Interferon Antagonists Induced by Newcastle Disease Virus (NDV) (37003)

B. GALLIOT, M. C. MOREAU, N. RENARD, AND C. CHANY

Unité de Recherche sur les infections virales, Unité 43, Institut National de la Santé et de la Recherche Médicale, Hôpital St. Vincent de Paul, 74, Avenue Denfert Rochereau, Paris XIV°, France

Interferon (IF) is probably a major defense system against viral infection at the cellular level. IF production and action are subject to complicated regulatory mechanisms (1).

We have previously shown that the antiviral state induced by IF can be affected by a tissue antagonist (TAI) (2) present in normal tissues. TAI decreases the established antiviral state induced by IF, but requires the integrity of cellular protein synthesis.

In addition, as also reported previously, a great variety of unrelated viruses might induce metabolic changes in the cells which result in impaired IF production and decreased IF sensitivity (3–7). Thus, SV40, adenovirus, or MSV decreased IF sensitivity in nonpermissive cells. These data seem to indicate that the decrease of the antiviral state is due to an event related to the penetration, decapsidation of these viruses in the cell, but seem to be unrelated to their replication. Indirect evidence supports the hypothesis that a viral induced protein is responsible for the decrease of the antiviral state. That protein has been tentatively termed stimulon.

In this report, we study a similar factor present in egg allantoic fluids infected with the Herts 33 strain of Newcastle disease virus (NDV). Some of these data have been presented in preliminary reports (8, 9).

Materials and Methods. Tissue culture and medium. For cell growth, Eagle medium (MEM 0111) supplemented with tryptose phosphate broth (2.95 g/liter) and 10% heat-inactivated calf serum was employed. For maintenance of the cells the serum concentration in the medium was decreased to 2%. Mouse L cells, and the African green monkey cells, line BSC, were routinely maintained in the laboratory.

Viruses. The Indiana strain of vesicular stomatitis virus (VSV) was propagated in L cells. Infectivity assays were performed by routine plaque titration.

The Newcastle disease virus (NDV) employed were: the Hertsfordshire and the Herts 33 strains, received through the courtesy of Dr. Béla Lomniczy. Stock virus was prepared in 9-day-old embryonated chicken leghorn eggs and titered by the 50% egg infectious dose method (EID_{50}).

Interferon (IF) preparation. Human white blood cell (WBC) IF. WBC IF was prepared as described by Gresser and modified by Falcoff, Fournier and Chany (10). Human amniotic membrane (HAM) IF was prepared as described by Fournier, Falcoff and Chany (11). In both cases either the Hertsfordshire strain or the Herts 33 strain of NDV was employed.

Mouse IF. Mouse IF was obtained by infecting MSV-IF+ cells with the Hertsfordshire strain of NDV as previously described (12). These preparations were purified by one passage through a Sephadex G75 column.

IF titration. 1. By CPE inhibition. Serial twofold dilutions of interferon were incubated with L cells grown in vertical hemolysis tubes. After 18 hr of incubation the cells were washed and infected with VSV, m.o.i. $=1$. The titer of interferon preparation was evaluated by the dilution which inhibited approximately 50% of the cytopathic effect (CPE) in the cell population.

2. By viral yield inhibition. Serial twofold dilutions of interferon were incubated with

TABLE I. L Cells Were Incubated for 18 hr with Diluted IF 1:2, with HAM Stimulon, or Medium (a), or with Supernatant of Uninfected HAM (b).[a]

L cells	Expt:	VSV titer (PFU/0.5 ml) on L cells 1	2	3
a. IF 20 units				
+ HAM stimulon		7.2×10^5	1.4×10^7	2×10^6
+ medium		3.2×10^5	3.8×10^6	5×10^5
VSV challenge virus		1.6×10^7	1.2×10^8	1.3×10^7
b. IF 20 units				
+ supernate of uninfected HAM		4.7×10^5	5.5×10^5	
+ medium		3×10^5	5×10^5	
VSV challenge virus		4×10^7	3×10^7	

[a] Then the cells were washed and challenged with VSV. Viral yield was measured at the end of the second replicative cycle.

L cells for 18 hr. The L cells were then washed and infected with VSV, m.o.i. = 1, and incubated at 37° for 16 hr. The viral yield was determined by plaque titration and compared to suitable control cultures.

For the assay of mouse IF, L cells were employed, while BSC cells were used for human interferons.

Preparation of NDV-induced antagonists (stimulon). Nine-day-old embryonated leghorn eggs were infected with 0.1 ml of the Herts 33 strain of NDV containing 10^5 EID_{50}. After a 48 hr incubation period at 36.5° the eggs were transferred for 12 hr at 4°. The separation of the virus from stimulon in the allantoic fluid was obtained by successive serial filtrations through 0.45; 0.22; and 0.10/μm Millipore filters. The filtrate was then concentrated 10-fold by pressure dialysis and centrifuged in a Spinco L1 preparative ultracentrifuge rotor 30 (27,000/rpm for 18 hr). The supernatant was tested in 9-day-old embryonated eggs and did not contain any infectious virus. Mock preparations were obtained with the same technique using uninfected allantoic fluids from the same lot of eggs.

Antagonist preparations from the WBC or human amniotic membrane were obtained with the same techniques as IF preparations. However, in most instances, the amniotic membrane infected with the Herts strain of NDV did not induce IF in detectable amounts.

Results. The starting point of these investigations was the observation that many IF preparations obtained either in the human amniotic membrane (HAM) or in WBC infected with the Herts strain of NDV also contained a virus-induced antagonist of the antiviral action of IF, which we previously termed stimulon. No such activity was detected when the Hertsfordshire strain was used, either in WBC or in the amniotic membrane. The existence of stimulon was demonstrated as follows:

Murine IF (20 units) and undiluted amniotic human IF (containing stimulon) preparations were mixed in a 1:2 proportion. In controls, murine IF was mixed with tissue culture fluids from uninfected human amniotic membranes or with normal medium. The mixture was incubated with L cells for 18 hr at 37°, washed and challenged with VSV (m.o.i. = 1). The viral yield was titered after an incubation period of 16 hr at 37°. The results summarized in Table Ia show that the human amniotic stimulon decreased the antiviral state induced by the murine IF in L cells. Stimulon, unlike IF, was not cell species specific. Table Ib shows that fluid from uninfected HAM had not a similar effect.

Further experiments performed as follows showed that the stimulon contained in the IF preparation decreased the effect of the antiviral protein induced by IF in heterologous L cells. The L cells were treated with 20 units of purified IF for 4 hr at 37°. Then the IF

TABLE II. L Cells Were Treated for 4 hr with IF.[a]

L cells	Expt:	VSV titer (PFU/0.5 ml) on L cells 1	2
a. IF 20 units			
+ HAM stimulon		4.5×10^6	9.6×10^6
+ medium		3.7×10^4	3×10^5
Vsv challenge virus		6×10^7	2.8×10^7
b. IF 20 units			
+ WBC stimulon		2.6×10^6	3.7×10^6
+ medium		3×10^5	5×10^5
VSV challenge virus		4×10^7	4×10^7

[a] IF was then removed and HAM stimulon (a) or WBC stimulon (b) was added for 18 hr. In control preparations the stimulon was replaced by medium. The cells were then washed and challenged with VSV.

was removed and stimulon or control medium was added for a further 18 hr incubation period at 37°. The cells were then washed and infected with VSV (m.o.i. = 1). After 1 hr adsorption the unadsorbed virus was removed and the preparations incubated for a further 16 hr. Then the cells were frozen at —80° before testing. The yield of the challenge VSV was measured by plaque titration in L cells.

Similar experiments were performed with stimulon preparations obtained from WBC as described in Materials and Methods. The results shown in Table IIa and b indicate that the antiviral state induced by IF in L cells was significantly reduced by stimulon preparations from HAM and WBC.

In allantoic fluids of embryonated eggs infected with the Herts 33 strain, no chick IF was found in detectable amounts, when tested in suitable assay systems. Stimulon was, however, produced. Stimulon could be easily separated from the inducing virus after serial filtration on Millipore membranes as described in Materials and Methods, and assayed in L cells (Table III). Because of the simplicity of the method and the possibility of producing large quantities of crude material, stimulon induction in embryonated eggs was studied in further experiments.

Lack of direct effect of NDV stimulon on IF. NDV stimulon and different concentrations of mouse IF were mixed 1:2 and incubated either at 37 or at 4° for 1 hr. The mixture was then inoculated in L cells for 4 hr and the medium was removed, and the cells were challenged with VSV. There was no direct effect of stimulon either on IF itself or on the establishment of the antiviral state (Table IV).

Effect of uv irradiation of NDV on stimulon production. Two series of 9-day-old-embryonated eggs were treated either with the

TABLE III. L Cells Were Treated for 4 hr with IF.[a]

Expt	L cells	VSV titer (PFU/0.5 ml) on L cells: Cells with IF	Cells without IF
1	NDV stimulon	2.8×10^5	3.5×10^7
	Control allantoic fluid	9×10^4	3.6×10^7
	Medium	3.8×10^4	3.8×10^7
2	NDV stimulon	1.7×10^6	2×10^7
	Control allantoic fluid	2×10^5	1.2×10^7
	Medium	1.2×10^5	2×10^7
3	NDV stimulon	2.6×10^5	2×10^7
	Control allantoic fluid	7×10^4	1.8×10^7
	Medium	6.8×10^4	2.1×10^7
4	NDV stimulon	10^5	3.3×10^7
	Control allantoic fluid	3.8×10^4	3.2×10^7
	Medium	1.5×10^4	4.7×10^7

[a] After the removal of IF the cells were treated with different egg allantoic fluid preparations, as described in Materials and Methods, for 18 hr and then challenged with VSV.

TABLE IV. Effect of a Constant Amount of NDV Stimulon on the Antiviral State Induced by Variable Amounts of IF.[a]

Murine interferon (units/ml)	VSV titer (PFU/0.5 ml on L cells)		
	Stimulon	Control allantoic fluid	Medium
0	ND[b]	ND	3.3×10^7
8	5.4×10^6	4.3×10^6	7.8×10^6
16	1.3×10^6	1.1×10^6	2.3×10^6
32	2.5×10^5	3.4×10^5	2.4×10^5

[a] IF and stimulon were mixed 1:2 and incubated at 37° for 1 hr. The mixture was then added to L cells for 4 hr. The mixture was then removed and the cells were challenged with VSV.

[b] ND = not done.

infectious Herts 33 strain or with uv irradiated virus (20,000 ergs/mm^2). This amount of irradiation inactivated completely the infectivity of NDV. Control allantoic fluid was either used as such or inactivated under similar conditions. Figure 1 illustrates the dose–response relationship of serial dilutions of these different preparations in the presence of a constant amount of murine IF (20 units). Ultraviolet irradiation of the virus completely abolished its capacity to induce stimulon in the egg allantoic fluid. In control preparation, a low interferon antagonist activity was observed, uneffected by uv irradiation.

General physicochemical properties of NDV-induced stimulon. As shown in Table V, heating at 56° for 1 hr did not significantly affect the anti-interferon activity of stimulon.

When frozen at —20 or —80°, the biological activity was maintained unchanged for several months.

The active product did not diffuse after pressure dialysis through membranes of a porosity of 48 Å.

When centrifuged for 18 hr in a Spinco preparative ultracentrifuge, rotor 30, at 27, 000 rpm (90,000*g* for 18 hr), all the activity was recovered in the supernatant.

When NDV stimulon or control allantoic fluid were incubated for 1 hr at 37° with trypsin at a final concentration of 250 units/ml a slight inhibition of activity occurred in some 10 subsequent experiments performed (unpublished data).

Discussion and Summary. NDV induces in human, mouse and chick cells the synthesis of a substance termed stimulon which decreases the antiviral state induced by interferon. Similar substances were previously described in cells infected with a number of oncogenic and nononcogenic viruses (13), and also in L cells infected with NDV. These substances have to be distinguished from tissue antagonists of interferon (TAI) present in normal tissues. Stimulon has no direct effect on interferon or on viruses. Both stimulon and TAI affect, however, the antiviral state after its establishment. Experiments using antimetabolites (14–16) and somatic monkey–mouse hybrid cells (17) have established that IF is not directly responsible for the antiviral state,

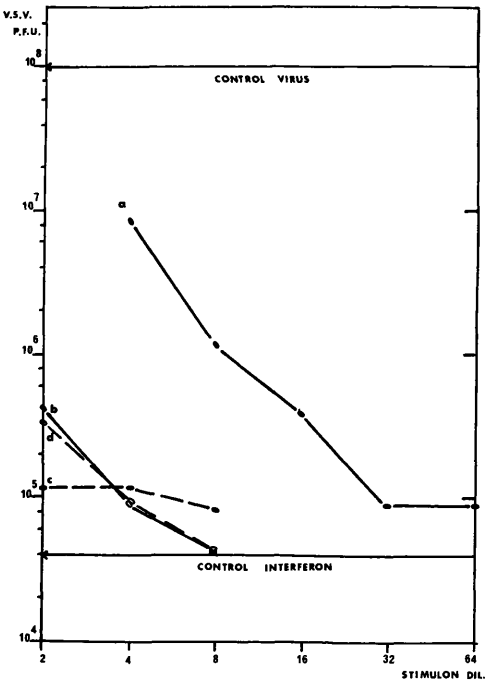

FIG. 1. Effect of uv treatment on stimulon induction in allantoic fluid. Dose–response relationship in the presence of a constant amount of IF. (a) Allantoic fluid from infectious NDV; (b) allantoic fluid from uninfected eggs; (c) allantoic fluid from eggs infected with uv-inactivated NDV; (d) uv irradiated control allantoic fluid.

TABLE V. Effect of Heating at 56° for 1 hr on Activity of NDV Stimulon.[a]

Expt	L cells	VSV titer (PFU/0.5 ml) on L cells	
		Cells with IF	Cells without IF
1	NDV stimulon	2.6×10^6	3.3×10^7
	NDV stimulon 56°	2×10^6	3.8×10^7
	Control allantoic fluid	4×10^5	2×10^7
	Control allantoic fluid 56°	1.5×10^5	3.2×10^7
	Medium	7.2×10^4	4×10^7
2	NDV stimulon	2×10^6	2×10^7
	NDV stimulon 56°	1.6×10^5	2×10^7
	Control allantoic fluid	5×10^4	1.7×10^7
	Control allantoic fluid 56°	7.2×10^4	2.2×10^7
	Medium	6.8×10^4	2.1×10^7

[a] L cells were treated for 4 hr with IF. After the removal of IF the cells were treated with different egg allantoic fluid preparations for 18 hr and then challenged with VSV.

but acts through a second protein. Both stimulon and TAI act on the antiviral activity of this second protein. Many of the basic physicochemical properties of the substance described here resemble a glycoprotein rather than a protein. The antiinterferon activity induced by NDV in this system could be related to breakdown cellular products, to virus induced proteins, or to a mixture of the two. Since the viral envelope could contain cellular and viral antigens, it seems possible that at least some of the TAI and stimulon, or both, are integrated in the envelope of the virion. This problem is under current investigation.

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Received Sept. 7, 1972. P.S.E.B.M., 1973, Vol. 142.