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Species Specificity of Interferon Action: Maintenance and Establishment of the Antiviral State in the Presence of a Heterospecific Nucleus

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The expression of the interferon-induced antiviral state was studied in heterokaryons and cytoplasmic hybrids (cybrids). An autoradiographic assay for the antiviral state, in which the percentage of cells containing vaccinia viral DNA factories was determined, was used. The expression of the antiviral state was dominant in homokaryons and heterokaryons formed by fusion of interferon-treated cells with untreated cells. Cytoplasts derived from treated cells conferred resistance to virus growth on cybrids formed by fusing such cytoplasts with untreated cells. Treatment of L cell × HeLa cell heterokaryons with human interferon or mouse interferon was much less effective in inducing a detectable antiviral state than was similar treatment of parental cells with homospecific interferon. The antiviral state was fully induced when heterokaryons were treated simultaneously with both types of interferon. Cybrids formed by fusing L cell cytoplasts with HeLa cells or HeLa cytoplasts with L cells did not enter a detectable antiviral state after treatment with interferon specific for the cell type of the enucleated parent. However, treatment of cybrids with interferon specific for the cell type of the nucleated parent was effective in inducing a detectable antiviral state.

The interferons are glycoproteins that are produced and released by cells in response to viral infections and certain other agents (e.g., polyinosinic acid-polycytidylic acid and other double-stranded RNAs). The interferon released by treated cells can interact with untreated cells and cause them to enter an antiviral state in which they are resistant to viral infection. Interferon action involves binding of interferon to the cell membrane, an event that causes a number of alterations in the plasma membrane (6, 8). However, the relationship of these changes to establishment of the antiviral state is unclear. Induction of the antiviral state by interferon requires the presence of genetic information and does not occur in cells that are exposed to interferon after enucleation (18). After establishment of the antiviral state, however, the cell nucleus is no longer necessary for its maintenance (18). It has been reported that in human × mouse hybrid cells the presence of human chromosome 21 alone is sufficient for induction of the antiviral state by human interferon, but the sensitivity of such hybrids to human interferon is significantly less than that of the parental human cell and somewhat less than that of hybrid cells containing more human chromosomes (20). Induction of the antiviral state by interferon also requires transcription and probably protein synthesis and has been correlated with an increase in the intracellular level of cyclic AMP (9, 16, 23).

The induction of the antiviral state by interferon appears to be a species-specific phenomenon in most cases; that is, interferon produced by cells of one species will induce the antiviral state in cells of the same species but not in cells of different species (24). This species specificity is not universal, however. For example, interferon produced by human amnion cells induced the antiviral state in rat embryo fibroblasts (6). It is unknown whether the species specificity of interferon action is a result of the specificity of surface receptors for interferon or whether it is dependent upon some other event that occurs after the initial binding of interferon. The finding that there is significant binding of some interferons to cells in which they do not induce an antiviral state and also that there are significant alterations in the plasma membrane in these cases suggests that species specificity of interferon action does not result simply from specificity of interferon binding (14).

The experiments described in this paper were designed to answer the following questions regarding the species specificity of interferon action in human and mouse cell lines. (i) Can a cell

that has been induced by interferon to enter the antiviral state transfer resistance to virus growth to a cell of a different species? (ii) Can a cytoplast (i.e., an enucleated cell) derived from a cell in the antiviral state transfer resistance to virus growth to a cell of a different species? (iii) Are heterokaryons formed by the fusion of cells of different species able to express the antiviral state in response to treatment with interferons derived from cells of either species? (iv) Can cytoplasmic hybrids formed by the fusion of a whole cell of one species with a cytoplast of a second species enter the antiviral state when treated with interferon derived from cells of either species?

MATERIALS AND METHODS

Cells. L929 and HeLa cells were grown in Eagle minimal essential medium containing nonessential amino acids and supplemented with 10% (vol/vol) fetal bovine serum, 50 μg of streptomycin per ml, and 50 IU of penicillin per ml (EMEM). The cell lines were treated monthly with gentamicin or kanamycin or both to suppress any mycoplasmal contamination.

Preparation of vaccinia virus. Confluent HeLa cells in large bottles were infected with vaccinia virus (WR strain) at a multiplicity of infection of 2 PFU/cell and incubated for 2 to 3 days. The cells were generally detached from the growth substrate at that time and were collected by centrifugation from the growth medium. The cells were broken by Dounce homogenization in EMEM diluted 1:10 with water, and nuclei were removed by centrifugation (500 × *g*, 10 min). The supernatant suspension was centrifuged at 40,000 × *g* for 1 h, and the pellet of crude virus was suspended in phosphate-buffered saline minus magnesium, supplemented with 1% bovine serum albumin (1 ml/10^7 original cells). After suspension, the virus was sonicated, dispensed in 1-ml portions, and stored at −70°C.

The virus was titrated in one of two ways: (i) by plaque assay on a human cell line (T98G) (21) or (ii) by scoring of cytoplasmic DNA factories in L cells and HeLa cells, using autoradiographic techniques, after infection with vaccinia and labeling with [^{3}H]thymidine as described below (assay of the antiviral state). L cells and HeLa cells that had been plated on glass cover slips were treated with various dilutions of the vaccinia preparation. We used a dilution of the virus which gave 40 to 60% infection as determined by method ii. The calculated multiplicity of infection, determined by the combination of titration methods, was 5 to 10 PFU/cell.

Preparation and titration of interferons. Crude interferon was prepared by the addition of Newcastle disease virus (10 hemagglutinating units per 10^6 cells) to monolayer cultures of L cells (mouse interferon) or T98G cells (human interferon). The supernatant fluids were collected 24 h later, dialyzed against pH 2 buffer for 5 days, dialyzed to pH 7 for 1 day, and titrated with an assay which measures the inhibition of viral nucleic acid synthesis (4).

Both mouse and human interferons were concentrated by ammonium sulfate precipitation. The cells were induced with UV-inactivated Newcastle disease virus in EMEM containing 2% serum. Solid ammonium sulfate was added to the supernatant fluids to 80% saturation. The precipitate was removed by centrifugation at 15,000 × *g* for 20 min and dissolved in Earle balanced salt solution (1/10 volume of the original starting volume). The solution was then dialyzed against pH 2 buffer and otherwise treated and titrated as was the crude interferon.

National Institutes of Health (NIH) mouse reference interferon no. G 002-904-511 was titrated in this system at 6,617 ± 3,026 U in five independent titrations. The range was from 6,300 to 12,500 U. NIH human fibroblast interferon reference no. G 023-902-527 was titrated in this system at 7,884 ± 2,720 U in five independent trials. The range was from 6,300 to 12,580 U.

Interferon from mouse L cells was inactive on T98G cells (e.g., a preparation with 4,466 U of activity on L cells had fewer than 100 U of activity on T98G cells). Interferon from T98 cells was inactive on L cells (e.g., a preparation having 3,388 U of activity on T98G cells had fewer than 100 U of activity on L cells). Although we do not know the kinds of interferon produced by T98G cells, the preparation displayed interferon activity on human cells and was species specific, the only requirements necessary for interpretation of the experiments presented here.

Enucleation of cells. Cells to be used in the experiments described were grown in Falcon tissue culture flasks in EMEM, to which was added latex spheres of approximately 1 or 2 μm in diameter (Fullam, Inc.). DEAE dextran (8 μg/ml) was also added to the cultures of HeLa cells to facilitate their uptake of the latex spheres. After 24 h of growth in medium containing spheres, the cells were either trypsinized and used in the fusion studies or were enucleated by the large-scale technique (25). Conditions for enucleation were as follows. Standard growth medium was replaced with growth medium supplemented with 10 μg of cytochalasin B per ml (CB medium), and the cells were subjected to centrifugation at 16,300 × *g*. L cells were routinely 95 to 98% enucleated; HeLa cells were 80 to 90% enucleated. After centrifugation, CB medium was replaced with standard growth medium, and the enucleated cells (cytoplasts) were allowed to recover from the effects of the CB medium before being trypsinized and fused with whole cells. In a few experiments, cytoplasts were not trypsinized before fusion with whole cells. In those cases, cells were grown and enucleated on plastic disks cut from tissue culture ware. Cells on the plastic disks were enucleated at 20,400 × *g* in plastic tubes containing 8 ml of CB medium.

Cell fusion. Cellular fusions and cell × cytoplast fusions were performed as follows. The different cell types were mixed in modified Hanks solution (Hanks solution minus glucose and bicarbonate buffered with 0.01 M Tris to pH 7.6) and then chilled in an ice water bath. UV-inactivated Sendai virus was added to a final concentration of 100 hemagglutinating units per ml and allowed to adsorb for 3 min in the ice water bath.

The final volume of the fusion mixture was approximately 0.5 ml. The cell-virus mixture was then incubated in a 37°C water bath for 30 min. After fusion, standard growth medium was added to the fusion mixture and cells were removed by centrifugation, resuspended in growth medium, and plated on glass cover slips in 35-mm tissue culture dishes.

Cells that were enucleated on plastic disks were fused to whole cells in a mixed monolayer-suspension system. Sendai virus (0.1 ml, 1,000 hemagglutinating units per ml) was added to chilled cytoplasts for 20 min at 4°C and then removed. Whole cells were then added (ca. 2×10^5 cells in 0.1 ml), and the disks were incubated for an additional 20 min at 4°C. Prewarmed medium (2.5 ml) was then carefully added, the fusion mixture was incubated at 37°C for 20 min, and the cover slips were vigorously washed and placed into normal growth medium or growth medium containing interferon.

Assay of the antiviral state. The antiviral state was assayed by scoring the presence or absence of cytoplasmic DNA factories after cells were infected with vaccinia virus, labeled with [^{3}H]thymidine, and subjected to autoradiography.

Cells that had been plated on glass cover slips in 35-mm dishes were washed twice with calcium-free phosphate-buffered saline. Vaccinia virus was added to the dishes in 0.6 ml of calcium-free phosphate-buffered saline. Virus was allowed to adsorb for 30 min at 37°C and was then removed, and the cells were washed with calcium-free phosphate-buffered saline. Cells were incubated in standard growth medium for 1 h and then in growth medium containing [^{3}H]thymidine at a concentration of 2 μCi/ml (40 Ci/mmol) for 2.5 h. The cells were then fixed with ethanol-acetic acid (3:1), air dried, hydrolyzed with 1 N HCl, air dried again, and the cover slips were attached to glass slides. Mounted cover slips were dipped in NTB-2 emulsion (Kodak), exposed for 5 days, developed with D19 or Dektol (Kodak), and stained with Giemsa. The slides were examined with a light microscope, and individual cells were scored for the presence or absence of DNA factories. When whole cell × cytoplast hybrids were being scored, only those cells which clearly contained a single nucleus and latex spheres of both sizes were counted. When homokaryons or heterokaryons were being scored, cells containing two or more nuclei and latex spheres of both sizes were counted. The number of cells scored for each experiment varied, depending on the quality of the individual slides and the resultant ease with which they could be scored. In the experiments described below, the number of cells counted for each experimental condition was 150 to 400 for whole cells, 100 to 350 for heterokaryons, and 50 to 200 for cytoplasmic hybrids (cybrids).

Statistical analysis. We compared the frequencies of viral infection (determined by the assay described above) in the different cell populations with contingency tables. In essence, we tested the hypothesis that the frequency of infection was independent of the cellular treatment or cellular constitution. The rejection of this hypothesis at the 0.05 level of significance was interpreted as a difference between the cell populations. The populations compared and the results of the analysis are noted throughout the text, tables, and figures that follow.

RESULTS

Properties of the assay system for the antiviral state. The experiments that we wished to perform seemed to require an examination of the resistance to virus growth in subpopulations of cells in a mixture of cell types. Therefore, we chose an autoradiographic assay for the antiviral state in which the different populations of cells could be identified and their resistance to virus growth could be, at least qualitatively, determined. We chose to use vaccinia virus as the challenge virus and to use the number of cells displaying cytoplasmic DNA factories as an estimation of their ability to be infected by the virus (determined as described above).

From the results of our early experiments, it was apparent that the expression of the antiviral state in our L cells and HeLa cells was not an all-or-none phenomenon. With each cell line, the number of cells displaying cytoplasmic DNA synthesis was dependent upon the concentration of interferon with which the cells were treated, the concentration of the virus with which they were infected, and the time in the infectious cycle during which the cells were allowed to incorporate radioactive thymidine. From these early experiments, we chose to treat L cells with more than 100 U of mouse interferon per ml and HeLa cells with more than 80 U of human interferon per ml. In all experiments we used a multiplicity of infection of 5 to 10 for vaccinia viruses (see above), and we allowed the cells to incorporate radioactive thymidine from 1 to 3.5 h after virus adsorption. We chose these conditions because of the following: (i) the interferon-treated cells were reproducibly less infected with the virus, (ii) a substantial fraction of untreated cells was detectably infected in the assay system, and (iii) the difference between interferon-treated and untreated cells was most apparent.

An example of the results of such an early experiment is shown in Fig. 1. In this experiment, five cover slip cultures of L cells (2×10^5 to 3×10^5 cells per culture) were treated with 1,500 U of mouse interferon per ml for 18 h, and similar cultures were left untreated. The cells of each culture were infected with vaccinia virus, and individual cultures were radioactively labeled during the time intervals shown in Fig. 1. The most apparent difference between interferon-treated and untreated cultures was observed when virus-infected cultures were radioactively labeled from 1 to 3 and from 2 to 4 h

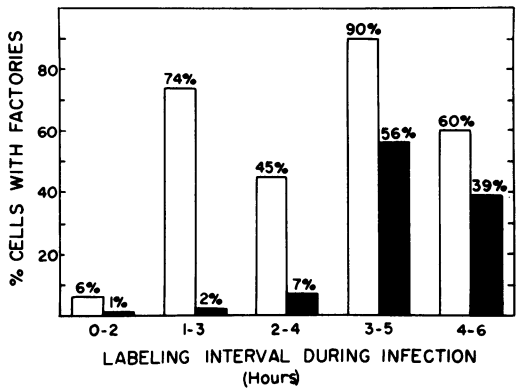

FIG. 1. *Effect of labeling period on the observed level of infection in mouse interferon-treated and untreated L cells. L cells were treated overnight with mouse interferon (1,500 U/ml, 2 ml)* (■) *or left untreated* (□). *They were then infected with vaccinia virus, as described in the text, and labeled with [^{3}H]thymidine for different periods during infection. The time intervals indicated are measured from the time of washing after the 30-min adsorption period. Interferon-treated cells were statistically different from untreated cells ($P < 0.01$) for all time intervals, except 0 to 2 h.*

after viral adsorption. However, the difference between interferon-treated and untreated cultures was statistically significant ($P < 0.01$) for all labeling intervals, except the 0- to 2-h interval, when virus replication appeared to be just starting. Similar results were found for HeLa cells (unpublished data).

The species specificity for induction of the antiviral state was evident with our assay system. Treatment of mouse L cells with 500 U of human interferon did not produce a statistically significant difference in the number of cells infected. Treatment of our HeLa cells with 1,500 U of our mouse interferon preparation did produce a slight inhibition. However, there was still a substantial, statistically significant difference with our HeLa cells between treatment with mouse interferon (1,500 U/ml) and treatment with human interferon (120 U/ml), as shown in Table 1 (also see legends to Fig. 5 and 6).

Because a number of our experiments involved testing the effect of interferon on fused cells, we were interested in determining the effect of Sendai virus on both vaccinia virus infection and induction of the antiviral state by interferon. We found that when L cells were treated with Sendai virus and infected by vaccinia virus on the next day, treatment with Sendai virus did not significantly affect the level of infection by vaccinia virus at two different concentrations of virus, nor did it alter the protection given by interferon treatment when cells were incubated overnight in interferon (1,500 U/ml) after the Sendai virus treatment. In all of the experiments reported here, vaccinia virus infection occurred within 24 h of treatment with Sendai virus. (When cells were infected with vaccinia virus on day 2 after Sendai virus treatment, however, the level of infection was decreased about twofold, although the relative degree of protection given by interferon treatment was not significantly altered [unpublished data]. This result may indicate that treatment with Sendai virus may induce an antiviral state in these cells that is detectable in this assay system by day 2 after Sendai virus treatment.) Moreover, treatment of both L cells and HeLa cells with Sendai virus did not result in the production of detectable levels of interferon, assayed by the inhibition of Semliki Forest virus nucleic acid synthesis (unpublished data).

TABLE 1. *Cross-species activity of interferons used*

Type of cell	% of cells with DNA factories		
	No interferon	Human interferon (500 U/ml, 2 ml)	Mouse interferon (1,500 U/ml, 2 ml)
HeLa	82[a]	7[a]	63[a]
L	93[b]	89[a, b]	28[a]

[a] All of these values are statistically different from each other ($P > 0.05$).

[b] All of these values are not different from each other.

Test of the dominance of the antiviral state after fusion with homologous or heterologous whole cells. L cells labeled with latex spheres were treated overnight with mouse interferon (1,000 U/ml, 4 ml) and then mixed or fused with untreated L cells labeled with spheres of a different size. After mixture or fusion, the cells were plated, allowed to attach to glass cover slips for 1 to 1.5 h, infected with vaccinia virus, and assayed as described above. Figure 2A shows three homokaryons after such treatment. Mixed and fused preparations were scored for infection of homokaryons containing latex spheres of both sizes and for infection of parental cells (cells containing a single bead size). As shown in Fig. 2B, such homokaryons were protected from vaccinia virus infection to the same degree that interferon-treated whole cells were. The percentage of treated and untreated cells that were infected by vaccinia virus was not statistically significantly different for the mixed and fused preparations, indicating that the cells appeared to exhibit protection from vaccinia virus infection because of the prior interferon treatment rather than exposure to Sendai virus.

HeLa cells were subjected to the same type of treatment. One population of HeLa cells was treated with human interferon (438 U/ml) for 18 h and mixed or fused with an untreated population of HeLa cells containing a different size of latex spheres as a cytoplasmic label. After the cells were plated and attached (ca. 1 to 1.5 h), they were infected with vaccinia virus and assayed as described above. The results of such an experiment (Fig. 3) were similar to those for L cells (Fig. 2B). However, the homokaryon population formed by fusion of untreated and treated cell populations, although less infected with vaccinia virus than was the untreated cell

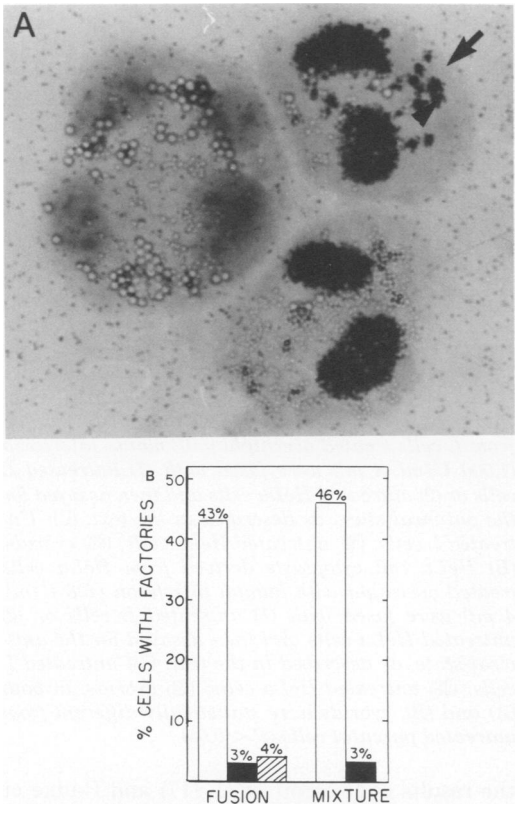

FIG. 2. *Transfer of the antiviral state between homologous L cells. L cells were treated overnight with mouse interferon (1,000 U/ml, 4 ml) or left untreated. Treated and untreated cells were mixed or fused and then assayed for the antiviral state, as described in the text. (A) Photograph of three homokaryons. Arrow indicates cytoplasmic viral DNA factories. (B) Graph showing the percentage of parental cells and homokaryons containing DNA factories. (□) Untreated L cells; (■) mouse interferon-treated L cells; (▨) homokaryons composed of mouse interferon-treated and untreated L cells. Homokaryons were statistically different from the population of untreated L cells (P < 0.05).*

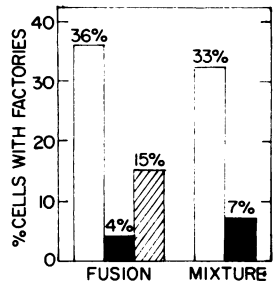

FIG. 3. *Transfer of the antiviral state between homologous HeLa cells. HeLa cells were treated overnight with human interferon (438 U/ml, 4 ml) or left untreated. Treated and untreated cells were mixed or fused and then assayed for the antiviral state, as described in the text. A bar graph is shown of the percentage of parental cells and homokaryons containing DNA factories. (□) Untreated HeLa cells; (■) human interferon-treated HeLa cells; (▨) homokaryons composed of human interferon-treated and untreated HeLa cells. The homokaryons were statistically different from both parental populations of cells (P < 0.05).*

population ($P < 0.05$), was more infected than was the population of interferon-treated cells alone ($P < 0.05$).

In a second type of experiment, interferon-treated or untreated cells labeled with latex spheres were mixed or fused with untreated cells of the other species labeled with latex spheres of a different size. The preparations were plated, allowed to attach for 1 to 1.5 h, and assayed as described above. Mixed and fused preparations were scored for viral infection of mononucleated cells of each parental type, and fused preparations were scored for infection of heterokaryons (binucleated cells containing latex spheres of both sizes in the cytoplasm) as well. The results of several different experiments are shown in Table 2. The percentage of cells in each parental population was not significantly different in mixed or fused preparations (cf. row 1, columns 1 and 2, 3 and 4, and 5 and 6, and row 2, columns 1 and 2, 3 and 4, and 5 and 6).

Heterokaryons involving cells which had been treated with interferon were significantly more resistant to virus infection than was the untreated cell population (cf. column 4, rows 2 and 3, and column 6, rows 1 and 3). In contrast, heterokaryons of untreated parental cells were not significantly protected (cf. column 2, rows 1 and 3 and 2 and 3).

Attempts to transfer virus resistance between homologous and heterologous cytoplasts and whole cells. Cells cytoplasmically labeled with latex spheres and treated for 16 to 18 h with homospecific interferon (1,000 U of

TABLE 2. *Dominance of antiviral state in heterokaryons*

Type of cell	% of cells with cytoplasmic DNA factories in following type of expt[a]:					
	1 (L + H)	2 (L × H)	3 (L^b + H)	4 (L^b × H)	5 (H^b + L)	6 (H^b × L)
L	38	43	0.5	1[b]	43	46[c]
HeLa	51	52	49	52[c]	5	3[c]
Heterokaryons		52		13[c]		22[c]

[a] L, L cell; H, HeLa cell; ×, Sendai virus-mediated fusion; +, mixture.

[b] Treated overnight with homospecific interferon (mouse interferon, 1,000 U/ml, 4 ml; human interferon, 437 U/ml, 4 ml).

[c] All values in columns 4 and 6 are significantly different ($P < 0.01$). Values for L cells and HeLa cells are not significantly different ($P > 0.05$) between columns 1 and 2, 3, and 4, and 5 and 6.

mouse interferon per ml, 4 ml for L cells; 438 U of human interferon per ml, 4 ml for HeLa cells) were enucleated as described above. The cytoplasts were mixed or fused with untreated L cells or HeLa cells labeled with latex spheres of a different size. The mixed or fused preparations were plated for 1 to 1.5 h to allow attachment of cells, infected with vaccinia virus, and assayed as described above. Preparations were then scored for vaccinia virus infection of mononucleated, parental cells and cybrids (mononucleated cells containing latex spheres of both sizes in the cytoplasm). The results for fused preparations are shown graphically in Fig. 4. Cytoplasts from interferon-treated L cells conferred resistance to vaccinia virus infection to untreated L cells and untreated HeLa cells when fused to them. Likewise, cytoplasts from interferon-treated HeLa cells conferred resistance to vaccinia virus infection when fused to untreated L cells and untreated HeLa cells. The infection of untreated parental cells was not statistically different ($P < 0.01$) in mixed or fused preparations (data not shown; typical results were similar to those shown in Fig. 2B, Fig. 3, and Table 2).

To verify that cytoplasts from interferon-treated cells had conferred viral resistance to untreated cells, we conducted additional experiments. First, we confirmed the results of Prescott et al. (17), showing that cytoplasts can be infected by vaccinia virus, and the results of Radke et al. (18), indicating that cytoplasts derived from interferon-treated cells continue to express resistance to vaccinia virus infection. The results of such an experiment are shown in Table 3. Interferon-treated (600 U of mouse interferon per ml, 4 ml, overnight incubation) and untreated L cells were enucleated (see above) and plated or plated as whole cells, allowed to attach for 1 to 1.5 h, infected with vaccinia virus, and assayed as described above. In this experiment, cells and cytoplasts were radioactively labeled between 1 and 6 h after the period of viral adsorption. As shown in Table 3,

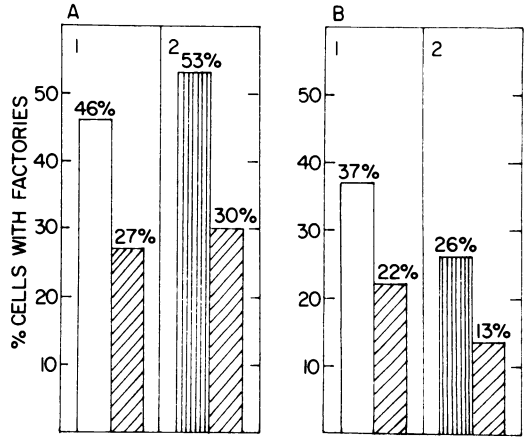

FIG. 4. *Transfer of the antiviral state from cytoplasts to whole cells. (A) L cell cytoplasts derived from L cells treated overnight with mouse interferon (1,000 U/ml, 4 ml) were fused with (1) untreated L cells or (2) untreated HeLa cells and then assayed for the antiviral state, as described in the text.* (□) *Untreated L cells;* (▥) *untreated HeLa cells;* (▨) *cybrids. (B) HeLa cell cytoplasts derived from HeLa cells treated overnight with human interferon (438 U/ml, 4 ml) were fused with (1) untreated L cells or (2) untreated HeLa cells and then assayed for the antiviral state, as described in the text.* (□) *untreated L cells;* (▥) *untreated HeLa cells;* (▨) *cybrids. In both (A) and (B), cybrids were statistically different from untreated parental cells ($P < 0.05$).*

the results of Prescott et al. (17) and Radke et al. (18) concerning infection of cytoplasts and maintenance of the antiviral state were confirmed.

The second type of control was a repeat of the fusion of cytoplasts to whole cells. In this case, however, the cytoplasts were derived from cells not treated with interferon. The results of such an experiment are shown in Table 4. The cybrids were not more resistant to vaccinia virus infection than were the parental whole cells.

Effects of treating HeLa cell × L cell heterokaryons with human or mouse or both

TABLE 3. *Effect of enucleation on infection with vaccinia virus and maintenance of the antiviral state in L cells*

Cell type	Cells infected (%)	
	Untreated	Mouse interferon treated[a]
Whole	35 (245/710)	9 (63/689)
Cytoplasts	36 (268/735)	10 (67/672)

[a] Cells treated overnight in 600 U of mouse interferon per ml (4 ml).

TABLE 4. *Infection of parental cells and cybrids*

Type of cell	% of cells with cytoplasmic DNA synthesis[a]			
	$L_c \times L$	$L_c \times H$	$H_c \times L$	$H_c \times H$
L	51		41	23
HeLa		45		
Cybrids	56	56	43	31

[a] Subscript "c" indicates the source of the cytoplasts. L, L cells; H, HeLa cells; ×, fusion.

interferons. We examined the ability of HeLa cell × L cell heterokaryons to express the antiviral state when treated with human or mouse interferon. HeLa cells and L cells were labeled with latex spheres of different sizes, fused, and plated into medium containing (i) no interferon, (ii) human interferon, (iii) mouse interferon, or (iv) human and mouse interferons. The cultures were incubated overnight, infected with vaccinia virus, and assayed for viral infection as described above. The results of two such experiments are shown in Table 5. Both human interferon and mouse interferon induced a readily detectable antiviral state in homospecific whole cells, but were unable to protect heterokaryons to a comparable extent. As shown in Table 5, there was often no statistically significant difference between the effect on heterokaryons and the heterospecific cell type (experiment 1, columns 2 and 3). With very large concentrations of interferon, the heterokaryon appeared to respond to a homospecific interferon (Table 5, experiment 2, column 2). However, treatment of the fused cellular preparations with interferon specific for both species produced an antiviral state in all cellular populations (Table 5, column 4); that is, in heterokaryons, the two species of interferon appeared to act synergistically.

Effect of treating cybrids with interferon specific for either species. Cybrids were formed by the fusion of whole L cells with HeLa cell cytoplasts or whole HeLa cells with L cell cytoplasts. The parental cells were cytoplasmically labeled with latex spheres of different sizes, as described above. The resulting preparations were treated overnight with human interferon or mouse interferon or were left untreated. Cells were then infected with vaccinia virus, assayed, and scored as described above. In each experiment, whole cells from which the cytoplasts were derived were similarly treated and assayed to determine the effectiveness of the interferon homospecific for that cell type. Figure 5 shows the results of an experiment involving whole HeLa cells and L cell cytoplasts, and Fig. 6 shows the results of an experiment involving whole L cells and HeLa cell cytoplasts. In both experiments, cybrids entered the antiviral state in response to treatment with interferon homospecific for the whole cell involved in the fusion, but did not enter the antiviral state in response to treatment with interferon homospecific for the cytoplast involved in the fusion.

We were concerned that these results might be due, at least in part, to loss of interferon binding sites from cytoplasts when they were trypsinized from flasks after enucleation. To eliminate this potential problem, we performed experiments in which cells were enucleated on plastic disks, fused to whole cells, and treated with interferon before being trypsinized and plated onto glass cover slips. Cybrids formed by this technique still failed to respond significantly to interferon homospecific for the cytoplasts involved in the fusion (unpublished data).

TABLE 5. *Effect of interferons on heterokaryons*

Cell type[a]	% of cells with cytoplasmic DNA factories with following type of interferon:			
	1 (No)	2 (Mouse)	3 (Human)	4 (Combination)
Expt 1				
L	57^{b}	$10^{c,d}$	$39^{b,e}$	21^{b}
HeLa	55^{b}	$41^{b,c}$	$8^{e,f}$	26^{b}
Heterokaryons	61^{b}	$36^{b,d}$	$33^{b,f}$	24^{b}
Expt 2				
L	52^{b}	$13^{g,h}$	45^{b}	12^{b}
HeLa	47^{b}	$38^{g,j}$	$19^{i,k}$	14^{b}
Heterokaryons	49^{b}	$22^{h,j}$	$44^{b,k}$	9^{b}

[a] In experiment 1, mouse interferon was 2,900 U/ml, 2 ml; human interferon was 1,500 U/ml, 2 ml; combination was 1,450 U of mouse interferon per ml and 750 U of human interferon per ml, 2 ml. In experiment 2, mouse interferon was 5,000 U/ml, 2 ml; human interferon was 570 U/ml, 2 ml; combination was 5,000 U of mouse interferon per ml and 570 U of human interferon per ml, 2 ml.

[b] These values are not statistically different ($P > 0.05$) in columns.

[c–k] These values are statistically different ($P < 0.05$).

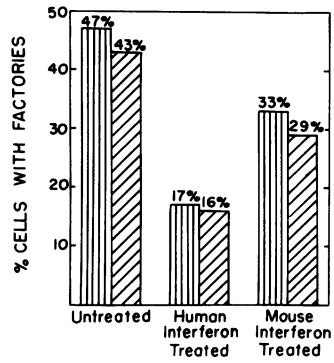

FIG. 5. *Interferon treatment of cybrids formed from untreated parental HeLa cells and L cell cytoplasts. HeLa cells were fused with L cell cytoplasts, and the resulting cell preparations were treated overnight with human interferon (104 U/ml, 2 ml) or mouse interferon (6,309 U/ml, 2 ml) or left untreated. The preparations were then assayed for the antiviral state, as described in the text. (■) HeLa cells; (▨) cybrids. In no instance was there a statistical difference between parental cells and cybrids receiving similar treatments with interferon ($P > 0.05$). Untreated control mouse L cells were 55% infected; those treated with the same concentration of mouse interferon were 2% infected, and those treated with the same concentration of human interferon were 58% infected.*

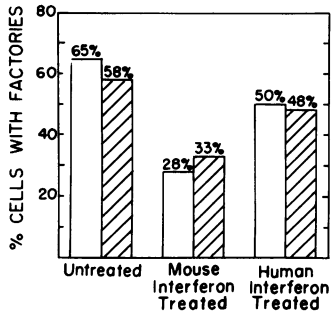

FIG. 6. *Interferon treatment of cybrids formed from whole L cells and HeLa cytoplasts. L cells were fused with HeLa cell cytoplasts, and the resulting cell preparations were treated overnight with human interferon (507 U/ml, 2 ml) or mouse interferon (150 U/ml, 2 ml) or left untreated. (□) L cells; (▨) cybrids. In no instance was there a statistical difference between parental cells and cybrids receiving similar treatments with interferon ($P > 0.05$). Untreated control HeLa cells were 71% infected; those treated with the same concentration of human interferon were 8% infected, and those treated with the same concentration of mouse interferon were 50% infected.*

DISCUSSION

We examined the expression of the antiviral state (i) in heterokaryons and cybrids formed by using one parental cell (or cellular fragment) in the antiviral state and (ii) in heterokaryons and cybrids treated with interferon after their formation. We used an autoradiographic assay system for the antiviral state to permit the simultaneous analysis of several different cell populations.

We wish to make several points about the assay system. First, a statistically significant difference ($P < 0.01$) is found between interferon-treated and untreated cell populations over a fivefold range of interferon concentrations and a fourfold range of vaccinia virus concentrations which we tried in the assay system (unpublished data). The conditions that we used here are rather arbitrary. Second, most of the experiments that we performed involve a comparison of cell populations. These populations are present in the same cover slip culture, and variations in experimental conditions should be identical for each population. Last, we used the assay only in a qualitative manner; that is, we cannot deduce, in a quantitative fashion, the level of the antiviral state. We can only deduce a statistically significant difference in the number (percentage) of cells with detectable cytoplasmic DNA synthesis.

Two previous autoradiographic studies offered conflicting results concerning the effects of interferon on vaccinia virus infection. The results of the first study indicated that the number of cells showing cytoplasmic factories can be reduced by interferon treatment (10). The results of the second study indicated that the size of the factories can be reduced, but that the number of cells infected is not nearly so dramatically affected (15). As shown in Fig. 1, when different periods for [^{3}H]thymidine incorporation are used, the apparent level of protection detected after interferon treatment can vary dramatically. The choice of labeling period and the duration of exposure during autoradiography are important for a clear-cut distinction of cells in the antiviral state from cells not in the antiviral state and may account, in part, for the apparent discrepancies in the earlier studies.

Dominance of the preestablished antiviral state. We have shown that the antiviral state, as assayed by the reduction in autoradiographically detectable vaccinia virus cytoplasmic DNA factories, is a dominant condition in homokaryons and heterokaryons (Table 2). An interferon-dependent transfer of virus resistance between L cells and human cells has been described previously (2). In this described transfer, requirement for metabolic activity of the recipient cells was demonstrated. Some of the effects which we have observed may be caused by similar mechanisms. However, we believe that the inhibition is caused primarily by cytoplasmic

factors acting directly because (i) the cells are washed free from interferon and are trypsinized before being fused and (ii) the assay for the antiviral state occurs within 1.5 h after cell fusion. However, we cannot totally exclude that some of the effects that we observe are dependent upon the metabolic activity of the nucleus of the untreated cell. Because of the short time interval between the time of cell fusion and assay of the antiviral state, we also do not believe that the elution of interferon from treated cells and its utilization by untreated cells account for our results. The lack of protection in the population of untreated cells in our preparations is also taken as evidence against this latter hypothesis.

The inhibition of protein synthesis in interferon-treated cells has been correlated with the production of a unique nucleotide (pppA2'p5'A2'p5'A) and with the appearance of a protein kinase that phosphorylates one of the initiation factors in protein synthesis (1, 11–14, 26). The fact that the same cytoplasmic characteristics of the antiviral state have been found in cells of different species suggests that the mediators of the antiviral state may not be species specific. The dominance of the antiviral state shown in this report is consistent with this hypothesis. The dominance of the antiviral state also indicates that there are no factors in untreated cells that interfere with the expression of the antiviral state. Such a lack of interference has been suggested from the results of cell-free studies, but had not been previously tested in intact cells.

Our results also show that the factors responsible for expression of the antiviral state as detected by the vaccinia virus assay are located in the cytoplasm and are transferred by cytoplasts (Fig. 4). The lack of protection in mixed preparations or in cybrids formed from cells not treated with interferon (Table 4) shows that the antiviral condition which is transmitted is dependent upon the prior treatment of cells with interferon.

Treatment of heterokaryons and cybrids with interferon. We tested the ability of heterokaryons to express the antiviral state after treatment with interferon specific for either parental cell type (Table 5). Heterokaryons generally did not show an increased resistance to vaccinia virus infection compared with cells of the species heterologous to that of the interferon (Table 5, columns 2 and 3 of experiment 1 and column 3 of experiment 2). At high levels of interferon, the heterokaryons were more sensitive than heterologous cells (Table 5, column 2 of experiment 2). We believe the heterokaryons would have responded to very high levels of human interferon, but such treatment was impractical with our preparation of human interferon. In the absence of a heterologous nucleus (i.e., in cybrids), the cybrids are fully responsive to lower levels of interferon (cf. Table 5 and Fig. 5). From these experiments we conclude that the expression of the antiviral state in heterokaryons is inhibited by the presence of a heterologous nucleus.

The nature of this apparent inhibition by a heterologous nucleus is not known. When heterokaryons are treated with both species of interferon simultaneously, the interferons appear to act synergistically (Table 5, column 4). A synergistic effect was described previously in hybrid cells and was attributed to a membrane effect (3, 6). A similar membrane-associated mechanism may be responsible for these results. This effect, however, would be dependent upon the continued presence of the heterologous nucleus.

There is considerable evidence that interferon exerts its antiviral effect from the exterior surface of the plasma membrane (for reviews, see references 6 and 8). Genetic data obtained from studies of somatic cell hybrids formed by fusions of mouse and human cells have shown that human chromosome 21 is involved in establishing the antiviral state in response to human interferon (22). Other data suggest that one product of human chromosome 21 is a specific cell surface receptor (19), although the latter conclusion has been questioned (7). One attractive hypothesis for the species specificity of interferon action is that species-specific interferon receptors are present on the plasma membrane. Upon interaction with interferon, the conformation of the plasma membrane is altered, ultimately resulting in the intracellular alterations which bring about derepression of the genes responsible for expression of the antiviral state. Just as the intracellular mediators of the antiviral state may not be species specific, the intracellular alterations which bring about derepression of genes responsible for expression of the antiviral state may not be species specific (5). Indeed, it has been suggested by one investigator that "the cell species specificity of interferon is solely due to the receptor site" (6).

In an attempt to verify this hypothesis directly, we formed cybrids between mouse and human cells and attempted to induce the antiviral state in the cybrids by treating them with interferon specific for the species from which the cytoplasts were derived. We reasoned that the cybrids would possess the cytoplasm and the plasma membrane (i.e., the interferon receptor

sites) of the species from which the cytoplasts were derived and would therefore be able to respond to interferon of that species.

In these experiments, we treated the cybrids with relatively low concentrations of interferon homospecific for the parental whole cells and relatively high concentrations of interferon for the cytoplast parent in an attempt to detect a transfer of species specificity via the cytoplast. If the hypothesis were correct, the detected infection of the cybrid should have been different from that of the parental whole cell when treated with interferon specific for the cytoplast parent. As shown in Fig. 5 and 6, we did not detect such a difference, even at relatively high concentrations of interferon. We have, therefore, reached the conclusion that we cannot directly verify this hypothesis with these techniques.

In summary, we have been able to show that: (i) the expression of the antiviral state is dominant in homospecific and heterospecific "heterokaryons"; (ii) at least a portion of the antiviral state induced by interferon is localized in the cytoplasm and (iii) can be transferred via the cytoplasm to cells not treated with interferon; (iv) heterokaryons express the antiviral state less efficiently than do parental cells in response to a given concentration of interferon; (v) cybrids express the antiviral state as efficiently as does the parental whole cell in response to a given concentration of interferon; and (vi) cybrids do not express a detectable antiviral state when treated with interferon specific for the cytoplast parent.

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