Blocking of Interferon Production by Chromatographically Purified L Cell Interferon[1] (36981)

R. R. GOLGHER[2] AND K. PAUCKER
(Introduced by W. Henle)
Department of Microbiology, The Medical College of Pennsylvania, Philadelphia, Pennsylvania 19129

Exposure of cell cultures to interferon can modify their response to interferon inducers in two ways: Interferon may be liberated earlier or in larger amounts than in the absence of pretreatment (1–4), or the quantity of interferon produced is greatly diminished if not entirely blocked (1, 2, 5–7).

Studies conducted in the Newcastle disease virus–L cell system disclosed that the blocking property of interferon preparations was closely allied with their protective effect (7, 8), but preliminary experiments also suggested that interferon and blocker might be separate entities (9).

Recent developments in purification methods of L cell interferon (10) prompted a reinvestigation of the nature of a possible factor capable of blocking interferon production. The present experiments dwelt on the time of appearance, sensitivity to actinomycin D as well as attempts at chromatographic separation of blocking and interferon activities. In additional studies, chromatographically purified interferon was used for pretreatment of cultures to clarify some quantitative aspects of the blocking phenomenon as it pertains to live or irradiated viral and nonviral inducers.

Materials and Methods. Cell cultures. Suspended L cells were propagated as described earlier (11) with the following modification: The cells were grown in minimum essential medium (MEM) for suspension cultures, containing 10% calf serum, 2 m*M* L-glutamine and antibiotics. For attachment to glass, basal medium (Eagle) in Hanks' solution (BME), supplemented with 10% calf serum, 2 m*M* L-glutamine, 0.075% sodium bicarbonate and antibiotics, was substituted. The media used for growth of L(MCN) cells (12) and of FL human amnion cells (Flow Laboratories, Rockville, MD) were previously listed (10).

Viruses and infectivity tests. The conditions for propagation and ultraviolet irradiation of the Victoria strain of Newcastle disease virus (NDV), passage of the Indiana serotype of vesicular stomatitis virus (VSV) and viral infectivity assays were also detailed in an earlier report (10).

Poly I:C. Complexed riboinosinic–ribocytidylic acid polynucleotide (Miles Laboratories, Kankakee, IL) (poly I:C), and diethylaminoethyl (DEAE)-dextran (Pharmacia Fine Chemicals, Piscataway, NJ) were dissolved in 0.006 *M* phosphate buffered saline at pH 7.2 (PBS) and sterilized in a Millipore filter of 0.45 μm. Optimal concentrations for induction of interferon in L cells were 10 μg/ml of poly I:C admixed with 125 μg/ml of DEAE-dextran.

Production of interferon. L cells were induced by NDVuv and the interferon collected 18 hr later was concentrated and dialyzed at acid and neutral pH as outlined previously (10). Dialyses were carried out in the cold. Proteins were determined by the method of Lowry *et al.* (13) using crystalline bovine plasma albumin (BPA) as a standard. Control materials were prepared from noninduced cultures and subjected to the same regimen as interferon.

Chromatography. This procedure was carried out in a refrigerated column (Pharmacia

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[2] Fellow of the Rockefeller Foundation. Present address: Instituto de Ciencias Biologicas da UFMG, Caixa Postal 2486, Belo Horizonte, Brazil.

TABLE I. Blocking of Interferon Production in L Cells Pretreated with Exogenous Interferon Collected at Different Stages of Production.

Pretreatment with interferon[a]		Interferon induction[c]	
Collection (hr)	Units[b]	Titer/ml[d]	Percentage of control
6–8	2880	1920	8
10–12	3840	1920	8
16–18	2880	1920	8
None	—	23040	100

[a] Contact for 24 hr as described in the text.

[b] Expressed in NIH reference units.

[c] Cells were exposed to 500 PFU equivalents of NDVuv before inactivation.

[d] Determined on monolayers of L (MCN) cells by plaque reduction of vesicular stomatitis virus and corrected for reference standard.

K25/45) of carboxymethyl-Sephadex C-25. The column was equilibrated with 0.1 *M* phosphate buffer at pH 6, and the loading ratio was 1 mg of protein to 10 ml of gel volume. The flow rate was adjusted to approximately 60 ml/hr, and interferon was eluted in a rising pH gradient at constant molarity of the buffer. Fractions of 5 ml each were collected in a refrigerated cabinet and BPA (0.5% w/v) was added for stabilization. Specific activities of chromatographed interferon were in excess of 1×10^6 NIH reference units/mg protein (exclusive of the BPA added). Samples were sterilized under ultraviolet light for 2 min.

Assay of interferon. Potency of interferon was measured by reduction of VSV plaques on monolayers of L(MCN) cells as previously reported (10). The reciprocal of the highest dilution which reduced the number of plaques found in controls by half was taken as 1 unit. Internal and external standards accompanied each titration. Values were corrected and expressed in terms of the reference interferon supplied by the Research Reference Reagents Branch of NIH.

Blocking tests. To series of 150×16 mm silicone-stoppered test tubes containing $4–6 \times 10^6$ L cells in the form of a pellet, 1-ml amounts of interferon or appropriate control material were added, and the cells were suspended in this volume. The mixture was agitated for 1 hr at 37° on a rotary shaker (Arthur H. Thomas Co., Cat. No. 2995-G10) at 150 rpm. The volume was then made up to 10 ml by addition of growth medium, and the cultures were incubated for 25 hr on a roller drum revolving at 39 rpm. The cells were washed once in 10 ml Hanks' solution by centrifugation at 500*g* and the pellets were exposed to the various inducers.

One milliliter amounts of inducer at the concentrations specified in the text were added to individual tubes and the cells were kept in suspension on the rotary shaker as before. The adsorption time for NDV or NDVuv was 1 hr, and for poly I:C admixed with DEAE-dextran (14), 4 hr. At that time the cells were washed once again in 10 ml Hanks' solution, resuspended in the same amount of growth medium and returned to the roller for an additional 24 hr. Following removal of the cells by centrifugation, the supernates of virus-induced cultures were dialyzed for 3 days against HCl–KCl buffer at pH 2, then against PBS, prior to titration for interferon activity. Dialysis against low pH was omitted in the case of cultures stimulated by poly I:C.

To titrate blocking potency, selected interferon preparations were tested in serial dilutions against NDVuv at an input multiplicity of 300–500 PFU equivalents. In view of the variations encountered, a blocking effect was considered significant only when the titer of interferon produced in cultures pretreated with interferon was 25% or less than that obtained in controls.

Results. Blocking by nonpurified interferon preparations. Comparison of interferon liberated at varying times after induction. In the NDVuv–L cell system interferon was shown to increase in the medium at an exponential rate between 4 and 12 hr following contact with NDVuv before gradually declining (8). An attempt was made to see whether blocking and interferon activities accumulated in the culture fluids simultaneously or whether they appeared at different times.

Interferon was induced by NDVuv in roller bottle cultures of L cells as described. At 6, 10 and 16 hr following stimulation, the cultures were washed twice with Hanks' solution, refed with 10 ml of serum-free growth

TABLE II. Inhibition of Release of Blocking and Interferon Activities by Actinomycin D.

Pretreatment of cells[a]		Interferon induction	
Source of Interferon	Units[b]	Titer/ml[c]	Percentage of control
Act. D-exposed cultures[d]	2400	480	17
	240	1560	55
	24	2820	100
Normal cultures	2400	240	9
	240	960	34
None			
(Act. D control)[e]	—	2820	100
(medium control)	—	2820	100

[a] Contact for 24 hr as described in the text.

[b] Expressed in NIH reference units.

[c] Determined on monolayers of L (MCN) cells by plaque reduction of vesicular stomatitis virus and corrected for reference standard.

[d] Concentration 3 μg/ml for 1 hr. Interferon production was inhibited 95%.

[e] Control for residual actinomycin D after dialysis. Interferon was stimulated by NDVuv, using 300 PFU equivalents.

medium and interferon was collected during the ensuing 2-hr time intervals. The three 2-hr collections were thus representative of interferon liberated during early, intermediate and late intervals after induction. The titers were adjusted to comparable levels, and the 3 materials, as well as a corresponding control, were examined for the capacity to block stimulation of interferon by NDVuv. Table I shows that pretreatment of cultures with any of the preparations reduced the subsequent interferon response to the same degree. Other experiments not reported here disclosed that the blocking effect could be diluted similarly with all 3 materials and blocking was reversed to the same extent by live NDV (see section on reversal of blocking). It was, therefore, apparent that no marked differences in blocking activity as related to interferon titer existed in materials harvested during variable intervals after stimulation.

Inhibition of blocking and interferon activities by actinomycin D. Since viral stimulation of interferon was shown to require unimpaired DNA-dependent RNA synthesis, (15), an experiment was undertaken to determine whether partial inhibition of interferon formation by actinomycin D would result in a comparable reduction of blocking activity. A series of roller bottles was first exposed for 1 hr to 3 μg/ml of actinomycin D (Merck Sharp and Dohme, Rahway, NJ) and after exhaustive washing the cultures were inoculated with NDVuv. Controls included materials from cells where exposure to either actinomycin or virus was omitted. Under the conditions described, production of interferon in actinomycin D-treated cultures was reduced to 5% of that elicited in normal cells. After adjusting interferon titers in both types of preparations to comparable levels, serial 10-fold dilutions in a selected range were tested for blocking of interferon production by NDVuv. Regular and actinomycin-containing media subjected to all dialysis steps served as additional controls. The results presented in Table II show that the blocking effect was closely associated with the interferon content of these materials, and that treatment with the antibiotic affected interferon and blocking activities to a comparable degree.

Blocking by chromatographed interferon preparations. Attempted separation of interferon and blocking activities. Chromatography on carboxymethyl-Sephadex was previously shown to allow considerable purification of NDVuv-induced L cell interferon (10). Therefore, efforts were made to determine whether during chromatographic segregation of extraneous proteins, interferon and blocking activities could be separated. An interferon preparation was subjected to ion exchange chromatography. Elution profiles of optical density and of interferon are recorded in Fig. 1. The first 20–30 fractions collected after loading of the interferon and before initiation of the pH gradient, were rich in nonreactive proteins and contained only low levels of interferon. With rising pH, this ratio was reversed. Interferon eluted over a fairly broad range of the gradient, but more than ⅓ of the elutable activity was confined to 2 sharp bands corresponding to positions of pH 7.3 and 7.7, respectively.

Selected fractions, comprising both interferon-free as well as interferon-containing re-

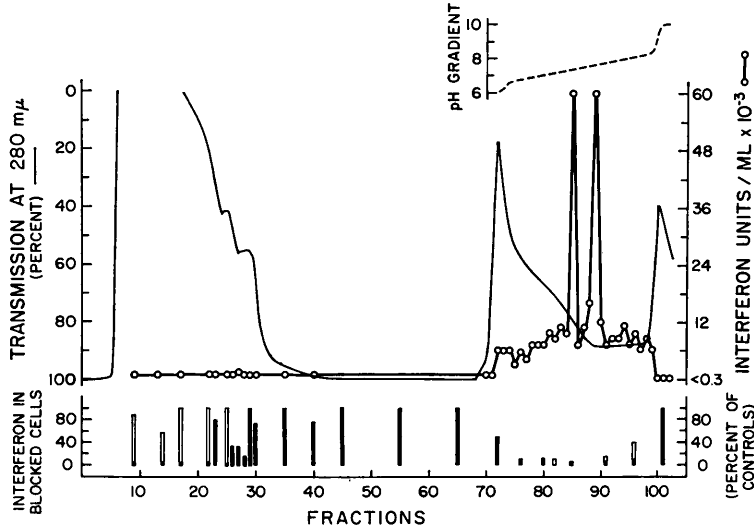

FIG. 1. Elution of interferon and blocking activities from carboxymethyl-Sephadex C-25. Total interferon activity recovered: 1.65 × 10^6 NIH reference units (about 30% of input). Specific activity of peak fractions: 1.2 × 10^6 units/mg protein (purified approx 60-fold). Blocking activities of individual fractions measured undiluted (closed bars) or in 1:10 dilution (open bars).

gions were then tested undiluted, and in some instances diluted 1:10, for blocking of interferon production by NDVuv. The titers obtained, listed as percentage of untreated controls, are included in the bottom portion of Fig. 1. With undiluted fractions (solid bars), a significant reduction of interferon titers was observed both in the initial (interferon-free) and terminal (interferon-positive) eluates, whereas the middle fractions were essentially devoid of any blocking effect. However, when relevant materials were diluted 1:10 (hollow bars), the blocking effect of early eluates had disappeared, whereas it was still marked in the interferon region. There was thus no indication from this and another similar experiment that an effective dissociation between blocking and interferon activities had occurred.

Comparison of blocking activities in crude and chromatographed interferon preparations. In a series of tests the blocking potencies of interferon preparations before and after chromatographic purification were quantitatively compared. Specific activities of initial materials ranged from 4.5 × 10^4 to 1.5 × 10^5 units/mg protein and from 1.3 to 3.6 × 10^6 units/mg protein for those subjected to ion exchange chromatography. Dilutions used for pretreatment of cells were adjusted to contain from 3000 to less than 1 unit of interferon/culture. A representative experiment is illustrated in Fig. 2. As shown, chromatographic purification did not diminish the blocking potency of interferon. In the case of both types of materials, the dose of interferon used for pretreatment had to be in more than 100- to 1000-fold excess of protective concentrations to elicit a marked inhibitory response to interferon induction by NDVuv. Blocking could not be clearly and reproducibly demonstrated with lesser amounts of interferon which behaved essentially like controls from noninduced cultures.

Reversal of blocking by NDV. Further studies were carried out to see whether blocking of interferon production was established with equal effectiveness against live and irradiated NDV. In the experiment presented in Fig. 3, cultures were exposed in the usual manner either to 3000 units of chromatographed interferon or to a corresponding control preparation. The cultures were subsequently infected with varying multiplicities of viable NDV as indicated in the figure and interferon titers were determined. It was found that the amount of interferon evoked in these cultures increased in direct propor-

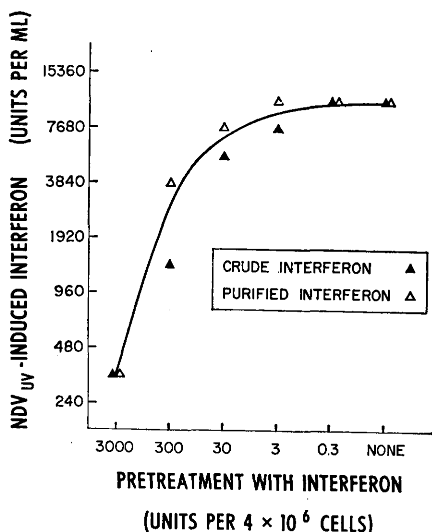

FIG. 2. Blocking potency of interferon before and after chromatographic purification. Sp. act, 4.5 × 10^4 and 1.3 × 10^6 units/mg protein for initial and final preparations, respectively. Pretreatment with interferon dilutions was for 24 hr before exposure to 300 PFU multiplicity equivalents of NDVuv. Crude and chromatographed media from noninduced cultures served as controls.

tion with the dose of NDV, ranging all the way from 4% to 50% of titers obtained in corresponding controls.

In another experiment, varying doses of NDV, before as well as after irradiation by ultraviolet light, were compared in their ability to reverse blocking established by pretreatment of the cells with chromatographically purified interferon. The results in Table III show that in two of the groups induction of interferon by NDVuv was reduced by more than 95%, but the effect was less pronounced with a suboptimal dose of inducer virus. In the case of live NDV, blocking was effectively manifested only against the lowest multiplicity of inducer virus. Larger doses partially overcame this effect.

Blocking of interferon induced by poly I:C. It was previously shown that pretreatment of L cells with chromatographically purified interferon resulted in a marked inhibition of interferon stimulated by poly I:C (16). In the following experiment an attempt was made to see whether an up to 25-fold increase in the optimal inducer concentration of poly I:C would result in a reversal of blocking similar to that observed with large doses of NDV.

Groups of cultures were inoculated with either 600 or 3000 units of a chromatographed interferon preparation or a corresponding control material. Following the requisite incubation, appropriate cultures were inoculated with varying amounts of poly I:C (admixed with DEAE-dextran) or with NDV as shown in Table IV. The results show that increasing concentrations of poly I:C failed to reverse the blocking state provoked by either dose of interferon used for pretreatment of the cultures. When 3000 units were employed to block the cells, the marginal dose of NDV was likewise inadequate to overcome this effect. However, blocking established by a 5-fold lesser amount of interferon was completely reversed by NDV under conditions where interferon induction by poly I:C remained inhibited.

Discussion. The blocking activity residing in crude L cell interferon preparations was previously shown to conform to a variety of criteria used to distinguish interferon from other viral inhibitors (17). On the basis of sedimentation behavior and stability at low pH (7), interaction with enzymes (4), with antiviral (7) and anti-interferon sera (9), as well as of host specificity (8, 9), the block-

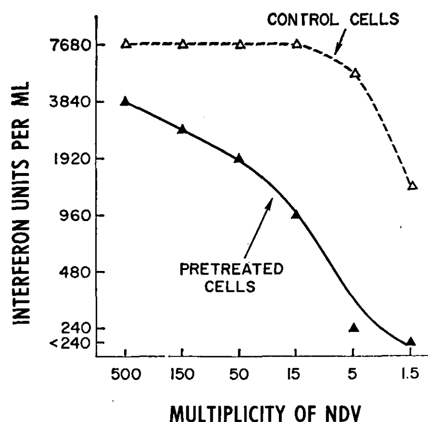

FIG. 3. Reversal of blocking effect by viable Newcastle disease virus. Cultures of 4 × 10^6 cells were pretreated for 24 hr with 3000 units of chromatographed interferon (2.8 × 10^6 units/mg protein) or corresponding material from noninduced cultures (control cells).

TABLE III. Reversal of Blocked Interferon Production by Live and Ultraviolet-Irradiated Newcastle Disease Virus.

Pretreatment with interferon[a]	Induction of interferon				
	Multiplicity of inducer[b]	NDV (titer/ml)[c]	%	NDVuv (titer/ml)[c]	%
+	5	240	6	180	19
+	50	1440	38	480	2
+	500	1920	50	480	3
—	5	3840	100	960	100
—	50	3840	100	23040	100
—	500	3840	100	15360	100

[a] Contact with 6000 NIH reference units of chromatographed interferon (sp act, 2.8×10^6 units/mg protein) for 24 hr as described in the text.

[b] Expressed as PFU equivalents before (NDV) or after ultraviolet irradiation (NDVuv).

[c] Determined on monolayers of L (MCN) cells by plaque reduction of vesicular stomatitis virus and corrected for reference standard.

ing property was provisionally attributed to interferon directly. To this list can now be added the requirement for cellular RNA synthesis since the liberation of both blocking and interferon activities was similarly inhibited by actinomycin D. The observation that both properties accumulate in comparable amounts in interferon materials collected during early, intermediate and late stages of release from induced cells, lends further, albeit circumstantial, evidence to a common identity. However, according to recent reports (18, 19) blocking and interferon effects were found to appear at different times *in vitro* as well as *in vivo* although the variations in blocking/interferon ratios cited may be considered as marginal.

The observations that antiviral protection developed much earlier than refractoriness (8), that responsiveness to interferon induction was regained sooner than susceptibility to viral infection (20) and that partially purified interferon preparations appeared to contain less blocking power than the initial material (9) hinted at a possible dissociation between interferon and blocking effects. However, the data presented in this report failed to support our earlier findings. In the course of approximately 60-fold chromatographic purification, the examination of individual eluate fractions in selected regions, covering a wide range of pH, gave no evidence that a clear separation of interferon and blocking effects could be achieved. Eluates of elevated specific activities (3.6×10^6 units/mg protein) contained as much blocking potency per unit of interferon as the initial nonpurified product. Attempts by others to achieve separation of interferon from blocker by electrophoresis (21) and chromatography (22) were similarly unsuccessful. However, in the absence of further studies with more extensively purified interferons, the existence in the NDV–L cell system of a blocker separate from interferon cannot be definitively ruled out. Whatever its nature may turn out to be, such a blocking substance would appear to differ from interferon depressors found in chick embryos infected with influenza, Newcastle disease, fowl plague (23) or Sendai viruses (24) as well as in normal allantoic fluid, heparin and capsular polysaccharide of *Klebsiella pneumoniae* (25), all of which could be readily distinguished from chick interferon by chemical and physical characteristics.

A close link between blocking and antiviral properties of interferon is further supported by studies with selected lines of mouse cells transformed by murine sarcoma virus, which vary in their sensitivity to interferon (26, 27), by analysis of dose–response relationships of antiviral protection and blocking in interferon-treated L cells (22) and the present findings that large amounts of infectious NDV may reverse the refractory state.

The response of cells to the antiviral effect of interferon ranges from complete suppres-

TABLE IV. Failure of Poly I:C to Reverse Blocking of Interferon Production.

Pretreatment with interferon[a]	Induction of interferon			
	Poly I:C[b] (μg/ml)	NDV multiplicity	Titer/ml[c]	Percentage of control
600	10	—	480	22
	50	—	300	16
	250	—	180	9
	—	30	3840	100
3000	10	—	360	17
	50	—	180	9
	250	—	120	6
	—	30	300	8
None	10	—	2160	100
	50	—	1920	100
	250	—	1920	100
	—	30	3840	100

[a] Contact for 24 hr as described in the text (interferon sp act, 3.6×10^6 NIH reference units/mg protein).

[b] Admixed with DEAE-dextran, 125 μg/ml.

[c] Determined on monolayers of L (MCN) cells by plaque reduction of vesicular stomatitis virus and corrected for reference standard.

sion of infection to diminished yields of virus per cell (28). Similarly, blocking of interferon production can be variable depending on the intensity of interferon treatment of the cultures (8, 9). With massive doses of interferon (1×10^{-3} units/cell applied for 24 hr) refractoriness is complete against NDVuv, poly I:C, as well as moderate amounts of infective NDV (16, 21, 22). Cells exposed to 10-fold lesser amounts of interferon remain refractory to stimulation by NDVuv and poly I:C but not by NDV. At that level of interferon pretreatment, inducibility of the cultures was not reinstated by excessive doses of NDVuv nor by up to 25-fold increases in optimal inducer concentration of poly I:C. Failure of others to observe reversal of blocking by NDV (22) may have been due to the limited range of virus multiplicities tested. However, overcoming of the blocking effect was shown to occur in interferon-treated chick cells exposed to elevated doses of heated Chikungunya virus (2), and in interferon-treated L cells stimulated subsequently by large amounts of NDV (29). Reversal of blocking appears, therefore, to involve both large concentrations of virions and full or partial integrity of the viral genome. On the other hand, NDVuv which does not initiate viral RNA synthesis (30), may not provide sufficient input nucleic acid to reverse the refractory state. The same reason may account for the inability of poly I:C, at greatly increased concentrations, to generate interferon in blocked cells even though, unlike in other studies (31), the level of 250 μg/ml employed displayed undiminished interferon-inducing potency in normal L cells.

Although treatment of L cells with interferon was previously shown to prevent stimulation of interferon by poly I:C as well as NDV or NDVuv (16, 21, 22), the control of interferon production by viral and nonviral inducers appears to be more complex in primary cultures of mouse (21), rabbit (32) and human derivation (33) as well as in another established line of L fibroblasts (29).

Summary. Pretreatment of L cells with mouse interferon diminishes their responsiveness to interferon induction (blocking effect). The blocking property of interferon preparations is liberated simultaneously with interferon from stimulated cultures. Both activities are reduced to the same degree by partially inhibitory concentrations of actinomycin D. Chromatographic purification of interferon (specific act, 1–3 $\times$ 10^6 units/mg protein), fails to dissociate a separate blocking

component from interferon. Nonpurified and chromatographed interferon preparations display the same blocking potency in relation to interferon content. Blocking can be partially or completely reversed by increasing the multiplicity of infectious NDV. Under the same conditions, reversal of blocking does not occur with excessive inducer concentrations of NDVuv and poly I:C.

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