSV plaque number		Percentage of clones at or above reconstituted population's percentage inhibition
	0–35	31–60	61–99	Mean of clones	Reconstituted population	
1	35	1	4	17	62	10
3.3	25	3	12	40	79	30
10	2	2	36	90	99	78
33	1	0	39	98	100	98

Table 3. Variation in the ability of L cell clones to transfer viral resistance to human WISH cells

Clone Number	Colonial Morphology	Log_{10} Inhibition of VSV Yield from*:	
		Clone	Cocultured WISH cells (transferred resistance)
1	Dense	4.3	1.1
2	"	3.5	0.8
3	"	4.0	0.5
4	"	3.5	0.5
5	"	>4.1	0.4
6	"	3.6	0.04
7	"	3.2	0
8	"	2.5	0.08
9	"	0.4	0.2
10	"	0.3	0.1
11	Contact Inhibited	1.9	0.3
12	"	0.3	0
13	"	0.1	0.08
14	"	0.1	0
15	"	0	0
16	Loose	3.4	0.7
17	"	3,0	0.4
18	"	1.7	0.2
19	"	3.0	0.1
20	"	0.7	0.1

* 1×10^5 cells of each clone with or without WISH cells (1×10^5) were treated overnight with 100 units/ml of mouse interferon and challenged with VSV virus yield were then determined, mouse interferon had no activity on WISH cells alone.

TABLE 4

Human Interferon Induction by Actinomycin D-Treated Mouse L Cells Co-Cultured with Nonsensitized Human Lymphocytes*

Cells	Interferon (u/ml)	
	Actinomycin D	Control
L-cells + human lymphocytes	600	1000
Human lymphocytes	< 10	< 10
L-cells	< 10	< 10

* Confluent cultures (10^5 cells/well) of mouse L cells in Micro Test II tissue culture plates were treated with actinomycin D (5 μg/ml) for 1 hr at 37°C. After washing three times to remove extracellular actinomycin D (Sigma), human lymphocytes (5×10^5 cells/well, prepared as described under Materials and Methods were added and cultures were incubated for 24 hr at 37°C in 4% CO_2. Supernatant fluids were then prepared and assayed for interferon as described under Materials and Methods. This level of actinomycin D (5 μg/ml) was sufficient to block [^{3}H]uridine incorporation into L cells by 95% in 30 min and to completely inhibit mouse interferon action on L cells.

Table 5. Human Leukocyte interferon induction by subcellular fractions of mouse L cells

Sonication (min)	Cell Destruction[a]	Fraction	Interferon (U/ml) No Lymphocytes	Interferon (U/ml) Lymphocytes
0	0	whole	0	30
		pellet	0	30
		supernatant	0	10
5	0	whole	0	20
		pellet	0	30
		supernatant	0	0
10	0	whole	0	30
		pellet	0	30
		supernatant	0	0
15	1+	whole	0	20
		pellet	0	30
		supernatant	0	0
20	4+	whole	0	20
		pellet	0	0
		supernatant	0	30

a 0-4+, 0=no destruction, 4+= total destruction.

Mouse L cells (1×10^6) were sonicated for the indicated time then centrifuged at 2,000xg for 5 min. Following centrifugation samples from the whole sonicated cell suspension, the pellet, and the supernatant were incubated with 1×10^7 nonsensitized human lymphocytes for 24 hrs. The supernatants from the lymphocyte cultures were assayed for interferon activity on human WISH cells.

Table 6. Mouse spleen cell interferon induction by subcellular fractions of human WISH cells.

Cell Treatment	Adherent cells	Centrifugation (xg)	Fraction	Interferon (U/ml) No Spleen Cells	Interferon (U/ml) Spleen Cells
None	-	2,000	Whole	0	10
			Pellet	0	10
			Supernate	0	10
None	+	2,000	Whole	0	30
			Pellet	0	30
			Supernate	0	10
Dounce Homogenized	+	2,000	Whole	0	30
			Pellet	0	20
			Supernate	0	30
Dounce Homogenized	+	10,000	Whole	0	40
			Pellet	0	30
			Supernate	0	20

Human WISH cells (1×10^6) were lysed by Dounce homogenization and the debris centrifuged at 2,000 or 10,000xg for 5 min. Following centrifugation, samples from the whole hemogenate, the pellet, and the supernatant were incubated with 1×10^7 mouse spleen cells for 24h. The supernatants from the spleen cell cultures were then assayed on mouse L cells for interferon activity.

Table 7. Characterization of the cell type that produces heterologous cell-induced leukocyte interferon.

Fraction	Rabbit Anti-Human Immunoglobulin + Complement	Human Interferon (units/ml)
Glass adherent (macrophages)	-	10
	+	10
Glass Nonadherent	-	600
	+	100
SRBC* Rosetting (T-cells)	-	10
	+	10
SRBC Non-rosetting	-	1000
	+	100

* SRBC, sheep red blood cells
Human leukocytes were fractionated as indicated and co-cultured with mouse L cells. Twenty four hours later supernatant fluids were assayed for interferon.

Table 8.

Interferon Production and Sindbis Virus Yields Following Co-cultivation of Various Cells with Human Peripheral Leukocytes

Human leukocytes and	Human Leukocyte	Interferon (U/ml) Mouse	Chick	Percent Inhibition of Sindbis Virus*
Mouse L-cells	3000	<3	—	99
Secondary mouse embryo cells	1000	<3	—	90
Primary chick embryo cells	300	—	<3	88
Diploid human skin muscle cells	<3	—	—	0
Human amnion WISH cells	1000	—	—	—

*Sindbis virus did not replicate in leukocytes

Leukocytes were added to confluent microtiter cultures (10^5 cell/well) of each cell type at a ratio of 10:1. Following 24h incubation fluids were harvested for interferon assay and the monolayers challenged with Sindbis virus (MOI = 50). Virus was harvested 24h later.

Table 9

Inhibition of Virus Yields Following Co-Cultivation of Human Peripheral Leukocytes with Mouse L-Cells

VIRUS	CULTURES	$TCID_{50}$	PERCENT INHIBITION
Sindbis	L-cells	$10^{7.5}$	
	L-cells + Leukocytes	$10^{6.2}$	95
Enterovirus 70	L-cells	$10^{4.8}$	
	L-cells + Leukocytes	$<10^{2.0}$	99.8
VSV	L-cells	$10^{6.6}$	
	L-cells + Leukocytes	$10^{3.1}$	99.9

Human leukocytes were co-cultured with mouse L-cells, 5:1 respectively, for 24 h. The cultures were challenged with approximately 200 $TCID_{50}$ and the fluids harvested for virus assay following 24h incubation.

Table 10

Relationship between IF production, inhibition of virus synthesis, and chromium release

Hours after addition of leukocytes	Human interferon (units/ml)	Percent inhibition of virus	Percent specific ^{51}Cr release at time IF is harvested
4	100	92	0.5
8	1000	93	0.8
12	5000	95	5.8
24	30000	99	30.5

Mouse L-cells pretreated with ^{51}Cr were washed prior to addition of human leukocytes. Interferon levels, virus inhibition, and percent specific ^{51}Cr release was determined at the indicated times. The effector to recipient cell ratio was 10:1.

Table 11. Effect of interferon treated human leukocytes on the *in vitro* anti-SRBC PFC response of mouse spleen cells.

Human leukocytes (cells/ml)	Human fibroblast interferon(U/ml)	PFC/culture ±SD
5.0×10^6	1000	0
5.0×10^6	None	110 ± 14
2.5×10^6	1000	40 ± 28
2.5×10^6	None	570 ± 269
1.0×10^6	1000	1020 ± 198
1.0×10^6	None	1400 ± 849
None	1000	1740 ± 820
None	None	2900 ± 1499

Table 12. Effect of interferon-treated mouse spleen cells on *in vitro* anti-SRBC PFC response of untreated cells*.

Interferon units/ml	No. treated spleen cells added	PFC/culture ± SD	PFC/10^6 viable cells ± SD
5,000	1×10^7	330 ± 297	156 ± 134
	3×10^6	1260 ± 594	853 ± 810
500	1×10^7	440 ± 452	190 ± 228
	3×10^6	2580 ± 481	1284 ± 478
Culture	1×10^7	3520 ± 453	2005 ± 1061
media	3×10^6	8200 ± 2942	3799 ± 1797

* C57Bl/6 spleen cells, 1×10^8/ml, were incubated with the indicated concentrations of interferon for 24 hr. After washing, the cells were added at 1×10^7 (100µl) or 3×10^6 (33 µl) to fresh spleen cell cultures containing 1.5×10^7 syngeneic cells in 1 ml. Sheep red blood cells (SRBC) were added and the cultures were incubated for 5 days, after which the direct anti-SRBC plaque-forming cell (PFC) response was determined.

Table 13. Effect of antibody to interferon on suppressor cell and suppressor factor activity*

Condition	Treatment	Anti-SRBC PFC/culture ± SD	% Inhibition
Suppressor cells	Anti-interferon	30 ± 14	99.7
(5×10^6/ml)	NRS	10 ± 14	99.7
	None	10 ± 14	99.9
Control cells	Anti-interferon	7980 ± 1216	25
(5×10^6/ml)	NRS	4600 ± 622	-3
	None	6120 ± 113	17
Suppressor factor	Anti-interferon	170 ± 113	98
(1:10 dilution)	NRS	150 ± 212	97
	None	60 ± 57	99
Control factor	Anti-interferon	10200 ± 792	4
(1:10 dilution)	NRS	6320 ± 4130	-41
	NOne	5140 ± 990	30
Interferon	Anti-interferon	6340 ± 1500	41
(100 U/ml)	NRS	1040 ± 962	77
	None	1840 ± 509	75
None	Anti-interferon	10680 ± 3564	-
	NRS	4480 ± 792	-
	None	7380 ± 537	-

Table 13. Legend

* Suppressor cells (interferon-treated) and control (untreated) cells were produced under conditions as described in Table 1 using 1000 units of interferon/ml with 24 hr incubation. Supernatants were obtained by incubating the washed cells at 1 x 10^8/ml at 37°C for 2 hr. Prior to addition to syngeneic cultures, suppressor cells (5 x 10^7/ml), control cells, supernatants, and the interferon (1000 U/ml) used for induction were incubated with equal volumes of a 1:20 dilution of anti-interferon serum, normal rabbit serum (NRS), or culture media for 1 hr at room temp. The values presented represent the final cell, supernatant, and interferon concentrations added to syngeneic cultures.

Table 14. Characteristics of the Hormonal Activity of Interferon and the Antiviral Activity of l-Noradrenaline on Mouse Myocardial Cells

Addition to Mouse Myocardial Cells:	Beat Frequency[a] (% of Control) ± S.D.	Minutes Required for[b] Maximum change in Beat Frequency	% Inhibition[e] of VSV Yield ± S.D.
Purified Interferon[f] (50 units/ml)	142 ± 8	2	ND
Purified Interferon (50 units/ml) + l-Noradrenaline (10^{-5} M)	161 ± 14	ND[c]	ND
Crude Interferon (50 units/ml)	151 ± 3	2	99 ± 1
Crude Interferon (50 units/ml) + Anti-Interferon Antisera (1:20 dilutions)	88 ± 18	>10[d]	ND
l-Noradrenaline [10^{-5} M]	ND	1	75 ± 3
l-Noradrenaline (10^{-5} M) + Actinomycin D (2 µg/ml)	ND	ND	0 ± 10

a Procedures were as described in Fig. 8. Crude interferon and anti-interferon antisera were mixed and incubated at room temperature for 30 minutes prior to addition to mouse myocardial cells.

b Times were derived from kinetics experiments as described in Fig. 3.

c Not done

d There was no change in beat frequency after 10 minutes.

e Procedures were as described in Fig. 1 Noradrenaline and Actinomycin D were added simultaneously.

f. $10^{8.5}$ units/mg protein.

TABLE 15. Soluble Interferon-Induced Transfer of Viral Resistance from Sonicated Mouse L 929 CEIls to Human WISH Cells

SAMPLE[a]	INTERFERON TREATMENT	TITER (Log_{10} PFU/0.1 ml)	% INHIBITION
L Cells 15'	3 U/ml	1.8×10^4	93.8
L Cells 30'	"	1.9×10^4	93.5
L Cells 60'	"	2.0×10^4	93.2
L Cells 90'	"	3.7×10^4	87.4
L Cells 90'	0 U/ml	29.5×10^4	----

a) L 929 cells @ 5×10^6 cells/ml in suspension were treated for the indicated times at 37°C with or without mouse fibroblast interferon. Following incubation, cells were sonicated for 1 min. and centrifuted at 3000 rpm for 5 min. to remove cell debris. Supernatants were placed on Human WISH cells and incubated overnight at 37°C. WISH cells were challenged with Vesicular Stomatitis virus at an input multiplicity of .01. 24 hours later, virus yields were assayed by microplaque reduction.

Table 16. Thermal stability of interferon induced transfer material*

Temperature	Hours at indicated temperature	% Inhibition of VSV Yield
-70°C	1	87
"	24	73
"	48	10
-20°C	1	88
"	24	65
"	48	10
20°C	1	70
"	24	50
"	48	0

* Interferon induced transfer material (mouse L cell) was produced by the standard procedure and incubated for 1,24, or 48 hrs at the indicated temperature. Transfer material was then thawed (if need) and placed on human WISH cells overnight. Cultures were challenged with VSV and virus yields were determined 24 hrs later.

TABLE 17. Antiviral Activity Present in the Supernatant Fraction of Sonicated Non-Interferon Treated, L-929 Mouse Cells[a]

SAMPLE[b]	TITER (Log_{10} PFU/0.1 ml)	% INHIBITION
L Cells 5'	7.0×10^4	54.8
L Cells 15'	2.9×10^4	81.3
L Cells 30'	4.6×10^4	70.0
L Cells 60'	7.0×10^4	54.8
L Cells 90'	3.3×10^4	78.7
L Cells Control	15.5×10^4	----

a) Antiviral activity has been noted in several species of cells, notably--Human WISH and HFS-4 Cells, Mouse Embryo Cells, and Baby Hamster Kidney Cells.

b) L 929 cells @ 5×10^6 cells/ml in suspension were incubated for the indicated times at 37°C. Following incubation, cells were sonicated for 1 min. and centrifuged at 3000 rpm for 5 min. To remove cell debris. Supernatants were placed on WISH cells and incubated overnight at 37°C. WISH cells were then challenged with Vesicular Stomatitis virus at an input multiplicity of .01. 24 hours later, virus yields were assayed by microplaque reduction.

TABLE 18. Vero Cell's Ability to Receive Control Transfer Material

SAMPLE[a)]	TITER (Log_{10} PFU/0.1ml)	% INHIBITION
L Cells 5'	15×10^5	50
L Cells 15'	14×10^5	53
L Cells 30'	12×10^5	60
L Cells 60'	5×10^5	83
L Cells 90'	8×10^5	73
Virus Control	30×10^5	--

a) CM was assayed on VERO cells in the same manner as WISH cells

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PUBLICATIONS

The following papers have resulted from this grant:

1st Year

1. Blalock, J.E. and S. Baron. 1979. Mechanisms of Interferon-Induced Transfer of Viral Resistance Between Animal Cells. J. Gen. Virol., 42:363-372.

2. Blalock, J.E. 1979. Cellular Interactions Determine Rate and Degree of Interferon Action. Infect. Immun., 23:496-501.

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4. Blalock, J.E., J. Georgiades and H.M. Johnson. 1979. Immune-type Interferon Induced Transfer of Viral Resistance. J. Immunol., 122:1018-1021.

2nd Year

5. Blalock, J.E. 1979. A Small Fraction of Cells Communicate the Maximal Interferon Sensitivity to a Population. Proc. Soc. Exp. Biol. Med., 162:80-84.

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