The Role of the Interferon System in Respiratory Syncytial Virus Infections[1] (36039)

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Respiratory syncytial (RS) virus is an important cause of severe respiratory disease in infants and children under 2 years of age (1). At present, there are no efficacious licensed vaccines or chemotherapeutic agents. Attempts to induce an effective immunity to this virus by means of a killed vaccine have not been successful and have in some instances apparently exacerbated subsequent natural infection (2). It has been shown that reinfection of children and adults commonly occurs even in the presence of circulating, virus-neutralizing antibody.

The interferon system is an important component of the body's nonimmune defenses against viral disease (3). Evidence indicates that interferon in the nasal secretions is an important factor in recovery from viral respiratory diseases. The peak interferon response comes very early in the course of infection, before detectable antibody, and apparently is responsible for the first subsiding of clinical symptoms and reduction of virus shedding (4, 5). Very low titers of interferon-like virus inhibitor have been detected in sera of patients suffering from acute respiratory disease due to RS virus (6). Gardner and coworkers (7) were unable to demonstrate *in vitro* stimulation of interferon by the RS virus in a human embryonic lung cell line, and they suggested that the abundant replication of this virus in young children might be related to the fact that this virus is a poor stimulator of interferon.

Because of these findings, we decided to study the interferon-inducing capacity and sensitivity of several strains of RS virus.

Materials and Methods. Virus. Several strains of RS virus were included in this study. Stock preparations of the Long strain (8) were produced in HEp-2 cells from stocks passaged more than 10 times in HEp-2. Bigelow, isolated in this laboratory in HEp-2 cells from the throat of a patient with upper respiratory disease, was passaged once in WI-38 cells. RS 11657, a strain attenuated by passage in cercopithecus monkey kidney cells (CMK) at low temperatures (4 passages at 28° and 12 at 25°, unpublished information, Lederle Laboratories) was passaged in HEp-2 and then WI-38 cells at 35° before use. Selected tissue culture fluids from the following strains, originally isolated from children with upper respiratory illness, which had been passaged at different temperatures in WI-38 cells for from 11 to 77 passages were assayed for the presence of interferon: CO49630, grown at 28°; RS-A_1-HK_5, grown at 33°; RS-64-3669, grown at 26 and 28°; and RS-65-463, grown at 26, 28, and 33°.

Tissue culture. HEp-2 established human epithelioid cells (Flow Laboratories), WI-38 human fetal lung fibroblasts (Dr. L. Hayflick, Stanford University, Stanford, CA.), human foreskin fibroblasts (HF-5 and HF-7) and rabbit kidney cells, produced as previously described (9), were grown in Eagle minimum essential medium (MEM) containing 10% fetal calf serum, nonessential amino acids and Earle's balanced salts. Cultures were maintained at 35° in a Wedco Model 2-17H incubator in an atmosphere of 5% CO_2 in air. Maintance medium consisted of MEM with 2% fetal calf serum, nonessential amino acids and Earle's salts. The diploid WI-38 and HF fibroblasts were always transferred 2 for 1 at regular intervals. WI-38 cells were used between passages 17 and 29, and HF cells between passages 10 and 35. Rabbit kidney cells were used at passage 2.

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TABLE I. Stimulation of Interferon in WI-38 Cells by Three Strains of RS Virus.[a]

Virus strain	Titer of interferon,[b] after incubation for (days): 1	2	3	4	5	6	7	8
Long	—	33	—	80	86	76	—	—
Bigelow	105	300	350	230	—	—	—	—
RS 11657	—	0	—	0	0	<10	10	17

[a] Multiplicity of infection = 0.01 pfu/cell.

[b] PR_{50} units measured on human foreskin cells against a VSV challenge.

Interferon production. Cultures of WI-38, HF, and HEp-2 cells were infected with RS virus at various multiplicities of infection (MOI). Incubation was at 35° except where indicated, and the culture fluids were assayed for interferon after inactivation of virus by treatment at pH 2 for 24 hr (10). Care was taken to keep the cell:media volume ratio equal in comparisons of interferon production.

Interferon assay. Interferon was assayed by its ability to reduce the number of plaques produced by vesicular stomatitis virus (VSV) on 60 mm plates of HF cells, as previously reported (10). One PR_{50} unit of interferon was that dilution which reduced the plaques by one-half in a challenge of 50 to 80 plaque-forming units (pfu)/plate, and the reciprocal of the dilution was the titer of a given preparation.

Inhibition of production of RS virus by interferon. To test the sensitivity of RS virus to the action of interferon, WI-38 cells were treated for 18–20 hr with from 0.4 to 6.0 PR_{50} units/ml of a human interferon preparation, HIF-3, in maintenance medium. HIF-3 was produced by exposure of HF cells to Newcastle disease virus (NDV) (9) and had a titer of 116 on WI-38 cells. The treated WI-38 cells were washed with Hanks' balanced salts solution (HBSS) and were then infected with a low MOI (0.01 pfu/cell) of RS strains. Virus was harvested from duplicate cultures by scraping the cell sheet with a rubber policeman, triturating vigorously with a small bore pipette, and pooling the culture fluids. RS virus yield was determined immediately, without freezing, using the microplaquing method of Coates *et al.* (11). This method employs monolayers of HEp-2 cells and methylcellulose-containing overlay, which is removed when syncytia are large enough to be counted as plaques. (This required 72 hr with the RS strains in this study). Cell layers are then fixed with formalin, stained with hematoxylin and eosin, and plaques are counted using a dissection microscope.

Results. Production of interferon by RS virus. Table I shows the yields of interferon from WI-38 cultures when infected with low MOI of 3 RS strains. All three strains stimulated measurable interferon titers but with peak titers occurring at different times of incubation. The Long strain reached a maximum titer of 86 after 5 days; whereas Bigelow produced a titer of 350 after 3 days. The low temperature adapted strain, RS 11657, stimulated very low titers only after 7 to 8 days incubation. These differences certainly reflect relative invasiveness of the strains to some extent, but the 11657 strain did not fail to infect the cells, as typical CPE was observed and a significant amount of infectious virus was produced.

TABLE II. Interferon Production by RS Strains at Lower Temperatures.

RS strain	Incubation temp (°)	No. of samples assayed	Av % reduction[a]	Range[b] (%)
RS-A_1-HK_5	33	15	33	0–94
RS-65-463	33	14	48	0–96
	28	14	6	0–30
	26	11	22	0–50
RS-64-3669	28	13	63	0–100
	26	10	65	0–98
CO 49630	28	13	20	0–100

[a] Reduction (%) of plaques when a 1:10 dilution of culture fluid was tested in the standard plaque assay.

[b] Highest and lowest reduction (%) in the group tested.

TABLE III. Stimulation of Interferon in HF-5 Cells by Four Strains of RS Virus.[a]

Virus strain	Titer of interferon after incubation for (days): 2	8
CO 49630	0	45
RS-A_1-HK_6	0	9
RS-64-3669	0	11
Long	0	18

[a] Multiplicity of infection estimated to be 0.05 to 0.001 pfu/cell.

In our laboratory, 4 strains of RS virus have been passaged in WI-38 cells at 33, 28, and 26° for periods up to 4 years and small samples of all passages have been stored at −70°. Culture fluids from 90 selected passage levels which had been stored up to 2 years were assayed for interferon at a 1:10 dilution by the standard plaquing method. Table II shows the average percentage reduction of plaques at this dilution and the range of percentage reduction for each strain and temperature group. All strains at all temperatures produced at least a small amount of interferon. Although this screening assay did not allow for plotting of actual titers, it could be estimated that five of the seven groups had at least one sample with a titer of 100 or more.

Four strains of RS virus were inoculated into human foreskin fibroblasts and HEp-2 cells at a low MOI; and the culture fluids were assayed for interferon at 2 and 8 days. In Hep-2 cells, only the Long strain stimulated any detectable interferon and this had a titer of <5. Table III shows the results in HF cells. No detectable interferon was produced at 2 days but all strains stimulated interferon by 8 days, titers ranging from 9 to 45.

The antiviral agent produced by WI-38 cells infected with Long, Bigelow, and RS 11657 strains of RS virus was interferon, as demonstrated by the fact that it was stable at pH 2 and was not sedimented by centrifugation at 100,000*g* for 1 hr. The agent was greatly reduced in activity by heating at 60° for 1 hr and was completely inactivated when undiluted culture fluid was incubated for 2 hr at 35° with 500 μg/ml of trypsin (purified 2× recrystallized, General Biochemicals). It was inactive when tested on L-929 mouse fibroblasts or dog arterial fibroblasts but did possess interspecific activity on rabbit kidney cells (see Table IV). Interspecific activity of human interferon, stimulated by NDV and other viruses, on rabbit cells has been reported by Desmyter *et al.* (12). Both RS virus and NDV are paramyxoviruses and apparently are capable of stimulating interspecific factor. Desmyter found that interspecific titers were often higher than homospecific titers. In our studies, the interspecific titers were lower both in RS- and NDV-induced interferon, but they were reproducible and readily detectable.

When WI-38 cells were infected with larger MOI of the Long strain of RS virus (as shown in Table V), less interferon was detected in the culture fluid at multiplicities of 1.0 and above than was detected at 0.1 and, previously, 0.01. In addition, when growth medium containing 10% fetal calf serum was substituted for the usual maintenance medium with 2%, no interferon was produced at a MOI of 1.0 and a titer of less than 10 at a multiplicity of 0.1. In addition, viable virus yields were increased up to tenfold.

In the cultures containing 10% serum, extensive, typical syncytial masses formed in the cell sheet. In the low serum cultures, cellular degeneration took place with formation of no, or very small, syncytia.

Sensitivity of RS virus to interferon action. Table VI shows the percentage reduction of virus yield from WI-38 cells which were pretreated with HIF-3 human interferon and subsequently infected with Long and RS 11657 strains of RS virus. In the case of

TABLE IV. Activity in Rabbit Cells of Human Interferon Induced by NDV and RS Virus.

Producing cell line	Virus	Titer on: HF-5	Rabbit kidney
HF-5	NDV	330	115
WI-38	RS (Long)	95	12
WI-38	RS (Bigelow)	195	28

TABLE V. Production of Interferon and Virus in WI-38 Cells by RS (Long).[a]

MOI[b]	10% Serum		2% Serum	
	Virus/culture	Interferon titer	Virus/culture	Interferon titer
3.0	—	—	2.4×10^5	<10
1.0	23.1×10^5	0	2.1×10^5	17
0.1	9.6×10^5	<10	1.2×10^6	170
Typical syncytia formed	Yes		No	

[a] Incubation time, 49 hr.
[b] Multiplicity of infection (pfu/cell).

both strains, virus yield was inhibited approximately 80% by a treatment of 5.8 units/ml, and only 0.4 unit/ml inhibited yields by 26 and 53% in Long and 11657, respectively. This represents an influence on the first two cycles of viral replication. The influence of interferon pretreatment appears to diminish after 4 days of incubation. This is probably because the virus in control cultures reached its peak first and its titer of infectious virus began to decrease first with further incubation at 35°. Cells in the interferon-treated cultures were in better condition longer and produced their peak titers of infectious virus later.

Discussion. It appears that the prolific multiplication of RS virus in infants and small children is not related to an inherent lack of interferon-stimulating potential in the virus. We can state that all RS strains which we have studied were capable of inducing at least small amounts of interferon in WI-38 cells at temperatures in the range of 26 to 35°. Virus yield reduction experiments showed RS virus to be sensitive to the antiviral action of interferon also, as has been reported in other studies (7, 13).

TABLE VI. Reduction of Yield of RS Virus in WI-38 Cells Pretreated with Interferon.[a]

Virus strain	Pretreatment (units/ml of interferon)	Reduction (%) of virus yield:[b]	
		2 days	4 days[c]
Long	0	—	—
	5.8	81	35
	1.5	42	24
	0.4	26	0
RS 11657	0	—	—
	5.8	79	50
	1.5	56	8
	0.4	53	0

[a] Pretreatment, 20 hr.
[b] Multiplicity of infection = 0.01 pfu/cell.
[c] Incubation time.

The concentration of serum proved to be an important factor in the production of interferon in WI-38 cells. The best yields of interferon were obtained from cultures infected with low MOI (0.01 to 0.1) of RS virus and containing 2% fetal calf serum. In infected cultures containing 10% fetal calf serum, little or no interferon was produced and the virus titer was increased up to tenfold. Large, typical syncytial masses were seen only in cultures with 10% fetal calf serum, but this was probably an effect of increased virus production rather than a cause as it has been reported that presence or absence of syncytia has little effect on total yield of infectious virus (14).

A similar reduction of interferon production in cell cultures incubated with 10% in contrast to 1.5% serum after addition of inactivated virus has been reported by Friedman (15). An increase in the rate of cellular protein synthesis, such as occurs in a confluent cell culture following a media change, especially with medium containing higher serum concentrations, does not necessarily lead to increased interferon yields and may decrease the yields greatly. It has been postulated that this increased protein synthesis may actually lead to accumulation of a stable interferon repressor in the cells (15).

In our study, the most recently isolated strain with the fewest passages in tissue culture was the most efficient stimulator of interferon. This suggests that the wild strain

RS virus which causes disease should stimulate effective interferon production in cells of the respiratory epithelium if the infected individual is competent to produce interferon. It has been suggested that younger animals are capable of producing less interferon than older ones, but this has not been conclusively proven, especially with reference to man (16). Studies involving interferon production by human leukocytes from individuals of widely varying ages have yielded conflicting results (17–19). Other cell and tissue culture studies have also led to different conclusions. Tissues obtained from young chick and mouse embryos generally have a poorly functioning interferon system (3) and foreskin fibroblasts cultured from older children were found to be superior in interferon production to fibroblasts obtained from newborn infants (20). However, in a recent study (21), although fibroblasts of adult origin produced somewhat more interferon in cell culture than did those of neonatal origin, fetal fibroblasts produced 4 to 6.5 times more interferon than either adult or neonatal cells.

Various organs and tissues from the same animal body differ in their ability to release interferon (21, 22). Since it is the cells in closest proximity to the infected, interferon-producing cells which subsequently become most resistant to virus (3), the ability of, in the case of respiratory viruses, the epithelial cells of the respiratory tract to produce interferon is of prime importance. Other studies indicate that heredity also has a profound effect on the efficient production of interferon (16, 17, 23). Another point which must be considered is the relative sensitivity of the cells of a tissue to the action of the interferon produced by infected cells. It may be that the cells of the respiratory tract of young children produce sufficient interferon upon infection with RS virus, but are not as sensitive to its action as are adult cells. It has been observed that human cell lines of embryonic origin are less sensitive to the action of exogenous interferon than are cell lines of neonatal origin (24). However, in a limited survey of interferon production in several human diploid cell lines (10), whereas a line of cells of embryonic origin was less sensitive to interferon action, there was no significant difference in the sensitivity of cells of neonatal and adult origin.

The interferon system may well be very important to the course of, and recovery from, disease caused by respiratory syncytial virus. It has been shown that this virus can spread from cell to cell, even *in vitro*, in the presence of excess neutralizing antibody in the overlay medium (25). The interferon system affords intercellular protection. Cells become resistant to viral attack and virus yields are reduced in infected cells even before free interferon is demonstrable in serum or nasal secretions. Circulating and secretory antibody reaches its peak several days later. If an individual was not competent to produce sufficient interferon in his respiratory tract in response to an RS virus infection, the cell-to-cell spread of the virus could produce overwhelming disease even in the presence of antibody. Further studies measuring interferon levels in nasal secretions from young children and adults suffering from viral respiratory disease, especially RS virus, are needed to clarify this point.

Summary. The ability of several strains of RS virus to stimulate interferon production *in vitro* was studied. All RS strains studied were capable of inducing at least low titers of interferon in WI-38 and HF fibroblasts. The concentration of serum in the medium was of importance, the best yields of interferon being produced in medium with 2% fetal calf serum and little or none in 10% fetal calf serum. RS virus was shown to be sensitive to the antiviral action of interferon in WI-38 cells. It appears that the prolific multiplication of RS virus in young children is not due to lack of interferon-stimulating potential of the virus. The possibility that the interferon system is less efficient in young children than in adults was considered.

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