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Effect of Exogenous Interferon on Rubella Virus Production in Carrier Cultures of Cells Defective in Interferon Production

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An established cell line (Vero) defective in interferon production was used to evaluate the role of interferon in chronic rubella virus infections of cell cultures. Inoculation of Vero cells with a low multiplicity of virus resulted in the development of carrier cultures which had the characteristics of a regulated infection. Although added interferon did not alter rubella virus production in carrier cultures of cells capable of producing interferon, such added interferon caused a dramatic reduction of virus production in the carrier cultures of Vero cells. There was a reduction of the fraction of cells producing virus in Vero carrier cultures, but not in carrier cultures of other cells upon incubation in the continual presence of rubella virus antibodies. In addition, the fraction of infected cells fluctuated in carrier cultures in Vero cells. The data indicate that interferon is not necessary for maintaining a chronic rubella virus infection in vitro and suggest an instability of the virus genome in Vero cells.

Rubella virus infection acquired in utero may result in a chronic viral infection. Available evidence suggests that a limited number of cells of the fetus are initially infected (15), and it has been postulated that these infected cells may give rise to clones of infected cells (13, 16). Although the contribution of interferon in establishing or maintaining the chronic infection is not known (1), chronic rubella virus infection of cells grown in vitro has been readily accomplished (4-6, 8, 12, 16, 19, 22), and it has been suggested that interferon may play a role in maintaining the infection (4, 8, 22).

Recently, cultures of a stable line of African green monkey kidney cells (Vero cells) were found to be defective in their ability to produce interferon although these cells responded to exogenous interferon (2). This cell line provides a system in which the role of interferon production in the chronic infection of cell cultures by rubella virus can be examined. This study describes some characteristics of Vero cell-carrier cultures and compares the effect of exogenous interferon on virus production in carrier cultures of human lung cells and Vero cells.

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MATERIALS AND METHODS

Tissue cultures. Vero cells, a stable line of African green monkey kidney cells, were used between the 152nd and 175th passages. BSC-1 cells, another stable line of African green monkey kidney cells, were obtained from laboratory stock and used between the 82nd and 105th passages. Human lung fibroblast cells were obtained from a fetus and used between the 17th and 20th passages. The Vero cells were grown with medium 199 containing 5% fetal bovine serum, 0.75 g of sodium bicarbonate per liter, 100 units of penicillin, and 100 μg of streptomycin per ml. Growth medium for BSC-1 cells and human lung fibroblast cells consisted of Eagle's medium supplemented with 0.75 g of sodium bicarbonate per liter, 10% fetal bovine serum, 100 units of penicillin, and 100 μg of streptomycin per ml. Maintenance medium for the three cell lines consisted of Eagle's medium with 1.5 g of sodium bicarbonate per liter, 2% fetal bovine serum, and antibiotics. When the cells were incubated in a 5% CO_2 atmosphere, the content of sodium bicarbonate was increased to 3.0 g/liter. The incubation temperature for growth of cultures and for virus replication experiments was 37 C.

Viruses. Rubella virus (R-1 strain) was used to establish carrier cultures in Vero cells and human lung fibroblast cells. The virus was assayed in BSC-1 cells by a previously described modification of the hemadsorption-negative plaque test (9, 14). Vesicular

stomatitis virus (VSV), Indiana serotype, was assayed in BSC-1 cells and Vero cells by plaque formation. The constituents of the agar medium and methods of plaque assay of this virus have been described previously (13).

Determination of the per cent of cells infected. The infectious-center assay method was used to make most of these determinations. The procedures for this assay were based on the method described previously (15, 16). Briefly, after the treatment of infected cultures with 0.2% trypsin solution containing 0.04% ethylenediaminetetraacetic acid (EDTA), the well-dispersed cells were washed twice with maintenance medium. After scoring the cell number by using either a model B Coulter counter or a hemocytometer, the cells were serially diluted in maintenance medium, inoculated onto BSC-1 cell monolayers in petri dishes, and allowed to adsorb for 60 min in a 5% CO_2 atmosphere at 37 C. Medium was then carefully added to the cultures and the subsequent procedure was identical to that of the assay for rubella virus in BSC-1 cells (14). The per cent of cells infected was calculated from the number of plaques obtained and the cell counts.

In some experiments the terminal dilution assay method was used. Each of the serially diluted cell suspensions was inoculated into five tube cultures of Vero cells and incubated for 4 days at 37 C. Then, the culture fluid from each tube was examined for the presence or absence of rubella virus by hemadsorption-negative plaque formation on BSC-1 cell monolayers in petri dishes. The number of cells needed to infect 50% of the culture tubes was estimated by the method of Reed and Muench (17).

Interferon production and assay. Interferon was prepared in BSC-1 cells by inoculating them with Newcastle disease virus, California strain. The methods used for partial purification and assay of interferon have been described elsewhere (1).

Isolation of cell clones from carrier cultures. After treatment of carrier culture cells with 0.2% trypsin solution containing 0.04% EDTA, dispersed cells were washed twice with Eagle's medium. The cell concentration was diluted in Eagle's medium supplemented with 20% fetal bovine serum and rabbit anti-rubella serum diluted 1:4 (16 units) to yield 100 cells per 60-mm petri dish. After 1-hr adsorption of cells to petri dishes in a 5% CO_2 atmosphere at 37 C, the medium was removed. The cells were then covered with Eagle's medium containing 20% fetal bovine serum, 0.75% agar, and a low concentration (0.75 g/liter) of sodium bicarbonate. The cells were incubated for 7 to 10 days in a 5% CO_2 atmosphere. Each colony of cells was then transferred to a new petri dish by a capillary tube.

RESULTS

Establishment of Vero cell cultures chronically infected with rubella virus. Infection of Vero cells with a low multiplicity of rubella virus resulted in the production of new virus progeny which rapidly spread to infect the majority of cells in the culture. At a multiplicity of 0.01 plaque-forming units (PFU)/cell, 40% of the cells were infected by 2 days after initial infection and 100% of the cells were infected by the ninth day after infection. In contrast, the same amount of virus added to human lung cells resulted in infection of 0.4% of the cells by the second day after infection, and 0.7% of the cells were infected by day 17. The Vero cell cultures infected with rubella virus underwent some cytopathic changes; however, the cell monolayers remained intact and cell multiplication continued. The infected cultures of Vero cells were carried through 10 subcultivations after which infected clones were selected and one of the clones was carried through an additional 28 subcultivations. Rubella virus was found in the fluid of the cultures during the 11 months of maintaining the cultures. The proportion of cells registering as infectious centers in the first 10 passages of the parent cultures varied between 50 and 100%. The proportion of cells registering as infectious centers in an infected clone was examined over the first seven passages and 55 to 95% were found to be infected.

To substantiate that a majority of the cells contained virus and to evaluate the susceptibility of noninfected cells of the carrier culture

TABLE 1. *Production of interferon and sensitivity to rubella virus of clones isolated from carrier cultures of Vero cells*

Clone designation	Virus carrier state	Interferon production[a]	Intrinsic interference[b]	Sensitivity to rubella virus[c]
1	+	NT[d]	NT	NT
2	−	<1:2	−	1.5×10^5
4	−	<1:2	−	3.0×10^5
7	+	<1:2	−	6.2×10^4 (5.8×10^4)
8	+	NT	NT	NT
9	+	NT	NT	NT
11	+	NT	NT	5.0×10^4 (5.2×10^4)
12	−	NT	NT	NT
14	+	<1:2	−	1.8×10^5 (1.8×10^5)
18	−	<1:2	−	2.8×10^5
23	−	NT	NT	NT
24	−	NT	NT	NT
Parent Vero BSC-1		<1:2 1:16	− +	3.0×10^5 NT

[a] Interferon production expressed as the dilution of culture fluid which inhibited 50% of vesicular stomatitis virus plaques. Interferon induced with Newcastle disease virus (NDV) in monolayers of cells grown in 240-ml (8-oz) prescription bottles and assayed as previously described (1).

[b] Intrinsic interference to NDV determined by method of Marcus and Carver (10).

[c] Expressed as the virus yield per milliliter after 4 days of incubation of culture infected with rubella virus at a multiplicity of 1 plaque-forming unit/cell. The figures in parenthesis indicate the titers of virus in control carrier cultures not superinfected with rubella virus.

[d] Not tested.

to rubella virus, cells of the 10th passage were monodispersed and cloned. Twelve clones were established; of the 12 clones, six were found to be infected (Table 1). The ability to support the replication of rubella virus was examined in three infected clones, in three virus-free clones, and in parent noninfected Vero cells by infecting the cells at a multiplicity of 1 PFU/cell and assaying the total virus yield after 4 days of incubation. The virus yield of the clones free of rubella virus was essentially the same as that of the parent Vero cells (Table 1), indicating that the cells in the carrier culture had not acquired genetic resistance to infection by the virus. Superinfection of the infected clones did not result in the production of additional virus.

Previously, it was shown that Vero cells were incapable of producing interferon, but were susceptible to exogenous interferon (3). Five clones, two infected and three virus-free, were examined for their ability to produce interferon as previously described. Intrinsic interference to Newcastle disease virus was also examined (10). BSC-1 cells and noninfected parent Vero cells were used as controls. The clones derived from the rubella-infected carrier cultures did not acquire the ability to produce interferon, nor did they exhibit intrinsic interference (Table 1).

Effect of exogenous interferon on rubella virus production in carrier cultures of Vero cells. The carrier state of rubella virus in cells capable of producing interferon does not appear to be altered by exogenous interferon (1). Experiments were designed to evaluate the influence of exogenous interferon on rubella virus replication in primarily infected and in chronically infected Vero cells. In addition to Vero cells which were infected with rubella virus at a multiplicity of approximately 0.01 PFU per cell and incubated for 10 days, one of the infected clones in the second passage after cloning was used. Human lung fibroblasts chronically infected with rubella virus were used as a control. The tube cultures were washed once with maintenance medium and divided into three groups. One group of cultures received 1 ml of medium containing 20 units of interferon, one group received medium containing antibodies to rubella virus, and the remaining cultures received maintenance medium only. Twenty-four hours later, culture fluids from four tubes of each group were pooled and assayed for infectious virus. Simultaneously, the number of cells producing virus was determined.

The results of these experiments in Vero cells are shown in Table 2. The extracellular virus content of cultures treated with interferon was about 50% less than cultures incubated with medium only. Incubation of the cultures in the presence of antibodies to rubella virus reduced extracellular virus infectivity by over 99%. The

TABLE 2. *Effect of exogenous interferon and antibody on the infection of Vero cultures with rubella virus*

Cultures	Determination	Expt 1		Expt 2		
		Cultures without interferon	Cultures with interferon[a]	Cultures without interferon	Cultures with interferon[a]	Cultures with antiserum[b]
Primary infected[c]	Extracellular rubella virus (PFU/ml)	4.0×10^5	1.3×10^5	1.2×10^6	5.5×10^5	6.8×10^3
	Total no. of cells per culture	3.8×10^5	3.0×10^5	2.5×10^5	2.5×10^5	2.8×10^5
	No. of cells producing virus per culture	2.4×10^5	2.1×10^4	2.0×10^5	3.5×10^4	2.2×10^5
	Per cent of cells producing virus	62	7	80	14	80
Cloned carrier[d]	Extracellular rubella virus (PFU/ml)	1.1×10^5	5.0×10^4	8.5×10^4	7.0×10^4	4.3×10^2
	Total no. of cells per culture	9.1×10^4	1.5×10^5	6.0×10^4	5.0×10^4	6.5×10^4
	No. of cells producing virus per culture	5.0×10^4	3.0×10^3	5.7×10^4	7.0×10^3	4.4×10^4
	Per cent of cells producing virus	55	2	95	14	68

[a] The concentration of interferon used was 20 units/ml.
[b] Antirubella rabbit immune serum (1:4 dilution; 8 units) was used.
[c] Vero cell cultures which were infected with rubella virus at a multiplicity of less than 0.01 plaque-forming units (PFU)/cell 10 days before evaluating the effect of interferon on virus production.
[d] Infected clones derived from Vero carrier culture at 10th passage.

number of viable cells in the cultures was not affected by 24 hr of incubation with either interferon or antibodies to the virus. The proportion of cells producing virus, as determined by infectious center assay, was markedly reduced in the cultures treated with interferon; the reduction was from 55 to 95% to 2 to 14%. No significant differences in the reduction of virus titer and the per cent of infected cells were observed between primarily infected cultures and the clones of the Vero carrier cultures. The presence of interferon had no effect on virus production or the proportion of virus-producing cells in infected cultures of human lung fibroblasts (Table 3).

Consistently, a greater reduction occurred in the proportion of cells producing virus than in extracellular virus after 24 hr of incubation of Vero cell cultures with interferon. This discrepant reduction was examined more closely. Cells from an infected clone of a carrier culture of Vero cells were grown in culture flasks. When confluent monolayers developed, they were washed, and two flasks received medium containing 20 units of interferon per ml, whereas two flasks received medium only. The cultures were incubated at 37 C and, at intervals, 1-ml samples of the culture fluids were removed and replaced with fresh media. The samples taken from the two flasks at each interval were pooled and frozen. After all samples were obtained, they were assayed simultaneously for infectious virus.

The release of rubella virus from cells incubated in the presence of interferon was the same as the release from control cells for the first 10 hr (Fig. 1). However, after 10 hr, further release of virus did not occur from cells treated with interferon. In cultures treated for 24 hr with interferon, there was less reduction in extracellular virus than there was in numbers of virus-producing cells. The discrepancy was the result of virus which matured and was released before the inhibition of virus synthesis by interferon was accomplished.

To substantiate that the reduction of virus synthesis was mediated by interferon, the effect of interferon was evaluated in the presence of dactinomycin which has been shown to prevent the virus inhibition mediated by interferon (20). Carrier cultures of Vero cells were divided into four groups. The cells were washed and one group received 1 ml of medium containing 20 units of interferon. Another group of cultures received 1 ml of medium containing 2 μg of dactinomycin—a concentration which was found to inhibit completely the antiviral effect of 20 units of interferon in a VSV-Vero cell system. Another group of cultures received interferon and dactinomycin, whereas the remaining cultures received only maintenance medium. After 24 hr of incubation, culture fluids from four replicate tubes in each group were pooled and assayed for infectious virus and the number of infected cells was determined by infectious center assay. The results are shown in Table 4. Extracellular virus titers and the per cent of cells producing virus in the cultures treated with interferon and dactinomycin were found to be similar to those of control cultures, whereas those of cultures treated with interferon but without dactinomycin were reduced to the degree noted in previous experiments. Dactinomycin was effective in preventing the inhibitory effect of interferon on virus production by cells of the carrier cultures.

Effect of the continued presence of interferon and antiserum on carrier cultures of Vero cells These carrier cultures were noted to have variable proportions of virus-producing cells; the percentage of cells producing virus at different passage levels varied from 55 to 100%. This suggests that cells free of virus arose in the Vero carrier cultures and that these cells subsequently became

TABLE 3. *Effect of exogenous interferon on the chronic infection of cultures of human lung cells*[a]

Determination	Expt 1		Expt 2	
	Cultures without interferon	Cultures with interferon[b]	Cultures without interferon	Cultures with interferon
Extracellular rubella virus (PFU/ml)	1.4×10^5	1.0×10^5	4.2×10^5	4.0×10^5
Total no. of cells per culture	5.5×10^4	9.6×10^4	2.2×10^5	2.4×10^5
No. of cells producing virus per culture	5.5×10^4	1.0×10^5	2.0×10^5	2.3×10^5
Per cent of cells producing virus	100	100	95	98

[a] Infected cultures were obtained by infecting human lung cells with rubella virus at a multiplicity of 0.01 plaque-forming units (PFU)/cell and subculturing seven times at intervals of 5 to 7 days (12).
[b] See Table 2.

infected. To examine this possibility, cells of an infected clone at the 15th and 17th passage were dispersed by trypsin and seeded into culture tubes at a concentration of 1.5×10^5 cells per tube. The cells were allowed to adsorb to the glass for 2 hr, at which time one-third of the tubes received 1 ml of medium containing 1:20 of pooled human serum which contained 320 hemagglutination-inhibiting antibody units against rubella virus. For comparison, a second group of tubes received medium containing 20 units of interferon per ml and the third group of tubes received medium only. Normal fetal bovine serum was added to the tubes containing interferon and medium only, to give the same final concentration of serum as the medium containing the human serum. The media in the tubes were changed every 2 days throughout the experiment. The total cell count, infectious virus in the fluid of the cultures, and the number of cells producing virus were assayed at intervals of 4 days.

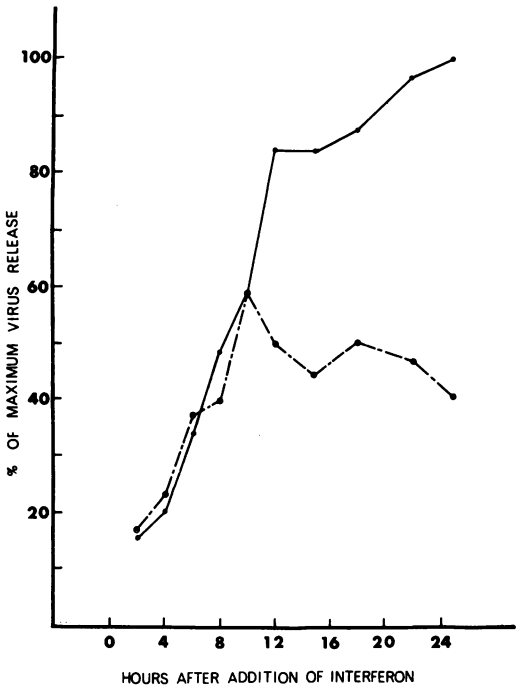

FIG. 1. *Release of rubella virus from Vero carrier cultures expressed as the per cent of the extracellular virus concentration in control cultures after 25 hr of incubation. Monolayers of Vero carrier cells were cultured in the presence (○) or absence (●) of 20 units of interferon per ml and samples of fluids were removed at intervals and assayed for virus.*

TABLE 4. *Effect of dactinomycin on the ability of interferon to reduce the per cent of cells producing virus in Vero carrier culture*

Treatment of culture	Expt 1		Expt 2	
	Extracellular rubella virus[a]	Per cent of cells producing virus	Extracellular rubella virus[a]	Per cent of cells producing virus
Maintenance medium only	6.0×10^5	100	1.0×10^5	100
Interferon[b]	2.5×10^5	15	5.0×10^4	21
Dactinomycin[c]	5.5×10^5	96	1.2×10^5	60
Dactinomycin + interferon	5.0×10^5	87	1.2×10^5	100

[a] Expressed as plaque-forming units per milliliter.
[b] Interferon used at a concentration of 20 units/ml.
[c] Dactinomycin used at a concentration of 2 μg/ml.

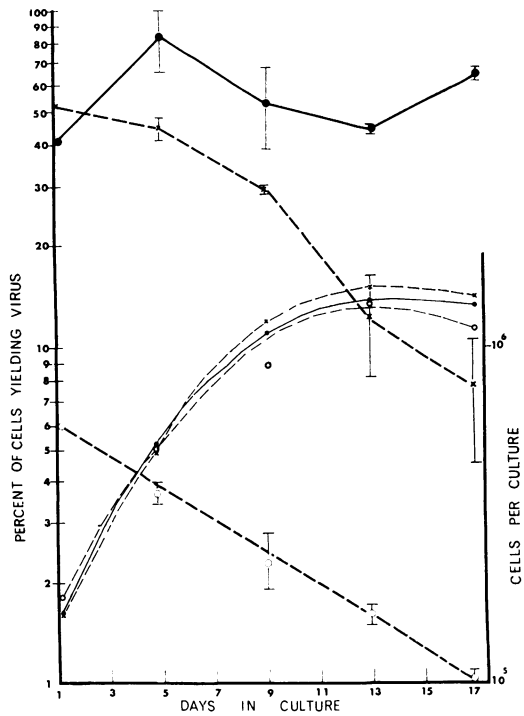

FIG. 2. *Effect of the continued presence of interferon and antiserum on rubella virus production in carrier cultures of Vero cells. Symbols: light lines, cell growth; heavy lines, per cent of cells producing virus; ●, medium only; ○, culture treated with interferon; and ×, culture treated with antiserum to rubella virus. Mean and range of percentage of infected cells are indicated.*

The results of the experiments are shown in Fig. 2. The replication of the cells was essentially the same in all three groups of cultures. In cultures receiving medium, the proportion of cells producing virus fluctuated between 40 and 80%

with the largest proportion noted during the phase of active cell replication. There was a dramatic decrease in the proportion of cells producing virus in cultures incubated in the presence of interferon. Only 6% of the cells registered as infectious centers after 1 day of incubation. Thereafter, a linear decrease in the per cent of cells producing virus was observed, amounting to about one-tenth of the remaining virus-producing cells per day. In contrast to the 30–50% reduction in extracellular virus observed in cultures treated with interferon for 24 hr, there was a 90 to 99% decrease in the cultures treated for 4 or more days.

The presence of antibodies to rubella virus in the culture fluid effected a 2 $\log_{10}$ or greater reduction in infectious virus in the extracellular fluid. Initially, the presence of antibodies to the virus did not affect the proportion of cells producing virus; however, the continued presence of antibodies resulted in a gradual reduction in the per cent of cells producing virus (Fig. 2) and by the 17th day only 8% of the cells registered as infectious centers.

DISCUSSION

Rubella virus is capable of entering a carrier state in established cell lines (8, 19) as well as in cell cultures of human fetal tissues (12). In vitro propagation of tissue from infected human fetuses, newborns, and from hamster lungs infected in vivo also results in chronically infected cultures (4–6, 16). None of the rubella virus carrier cultures described has required the addition of antiviral factors or antibodies to the medium. The fraction of cells releasing virus has ranged from 5 to 50% in the cultures of hamster lungs (4) to nearly all of the cells in cultures of human tissues (16). Essentially 100% of LLC MK_2 cells in a carrier culture of rubella virus contained viral antigen by 10 days after subculturing (8). Incorporation of antibodies to rubella virus or antiviral substances which inhibited viral penetration (amantadine HCl and ammonium acetate) was found not to reduce the fraction of infected cells (4, 8, 13). The infected cells appeared to divide, giving rise to infected daughter cells (8, 16), and the carrier cultures were found to be resistant to superinfection by some heterologous viruses but not by others (4, 8, 16). These characteristics are similar to those described for infection by certain other ribonucleic acid viruses, and carrier cultures with these features have been referred to by Walker (21) as having regulated infections.

The carrier state of rubella virus in Vero cells has somewhat similar characteristics. A large fraction of the cells were found to be infected, and neither antiviral substances nor antibodies to the virus were needed to maintain the carrier state. Single cells of the carrier culture gave rise to infected clones. In addition, we have demonstrated that virus-free clones derived from the carrier cultures are not genetically resistant to reinfection with rubella virus. Several authors have postulated that interferon may play a role in maintaining chronic infection by rubella virus in cell cultures (4, 8, 22). The ability to establish carrier cultures in Vero cells which are defective in their ability to produce interferon clearly indicates that interferon is not required for maintaining the chronic infection of cultures by rubella virus. The lack of effect of exogenous interferon on the carrier state in cells capable of producing interferon and responding to it could be interpreted in the same way, but it may also reflect the mechanism of chronic infection imposed upon cells in which the interferon system is fully active.

Several interesting differences between the chronic rubella virus infection in Vero cells and the chronic infection in other cells were noted. A considerable fluctuation of the proportion of Vero cells producing virus was observed. This was seen even in clones of Vero cells derived from a single cell. In contrast, the proportion of infected cells in chronically infected human fetal cell cultures remained constant. In addition, there was a reduction in the proportion of cells producing virus when carrier cultures of Vero cells were incubated continually in the presence of antiserum, whereas antiserum did not alter the chronic infection of other cells. There was also a dramatic reduction in the proportion of cells producing virus in the Vero carrier cultures incubated with exogenous interferon, whereas interferon did not alter virus production in chronically infected human fetal cells.

The unusual characteristics of the chronic infection in Vero cells suggest that the virus genome in these cells may be destroyed or deleted. Infected cells could lose their infectious state if the virus genome were unstable. An unstable virus genome could account for the fluctuating proportion of cells in the chronically infected cultures, for the loss of infection by part of the progeny of single infected cells, and for the continued sensitivity of the virus to exogenous interferon and to rubella virus antiserum. One might see a paradox between instability of the intracellular viral genome and the high viral yields accompanied by cytolysis which have been found in Vero cells (7, 18). However, loss of the viral genome by part of the cell population

may be the very condition which allows perpetuation of chronic rubella virus carrier cultures when infection is not restrained by the interferon system or eventually leads to cytolysis, or both. Additional data will be needed to clarify the cell-virus relationship in the chronic infection of cells which are defective in interferon production as compared to such infections in cells capable of producing interferon.

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