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Priming: a Nonantiviral Function of Interferon[1]

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No interferon is made by L cells when they are infected with MM virus. However, several thousand units of interferon are produced when interferon-treated L cells are infected with MM virus. We call the conversion of cells, from nonproducers to producers, priming. The time required for cells to become fully primed is dependent on the interferon concentration with which they are incubated. Primed cells produced interferon earlier than normal cells stimulated by other inducers. Cells which were exposed to interferon in the presence of inhibitors of protein synthesis became fully primed yet developed no virus resistance. Also, primed cells produced interferon in response to low concentrations of polyriboinosinic acid·polyribocytidylic acid that did not induce interferon in normal cells. Therefore, priming appears to be a function of interferon separable from its antiviral activity. Several other picornaviruses that failed to induce interferon in L cells, human embryonic lung cells, or monkey kidney cells did induce interferon when these cells had been primed by homologous interferons.

Pretreatment of cells with interferon can modify their production of interferon in three ways. It can (i) enhance the amount of interferon produced, (ii) bring about interferon production at an earlier time, or (iii) inhibit interferon production.

The ability of interferon pretreatment to enhance interferon yields was first reported by Isaacs and Burke (12) who found that interferon-treated chick chorioallantoic membranes produced significantly greater yields of interferon than did control membranes in response to live influenza virus. They referred to this enhancing effect of interferon pretreatment as "priming." In addition to the reports of priming by interferon, there are several reports showing that certain active or inactivated viruses also had an enhancing effect on interferon yields (2, 11, 19). Although the factor(s) responsible for the priming by viruses was not clearly resolved, at least some of the enhancement probably resulted from interferon induced by the priming agent. The term "priming" of interferon production has been set forth to imply priming by interferon per se (12), and it is in this sense that we use it. Enhancement of interferon yields by pretreatment with interferon was extended to chick embryo cells induced with Semliki Forest virus or Chikungunya virus by Friedman (6) and by Levy et al. (14). These investigators (6, 14) found that, besides enhancing the yield of interferon, interferon pretreatment caused interferon to be produced earlier than in normal cells. The earlier production of interferon by cells pretreated with interferon has also been observed in several types of human cells (24). In a series of studies in L cells, pretreatment with interferon has been shown also to bring about the earlier production of interferon when induced by either Newcastle disease virus (NDV) or polyriboinosinic acid·polyribocytidilic acid (poly rI·poly rC; references 15, 21-23, 25). In some instances, the yields were enhanced (23); in others they were unaltered or reduced (21, 22, 25). The resolution of these seemingly paradoxical results appears to lie in the ability of interferon pretreatment of cells to inhibit interferon production also. This was first reported by Vilcek (28) and Vilcek and Rada (29) who found that pretreatment of chick embryo cells with interferon inhibited the ability of the cells to produce interferon in response to tick-borne encephalitis virus. Subsequently, yields of interferon were found reduced when L cells which had been pretreated with interferon were induced with NDV (3, 21, 22, 25, 30) or with poly rI·poly rC (25, 30). The data of Lockart (16), which first showed the priming effect by interferon in L cells, probably were the first to suggest the solution to the foregoing paradox with respect to yields. He showed that pretreatment with small amounts of interferon resulted in enhanced yields, whereas pretreatment

[1] Contribution No. 1797.

with larger amounts had the opposite effect. This dose-dependent effect of interferon pretreatment was confirmed by Friedman (6).

The previous studies of the priming phenomenon have been complicated by the normal, unprimed interferon yields, and the enhancement of yields was frequently not great. We describe a better system in which to study the priming phenomenon, as the stimulus did not induce interferon production in unprimed cells. In a personal conversation with P. T. Allen, Brooks AFB, San Antonio, Tex., we were told that MM virus was able to induce only small amounts of interferon in L cells unless these cells were treated with interferon prior to infection. This serendipitous information led to our use of MM virus and to the following study to delineate more clearly the phenomenon of priming. Giron et al. (9) have also observed the priming effect with MM virus in this strain of L cells.

MATERIALS AND METHODS

Materials. Actinomycin D was obtained from Mann Research Laboratories, New York, N.Y. Cychoheximide and *p*-fluorophenylalanine (FPA) were obtained from Sigma Chemical Co., St. Louis, Mo. Puromycin dihydrochloride was obtained from Nutritional Biochemical Corp., Cleveland, Ohio, and poly rI·poly rC was obtained from Miles Chemical Co., Elkhart, Ind. International mouse reference interferon, catalog no. G002-902-026, was obtained from the National Institute of Allergy and Infectious Diseases, Bethesda, Md.

Tissue cultures. L cells were kindly provided by P. T. Allen, Brooks AFB, San Antonio, Tex., who grew them from a clone. These were grown in Eagle's minimal essential medium (MEM) modified for Spinner cultures supplemented with 10% fetal calf serum (FCS). Monolayer cultures for use in experiments were prepared from Spinner cultures by inoculating approximately 10^6 cells into 60-mm plastic petri plates in 3 ml of growth medium (GM) consisting of MEM with 10% FCS and antibiotics. Cultures were incubated overnight at 37 C in 5% CO_2 atmosphere.

Human embryonic lung diploid cells line 107 were obtained from Industrial Biological Laboratories, Inc., Rockville, Md. LLC-MK_2 cells, a stable line of rhesus monkey kidney cells, were provided by our colleague, S. Halperen. Both of these cells were grown in GM, and cultures for experiments were prepared by inoculating 60-mm plastic petri plates with approximately 10^6 cells in 3 ml of GM and incubating for 24 hr at 37 C in 5% CO_2 atmosphere.

Viruses. Stock suspensions of vesicular stomatitis virus (VSV) were prepared by inoculating monolayer cultures of the Vero line of African green monkey kidney cells, as described elsewhere (26).

NDV stocks were prepared by allantoic passage in 11-day-old chick embryos. Allantoic fluids were harvested 48 hr postinoculation and stored at −20 C.

ME virus stock was obtained from R. R. Rueckert, Univ. of Wisconsin, Madison.

Mengovirus stock was prepared in monolayer cultures of actinomycin D-treated L cells. Culture fluids harvested 24 hr postinfection were clarified by low-speed centrifugation, dispensed, and stored at −20 C.

Human rhinovirus type 2-HGP, equine rhinovirus (ERV-P), human rhinovirus type 14, human rhinovirus type 51, and poliovirus type 2, vaccine strain P712-ch 2 a, b, were obtained from our colleague, K. K. Lonberg-Holm, and had been passaged in HeLa cell cultures which do not seem to produce interferon (26).

Human rhinovirus type la was obtained from our colleague, B. D. Korant, and had been passaged in the HeLa cell cultures described above.

Echoviruses, types 7 and 12, were obtained from our colleague, S. Halperen, and had been passaged in LLC-MK_2 cell cultures.

MM virus was obtained from C. Gauntt, Univ. of Arizona, Tucson. Stocks were prepared in monolayer cultures of L cells which had been incubated for 30 min with 5 μg of actinomycin D per ml and washed three times immediately prior to infection. Medium was harvested at 24 hr postinfection and, after three freeze-thaw cycles, was clarified by low-speed centrifugation and stored at −20 C. This clarified suspension, referred to as crude virus, was homogenized with an equal volume of trichlorotrifluorethane at 0 C, to remove extraneous proteins. The aqueous layer was separated by low-speed centrifugation and was ultracentrifuged at 81,000 × *g* for 4 hr. Pelleted virus was soaked overnight in 0.02 M tris(hydroxymethylaminomethane (Tris) buffer (*p*H 7.5), sonically treated, and layered on cesium chloride gradient, specific gravity 1.25 to 1.45 g/cm^3. The virus-containing band was diluted in GM, dispensed, and stored at −20 C as purified MM virus stock.

Interferon production. Human interferon was kindly provided by S. Toy, Case Western Reserve Univ., Cleveland, Ohio, and preparation of rhesus monkey interferon was as described elsewhere (26).

Chicken interferon was prepared by inoculating monolayer cultures of chick embryo fibroblasts with Sindbis virus. Fluids were distributed into 100-mm petri plates (5 ml per plate) and exposed to an 8-w G.E. germicidal lamp for 5 min at a distance of 15 cm.

Mouse interferon was prepared by inoculating monolayer cultures of L cells with NDV, multiplicity of infection (MOI) 10. After adsorption at 37 C for 1 hr, inocula were removed, cultures were washed, and serum-free GM was added to cultures. Culture fluids harvested at 24 hr postinfection were clarified by low-speed centrifugation and assayed for interferon activity. This crude interferon had a specific activity of approximately 10^4 PDD_{50}-VSV/mg of protein. [Interferon titers are expressed as the reciprocal of the dilution depressing plaque numbers of VSV by 50% (PDD_{50}-VSV).] Crude interferon preparations were purified 10-fold by selective acid and ammonium sulfate precipitations to give interferon preparations with specific activity of approximately 10^5 PDD_{50}-

VSV/mg of protein. These preparations were purified by electrophoresis to give interferon preparations with specific activity of approximately 10^6 PDD_{50}-VSV/mg of protein, and these were further purified to give a specific activity of approximately 10^7 PDD_{50}-VSV/mg of protein. Details of these procedures will appear elsewhere (E. Knight, Jr., and W. E. Stewart II, *in preparation*). These preparations have been characterized to fit the criteria for interferons (18). Prior to storage at 4 C, all interferon preparations were exposed for 5 min at 15 cm to 8-w G.E. germicidal ultraviolet light.

Interferon assays. Interferons were assayed on L cells, human embryonic lung cells, or LLC-MK_2 cells by a plaque reduction method with VSV as challenge virus. Monolayer cultures of appropriate cells were incubated overnight with 2 ml of serial dilutions of interferon preparations and were washed twice with GM; 0.5 ml of virus suspension containing 50 to 100 plaque-forming units (PFU) was added. After adsorption for 1 hr at room temperature, inocula were removed, and plates were overlaid with 3 ml of medium containing 1% agar, 2% FCS, MEM, antibiotics, and 0.08% protamine sulfate, adjusted to *p*H 7.2 to 7.4 with $NaHCO_3$. Our PDD_{50}-VSV unit (*see definition above*) is equivalent to 7.5 units of international mouse reference interferon.

Extraction and assay of infectious RNA from MM virus. Infectious ribonucleic acid (RNA) from MM virus was extracted with phenol at 45 C in the presence of 0.2% sodium dodecyl sulfate, 0.02 M Tris (*p*H 7.5), and 0.002 M ethylenediaminetetraacetic acid (EDTA; 4), and was precipitated with ethanol at −20 C by the method reported by Nair and Lonberg-Holm (20). Precipitated RNA was dissolved in 0.02 M Tris, 0.002 M EDTA buffer (*p*H 7.5) and stored at −70 C. Infectivity assays were performed on L-cell cultures with RNA preparations diluted in 0.14 M LiCl, 0.01 M Tris, 0.001 M $MgCl_2$ (LTM) buffer containing 1.2 mg of diethylaminoethyl (DEAE) dextran/ml (DEAE-dextran-LTM buffer). RNA was adsorbed to cells at 37 C for 30 min. Plaques were developed by the second day and were similar to those produced by whole virus.

RESULTS

Development of the primed state by incubation with interferon. Previous workers investigating the priming phenomenon employed various times of exposure of cells and various concentrations of interferon, both seemingly arbitrarily chosen (6, 12, 14, 15, 21). We know of no published report which describes the kinetics of development of the primed state with respect to the concentration of interferon employed and the time. The L cells used in this study make no interferon in response to MM virus unless they are exposed to interferon and thus make this determination easy, as there is no background level of interferon with which one has to contend.

The rate of development of the primed state was therefore determined by using several concentrations of interferon. Monolayer cultures of L cells were exposed to various amounts of mouse interferon in 2 ml of GM for various periods at 37 C. Cultures were then washed three times and incubated for 1 hr at 37 C with MM virus, MOI 10. Inocula were removed; cultures were washed, refed with 3 ml of GM, and incubated for 24 hr, at which time the media were harvested for interferon assays (Fig. 1). Cells incubated with 100 units or more of interferon were fully primed by 2 hr, whereas those incubated with 10 units required at least 4 hr of pretreatment, and those incubated with 1 unit required at least 6 hr. Those cells treated with only 1 unit of interferon were not primed maximally, regardless of the time of exposure, and developed a state of priming which permitted only about 20% of the maximum yield of interferon brought about by larger doses of interferon. Also, with 24 hr of pretreatment, cells exposed to 100 units or more produced less than maximal responses. Results comparable to these have recently been found by Giron et al. (9).

Effect of multiplicity of inducing virus and the demonstration that the priming agent is interferon. Monolayer cultures of L cells were incubated overnight with either 10 units of mouse interferon in 2 ml of GM or GM alone. Cultures were then washed three times and incubated for 1 hr at 37 C with 0.5 ml of MM virus at MOI 0.1, 1, 10, or 100. Inocula were replaced with 3 ml of GM, and cultures were incubated for 24 hr at 37 C, at which time media were harvested and assayed for interferon. Little, if any, interferon was detectable

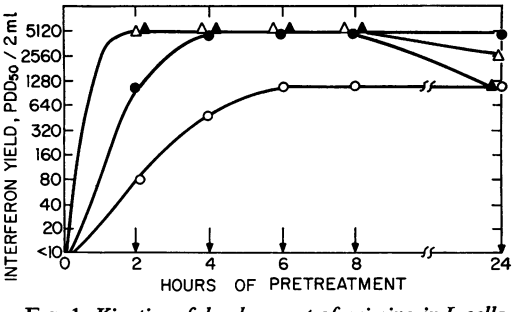

FIG. 1. *Kinetics of development of priming in L cells. Monolayer cultures of L cells were incubated with 1 (○), 10 (●), 100 (△), or 1,000 (▲) units of interferon, specific activity approximately 10^6 PDD_{50}-VSV/mg of protein, in 2 ml of GM for indicated periods. Cultures were then washed three times, infected with MM virus (MOI 10), refed with 3 ml of GM, and incubated for 24 hr at 37 C. Media were then harvested and assayed for interferon. Arrows indicate yields of interferon from cultures incubated with GM prior to infection with MM virus. Each point represents the average of two determinations.*

TABLE 1. *Interferon production in normal and interferon-treated L-cell cultures infected with MM virus*

Pretreatment[a]	MOI	Interferon yield[b]						
		1	2	3	4	5	6	7
None	0.1, 1, 10, 100	<10	90	30	<10	<10	<10	<10
Interferon	0.1	500	1,000	1,000	1,500	1,000		
	1.0	4,000	5,000	10,000	10,000	5,000		
	10.0	4,000	5,000	10,000	10,000	5,000		10,000
	100.0	4,000	5,000	10,000	10,000	5,000		
Interferon								
SA 10^4	10.0						4,000(1,000)[c]	
SA 10^5	10.0						4,000(1,000)	
SA 10^6	10.0						5,000(1,000)	
SA 10^7	10.0						4,000(1,000)	
Trypsinized interferon	10.0							<10

[a] Monolayer cultures of L cells were incubated overnight with 2 ml of GM or 10 units of mouse interferon in 2 ml of GM. These solutions were removed, and cultures were washed three times prior to inoculation with MM virus. Unless specified otherwise, interferon preparations had specific activities (SA) of approximately 10^6 PDD_{50}-VSV units (reciprocal of the dilution depressing plaque numbers of vesicular stomatitis virus by 50%)/mg of protein.

[b] After removal of inocula, cultures were washed and incubated for 24 hr with 3 ml of growth medium. Media were then harvested and assayed for interferon. Values represent PDD_{50}-VSV units/2 ml. In experiments 1, 2, and 3, crude MM virus inocula were used; in experiments 4, 5, 6, and 7, purified MM virus was used. In experiments 2 and 3, media from control cultures infected at multiplicity of infection (MOI) 1, 10, and 100 contained indicated interferon titers, whereas those infected at MOI 0.1 titered <10.

[c] Numbers in parentheses for experiment 6 indicate interferon yields from cultures which were incubated with 1 unit of specified interferon.

in culture fluids from normal MM virus-infected L cells at any MOI (Table 1). However, media from primed cultures contained significant titers of interferon. Similar titers resulted from virus multiplicities of 1, 10, or 100, whereas lower amounts of input virus resulted in less interferon. Since virus replication was inhibited, the lower yields of interferon at multiplicities of virus below 1 probably reflect the proportion of the population infected. Occasionally, interferon with titers between 30 and 90 units/2 ml was recovered from unprimed L cells when crude preparations of MM virus were used. However, MM virus which had been purified consistently failed to induce detectable levels of interferon. Even though they showed that enhanced interferon yields resulted from interferon pretreatment, Giron et al. (9) found that considerable amounts of interferon were produced in unprimed cells infected with MM virus. It seems possible that this induction in unprimed cells may be attributable to interferon in the MM virus preparations.

L-cell cultures were primed with interferon preparations having specific activities of approximately 10^4, 10^5, 10^6, and 10^7 PDD^{50}-VSV units/mg of protein. Notice that 1 and 10 units of interferon over the 1,000-fold range of purification primed cells to the same extent, respectively (Table 1). Also, interferon which had been inactivated, in terms of loss of antiviral activity, by incubation for 1 hr at 37 C with 0.25% trypsin also lost its priming activity. It is most likely, therefore, that the interferon molecules per se are responsible for priming.

Induction of interferon in primed L cells by infectious RNA from MM virus. Cultures that had been incubated with 10 units or more of interferon for at least 8 hr were inhibited in their virus-producing capacity greater than 99.9%, and these cultures displayed no cell destruction (Table 2). These cultures still produced interferon. This indicated that interferon induction occurred in protected cells in the absence of virus replication. However, it could be argued that the small amount of virus recovered from interferon-treated cells represented virus that was newly made, rather than merely residual inoculum, and that only this small amount of virus replication was

TABLE 2. *Induction of interferon by infectious ribonucleic acid (RNA) from MM virus in interferon-treated L cells*

Pretreatment[a]	MOI	Inducer[b]	Yields[c]	
			Interferon	Virus
Control	0.1	MM	<3	6×10^7
		RNA	<3	3×10^7
	1.0	MM	<3	8×10^7
		RNA	<3	2×10^7
Interferon	0.1	MM	100	2×10^3
		RNA	100	<10
	1.0	MM	800	6×10^3
		RNA	800	<10

[a] L-cell cultures were incubated for 18 hr with either 2 ml of growth medium (GM) or GM containing 100 units of mouse interferon. Cultures were then washed three times and inoculated with indicated inducer.

[b] Cultures were incubated for 30 min at 37 C with either 0.5 ml of diethylaminoethyl-dextran-LTM (0.14 M LiCl, 0.01 M tris(hydroxymethyl)-aminomethane, 0.001 M $MgCl_2$) buffer containing either 2×10^5 or 2×10^6 plaque-forming units (PFU) of MM virus or 2×10^5 or 2×10^6 PFU of infectious RNA from MM virus. Inocula were removed; cultures were washed twice, refed with 3 ml of GM, and incubated for 24 hr.

[c] Media and cells harvested at 24 hr postinfection were sonically treated and assayed for interferon and virus. Values represent average interferon titers in PDD_{50}-VSV (reciprocal of the dilution depressing plaque numbers of vesicular stomatitis virus by 50%)/2 ml from two experiments and virus yields in PFU/ml from a representative experiment.

needed to give interferon responses comparable to those induced by much larger amounts of virus replication. To rule out any virus replication, infectious RNA from MM virus was employed. Any virus recovered from these cultures would represent newly synthesized virions. L-cell cultures were incubated for 18 hr with 100 units of mouse interferon in 2 ml of GM. Cultures were then washed and inoculated with either purified MM virus, MOI 0.1 or 1.0, or the same MOI of infectious RNA extracted from purified MM virus. As seen in Table 2, neither infectious RNA from purified MM virus nor intact MM virus induced interferon in unprimed cells. However, primed cultures produced the same amount of interferon in response to both MM virus and infectious RNA from MM virus. The primed cultures produced interferon but no virus in response to infectious RNA. These data show that, if any, less than a complete replicative cycle is required for MM virus to induce interferon in primed L cells.

Actinomycin D sensitivity of interferon production in primed cells. The L cells employed in these studies require cellular RNA and protein synthesis to produce interferon in response to NDV or poly rI·poly rC (25). By using those stimulating materials, interferon production was sensitive to inhibition by actinomycin D until about 8 hr after induction. However, these and other strains of L cells, when induced with either NDV or poly rI·poly rC after having been treated with interferon, produce interferon earlier than normal L cells (15, 21, 22, 25), and these earlier primed responses become resistant to inhibition by actinomycin D by 4 hr after induction (25). It was, therefore, of interest to determine whether the interferon response induced by MM virus in primed L cells was an early response.

Cultures of L cells treated with 10 units of interferon for 6 hr to develop the maximally primed state were washed three times, inoculated with MM virus (MOI 10), and refed with 3 ml of GM. At 2-hr intervals, media were collected from duplicate cultures which were washed and refed with 3 ml of GM and incubated until the next interval. Interferon first became detectable in the medium about 4 hr postinoculation. When the kinetics of production was determined in cultures which were harvested at 2-hr intervals up to 14 hr after infection, the majority of the interferon was found to be produced in the final interval between 14 and 24 hr postinfection, during which time medium was left on the cultures (Fig. 2A). However, when cultures were first incubated for 14 hr postinoculation and then harvested for interferon at 2-hr intervals from 14 to 24 hr postinfection, virtually all of the interferon was found to be produced prior to 14 hr after infection (Fig. 2B). This phenomenon, suggesting a positive feedback requirement, has not been observed in these or other normal or interferon-treated cells induced with either NDV or poly rI·poly rC (25, 27). We are now studying it in greater detail. It was necessary, therefore, to determine the kinetics of interferon production by determining differential titers from cumulative titers obtained from medium incubated on duplicate cultures from time of infection to intervals indicated in Fig. 3. In primed L cells infected with MM virus, interferon was produced between 4 and 14 hr, as compared to a production course of 8 to 18 hr for this same strain of L cells inoculated with either NDV or poly rI·poly rC (25).

Cultures of L cells treated with 10 units of interferon for 6 hr to develop the maximally primed state were then washed three times, inoculated with MM virus (MOI 10) in GM or GM

containing 5 μg of actinomycin D per ml for 30 min at 37 C, and refed with 3 ml of GM. At times shown in Table 3, media were collected from duplicate cultures, which were washed and refed with 3 ml of GM containing 5 μg of actinomycin D per ml. This concentration of actinomycin D inhibited 95 to 99% of cellular RNA synthesis within 30 min, as determined by the use of radioactive uridine. The medium containing actinomycin D was removed after 30 min, and cultures were washed three times and refed with 3 ml of

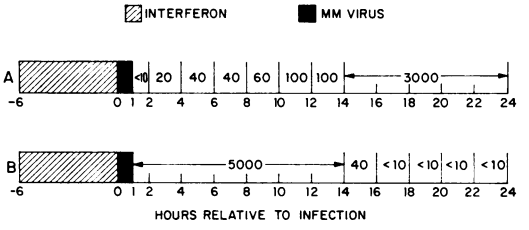

FIG. 2. *Amounts of interferon produced in interferon-treated L cells infected with MM virus during different intervals of time. L-cells cultures incubated for 6 hr with 10 units of interferon were washed three times, inoculated with MM virus (MOI 10), washed, and incubated with 3 ml of GM. At the indicated time intervals postinfection, medium was harvested for interferon assays. The cultures were washed and refed with 3 ml of GM. The numbers represent interferon units PDD_{50}-VSV/2 ml produced during the indicated interval of time.*

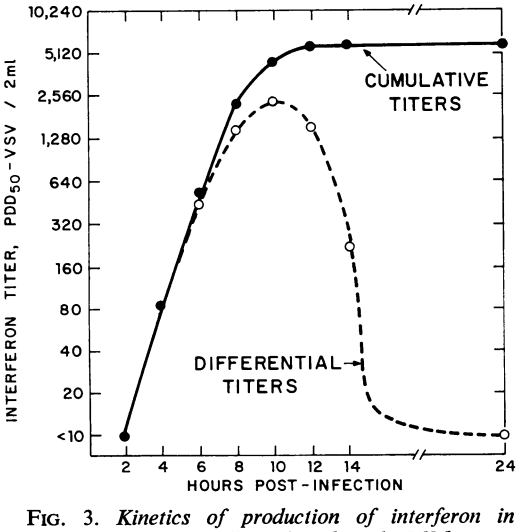

FIG. 3. *Kinetics of production of interferon in interferon-treated L cells infected with MM virus. Cultures of L cells treated with 10 units of interferon for 6 hr were washed three times, inoculated with MM virus (MOI 10), and incubated with 3 ml of GM. At indicated times, medium was harvested from duplicate cultures and assayed for interferon. Each point represents average from two separate determinations.*

TABLE 3. *Effect of actinomycin D on interferon production in interferon-treated L cells infected with MM virus*

Time of addition of actinomycin D[a]	Interferon yield (per cent control)[b]
0	0
2	0
4	45
5	90
6	95

[a] At indicated time, in hours, relative to addition of MM virus, culture fluids were harvested for interferon assays; 2 ml of growth medium containing 5 μg of actinomycin D per ml was added to duplicate cultures. Cultures were incubated for 30 min at 37 C, actinomycin D-containing medium was removed, and cultures were washed three times and incubated with 3 ml of growth medium. At 24 hr after inoculation of MM, medium was again harvested for interferon assay.

[b] Additive interferon yields determined as per cent of 24-hr interferon yields from cultures not treated with actinomycin D. Figures represent average per cent yield from two determinations.

GM. They were then incubated until 24 hr after infection, at which time the medium was again harvested, dialyzed, and assayed for interferon. The interferon response induced in primed L cells by MM virus became actinomycin D-resistant between 4 and 5 hr postinfection. It should perhaps be mentioned that normal, unprimed cultures similarly treated with actinomycin D after MM virus infection did not produce interferon. This suggests that the primed interferon response is induced more rapidly than that normally initiated by NDV or poly rI·poly rC in these same cells (25). With respect to the time at which interferon production became resistant to actinomycin D, the primed interferon response to MM virus appears to be similar to the earlier and primed response in L cells induced by NDV and poly rI·poly rC, but the latter gave reduced yields of interferon (25).

Lack of requirement for protein synthesis for the development of priming. L-cell cultures exposed to high concentrations of interferon became fully primed within 2 hr. However, cultures primed by this short exposure to interferon showed little, if any, reduction of virus replication. This suggested that priming was not a result of development of antiviral activity in the cells. Since cellular protein synthesis is required for cells to become resistant to viruses as a consequence of exposure to interferon (8, 13, 17), it was of interest to determine whether protein synthesis was also required to establish the primed state.

Monolayer cultures of L cells were incubated

for 2, 4, or 6 hr at 37 C with interferon (10 units/2 ml) or the same concentration of interferon in the presence of 200 μg of FPA per ml, 25 μg of cycloheximide per ml, or 50 μg of puromycin per ml. Cultures, along with appropriate control cultures, were then washed three times, infected with MM virus (MOI 10) and incubated for 24 hr with 3 ml of GM. The fluids were harvested for virus and interferon assays (Table 4). At the concentrations employed, cycloheximide and puromycin inhibited protein synthesis by greater than 95 and 80%, respectively, as determined by use of radioactive amino acids, and this inhibition was reversible after washing out the inhibitors. Cells incubated with interferon in the presence of the protein synthesis inhibitors became primed to the same extent as those exposed to interferon alone. Also, it is important to note that cells exposed to interferon in the presence of the protein synthesis inhibitors did not develop antiviral activity, whereas those exposed to interferon alone for longer than 2 hr developed some virus resistance as reflected by lower yields of virus. These data further support the interpretation that priming is a nonantiviral function of interferon.

The lack of the need for cellular protein synthesis for the priming activity to develop appears to be in direct opposition to the finding of (6) that chick embryo cells did not become primed to produce interferon in response to Chikungunya virus if they were exposed to interferon in the presence of FPA. Reasons for this apparent discrepancy are not clear but one cannot be certain that chick cells and L cells are similarly affected by their respective interferons.

Priming for interferon induction by a non-viral interferon inducer. The primed state seemed to sensitize the cells to be stimulated by an otherwise ineffective inducer (MM virus). Would cells in the primed state be sensitized in other ways? The earlier production of interferon has also been found to occur in interferon-treated cells induced with poly rI·poly rC (23, 25) and thus indicated that the primed state altered the cells response to a nonviral inducer.

We wondered whether primed cells would respond to a non-viral inducer insufficient to induce normal cells. We, therefore, exposed primed cells to an amount of poly rI·poly rC less than that necessary to induce interferon in unprimed L cells. Levels of poly rI·poly rC below those necessary to induce interferon production in normal L cells stimulated interferon production in primed L cells (Table 5). This interferon was detectable by 4 hr postinoculation. The normal interferon response induced by higher levels of poly rI·poly rC (2 to 25 μg) in this same strain of L cells does not become detectable until approximately 8 hr postinoculation (25). On the basis of the virus yield-reduction and protein synthesis inhibitor data, it could have been argued that interferon pretreatment could have inhibited a viral function that prevents interferon induction or production and not have lowered virus yields,

TABLE 4. *Effect of protein synthesis inhibitors on development of priming activity in L cells*

Pretreatment[a]	Length of pretreatment	Interferon production[b]	Virus yields[c]
None	2, 4, or 6 hr	<10	1.4×10^8
Cycloheximide	2, 4, or 6 hr	<10	1.5×10^8
p-Fluorophenylalanine (FPA)	4 hr	<10	1.5×10^8
Puromycin	4 hr	<10	1.4×10^8
Interferon	2 hr	600	1.8×10^8
Interferon + cycloheximide	2 hr	700	1.2×10^8
Interferon	4 hr	3,000	3.0×10^7
Interferon + cycloheximide	4 hr	3,000	1.6×10^8
Interferon + FPA	4 hr	3,000	1.4×10^8
Interferon + puromycin	4 hr	1,000	1.5×10^8
Interferon	6 hr	10,000	10^6
Interferon + cycloheximide	6 hr	10,000	1.8×10^8

[a] Monolayer cultures of L cells were incubated at 37 C for indicated intervals with 2 ml of growth medium (GM) or GM containing mouse interferon (10 units/2 ml), interferon plus 25 μg of cycloheximide per ml, interferon plus 200 μg of FPA per ml, interferon plus 50 μg of puromycin per ml, cycloheximide, FPA, or puromycin. After incubation for specified length of time, duplicate cultures were washed three times, infected with MM virus (multiplicity of infection 10), refed with 3 ml of GM, and incubated for 24 hr, at which time fluids were harvested for virus and interferon assays.

[b] Interferon titers = PDD_{50}-VSV units (reciprocal of the dilution depressing plaque numbers of vesicular stomatitis virus by 50%)/2 ml. Figures are average of three separate experiments.

[c] Virus yields in plaque-forming units per milliliter from a representative experiment.

TABLE 5. *Induction of interferon in interferon-treated L-cell cultures by subinducing doses of polyriboinosinic acid·polyribocytidilic acid*[a]

Pretreatment[a] (units of interferon)	Length of pretreatment (hr)	Interferon yield[b]
Control	2, 4, 8, or 16	<10
1	2	15
10		80
100		180
1	4	30
10		140
100		250
1	8	45
10		150
100		200
1	16	45
10		150
100		150

[a] Monolayer cultures of L cells were incubated for designated periods with either 2 ml of growth medium (GM) or indicated amounts of mouse interferon in 2 ml of GM. These solutions were removed and cultures were washed three times prior to inoculation with 1.0 ml of GM containing 0.5 μg of polyriboinosinic acid·polyribocytidilic acid and 100 μg of diethylaminoethyl-dextran. After cultures were incubated for 30 min at 37 C, inocula were removed, and plates were washed and refed with 3 ml of GM and incubated for 24 hr.

[b] Media harvested 24 hr postinoculation were assayed for interferon. Values represent average interferon titers in PDD_{50}-VSV units (reciprocal of the dilution depressing plaque numbers of vesicular stomatitis virus by 50%)/2 ml from two experiments.

a situation similar to the inhibition of viral RNA synthesis in the absence of inhibition of virus yields reported by Friedman (7). However, in view of these findings with poly rI·poly rC, it is unlikely that priming allows cells to produce interferon as a consequence of its inducing undetected antiviral activity.

Induction of interferon by other picornaviruses in normal and interferon-treated cells. L-cell cultures were incubated with 10 units of mouse interferon for 4 hr, washed and infected with MM virus, ME virus, mengovirus, human rhinovirus type 2, human rhinovirus type la, human rhinovirus type 14, human rhinovirus type 51, poliovirus type II, echovirus type 7, echovirus type 12, or ERV-P. Cultures were then refed with 3 ml of GM and incubated for 24 hr, at which time fluids were harvested for interferon assays (Table 6). None of these viruses induced demonstrable interferon in normal L cells, but several induced interferon in primed L cells. To determine the general ability of picornaviruses to induce interferon in primed or normal cells, we treated human embryonic lung cells with human interferon and LLC-MK_2 cells with monkey interferon. These cultures were then inoculated with MM virus, mengovirus, or poliovirus. The priming phenomenon was demonstrable in both of these cell types (Table 7).

Lack of priming activity of interferons on heterologous cells. Since development of antiviral activity is not required for cells to become primed by exposure to interferon, it seemed possible that interferons might be able to prime heterologous cells in which they induced no virus resistance. To determine this, human and monkey cells were exposed for 6 hr to mouse interferon, and L cells were incubated with chick or human interferon. Cultures were then washed three times, inoculated with MM virus (MOI 10), refed with 3 ml of GM, and incubated for 24 hr. Fluids were then harvested for interferon assays. No priming activity was observed in monkey or human cells which had been incubated with as much as 1,000 units of mouse interferon, and no priming activity was found in L cells incubated with up to 100 units of chick interferon or 100 units of human interferon. These results seem to be in agreement with those of Soloviev et al. (24), but in opposition to those of Levy-Koenig et al.

TABLE 6. *Interferon production in normal and interferon-treated L cells infected with several picornaviruses*[a]

Virus	Interferon response[b]	
	Control	Primed
MM	<3	12,500
ME	<3	15,000
Mengo	<3	1,500
Human rhino-1a	<3	1,750
Human rhino-2	<3	95
Equine rhino	<3	25
Polio II, human rhino-14, human rhino-51, echo 7, echo 12	<3	<3

[a] Monolayer cultures of L cells were incubated for 4 hr with either 2 ml of growth medium (GM) or GM containing 10 units of interferon. Cultures were then washed three times and infected with indicated virus at multiplicity of infection of 5 to 10, 1 hr at 37 C. Cultures were refed with 3 ml of GM and incubated for 24 hr; fluids were harvested for interferon assays.

[b] Values represent average titer in PDD_{50}-VSV units (reciprocal of the dilution depressing plaque numbers of vesicular stomatitis virus by 50%)/2 ml from two separate determinations for each virus.

TABLE 7. *Interferon production in normal and interferon-treated human embryonic lung cells and LLC-MK$_2$ rhesus monkey kidney cells infected with MM virus, mengovirus, or poliovirus type II*

Cells[a]	Virus	Interferon production[b]	
		Control	Primed
Human lung	MM	<3	500
	Mengo	<3	200
	Polio II	<3	100
Monkey kidney	MM	<3	100
	Mengo	<3	250
	Polio II	<3	50

[a] Monolayer cultures of the indicated cells were incubated with 10 units of homologous interferons in 2 ml of growth medium (GM) or with GM for 4 hr. Cultures were then washed three times, inoculated with indicated viruses at multiplicity of infection of 10, refed 3 ml of GM, and incubated for 24 hr. Fluids were then harvested for interferon assays.

[b] Values represent interferon units PDD_{50}-VSV (reciprocal of the dilution depressing plaque numbers of vesicular stomatitis virus by 50%)/2 ml.

(15), who reported that human interferon exhibited priming activity in L cells induced with NDV. However, they found this effect only when L cells were exposed to 400 units or more of human interferon. It appears, therefore, that if interferons are indeed able to prime heterologous cells, they are able to do so with a markedly reduced efficiency.

DISCUSSION

L cells must be primed to produce interferon in response to MM virus. The extent of this cellular modification is extremely dramatic. Since many picornaviruses are known to inhibit cellular RNA and protein synthesis (1), the most obvious explanation was that MM virus failed to induce interferon as a consequence of its ability to inhibit cellular synthesis but that this viral function was prevented by the antiviral activity induced by interferon. However, since full yields of interferon were produced by primed cells which exhibited no detectable or complete virus resistance and since cells were also primed to produce interferon in response to doses of poly rI·poly rC that did not induce interferon production in unprimed cells, interferon pretreatment seemingly enhances the sensitivity of the cells to inducing materials in some manner with no obvious relation to its antiviral activity. That this sensitization can come about in the absence of protein synthesis also seems to dissociate the primed state from the antiviral state.

Also, interferon-treated cells produce interferon earlier than normal cells regardless of the type of inducer employed (6, 14, 15, 21–23, 25). This and the data with actinomycin D suggests that the cells are altered so that the induction process proceeds more rapidly. It is as if the cells are "partially induced" by interferon pretreatment. A partial induction of a sequential induction process would explain both the earlier appearance of interferon and the ability of primed cells to respond to inducers that were insufficiently stimulatory to induce normal cells. The ability to skip one step of a sequential process would also serve to explain the observation. MM virus might be unable to induce an initial step in the sequence but able to induce a subsequent step, whereas interferon might be able to perform the initial step or permit the inducer to skip that step but not subsequent events. More work is required to determine the exact nature of the events.

It is also tempting to speculate that priming might play a role in the pathogenesis of viral infections. It would appear that certain viruses which induce only small amounts of interferon in their initial replicative cycle would produce considerably more in subsequent cycles of infection, since the interferon released during the first cycle would prime other cells to produce interferon earlier and in greater quantities. Goorha and Gifford (10) have recently reported a situation analogous to this in multiple-cycle in vitro infection of chick embryo cells with Semliki Forest virus, wherein a single-cycle infection induced small amounts of interferon but multiple-cycle infections gave considerably larger amounts of interferon.

Also, priming may have other uses. One of the main drawbacks to the application of the interferon inducers, poly rI·poly rC and others, has been the narrow margin between the dose producing desired antiviral effects and the dose causing toxic effects (5). Our data showing that interferon-treated cells are able to produce interferon in response to lower levels of poly rI·poly rC raise the question of whether administration of smaller doses of this inducer shortly after a priming dose of interferon might circumvent the problem of inducer toxicity.

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