Biochemical Analysis of Mutants of a Macrophage Cell Line Resistant to the Growth-inhibitory Activity of Interferon

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ABSTRACT While a multiplicity of cellular and biochemical effects are mediated by inteferons on cultured cells, the mechanisms involved in the direct growth-inhibitory activity of interferons remain problematic. We have previously found that variants in cAMP metabolism in a macrophage cell line, J774.2, were at least 50-fold less sensitive to the growth inhibitory activity of interferons (IFN) than the parental clone. To test the hypothesis that cAMP mediates the growth inhibition produced by IFN in these cells, interferon-resistant variants were selected and characterized with respect to cAMP synthesis and function. Approximately one-third of the IFN-resistant clones were found to be resistant to growth inhibition produced by cholera toxin, but not 8Br-cAMP. IFN was fully able to protect all of the interferon-resistant/cholera-toxin-resistant (IFN^r/CT^r) clones against infection by vesicular stomatitis virus and markedly stimulated 2′, 5′-oligodenylate synthetase activity. These IFN^r/CT^r variants were shown to have a defect in adenylate cyclase. The remaining IFN-resistant clones were fully susceptible to the growth-inhibitory effects of cholera toxin because their basal and stimulated adenylate cyclase activity is similar to that of the parental clone. IFN failed to protect these IFN^r/cholera-toxin sensitive clones against infection by vesicular stomatitis virus and failed to stimulate 2′, 5-oligodenylate synthetase, suggesting that they have defective or deficient IFN receptors. In addition, IFN failed to increase intracellular cAMP levels in both IFN^r/CT^r and IFN^r/cholera-toxin sensitive clones. These results provide firm genetic and biochemical evidence that the growth inhibitory effects of IFN on this cell line are mediated by cAMP.

Interferons (IFN)[1] exert a variety of biological effects in addition to their antiviral activity. They inhibit cell growth of primary and transformed cells (1–4), modify thymidine transport (5), induce the synthesis of specific enzymes, such as 2′,5′-oligoadenylate synthetase (6) and a cAMP-independent protein kinase (7), alter cytoskeletal and morphological properties of cells (8), and increase intracellular levels of cAMP (9). In studies of the effects of IFN on cells of the immune system, we and others have observed an increase in Fc receptor mediated phagocytosis in macrophages or macrophage-like cell lines, similar to that induced by treatment with cAMP (10–12). Upon exposure to IFN the intracellular concentration of cAMP increased concomitantly with phagocytosis. Further IFN failed to augment Fc receptor-mediated phagocytosis in adenylate cyclase and cAMP-dependent protein kinase variants selected from a macrophage-like cell line, J774.2, although it conferred full antiviral protection (12). These variants in cAMP synthesis and function in J774.2 were also resistant to IFN-mediated growth inhibition. If cAMP mediates the growth-inhibitory effect of IFN on cells, then one would predict that among clones selected for resistance to the growth inhibitory effects of IFN variants in adenylate cyclase or cAMP-dependent protein kinase activities would be found. Confirmation of this prediction is herein presented.

[1] *Abbreviations used in this paper:* CT^r, cholera-toxin resistant clones; CT^s, cholera-toxin sensitive clones; GppNHp, guanyl-5′-imidodiphosphate; IFN, interferons; IFN^r, interferon-resistant clones; VSV, vesicular stomatitis virus.

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MATERIALS AND METHODS

Cells: The cloned cell line J774.2 was originally established from mouse reticulum cell sarcoma derived by P. Ralph et al. (13) and shown to have a macrophage like phenotype (14, 15). Cells were maintained in Dulbecco's modified Eagle's medium (DME, Gibco Laboratories, Grand Island, NY) supplemented with 20% heat-inactivated horse serum (Gibco Laboratories), 10% NCTC 109 medium (Microbiological Associates, Walkersville, MD), 0.1 mM nonessential amino acids (NEAA, Gibco Laboratories), 1 mM sodium pyruvate, penicillin, and streptomycin at 37°C in a humid incubator containing 5% CO_2.

The mouse hepatoma cell line, E_3, was incubated in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco Laboratories), 5% NCTC, and 1% NEAA and trypsinized once a week. Primary rat embryo fibroblasts (REF) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco Laboratories).

Interferon: Mouse IFN (IFN $\alpha + \beta$, specific activity 1.3×10^7 IU/mg protein) was purchased from Lee Biomolecular Research Laboratories (San Diego, CA) and IFN α from Enzo Biochemicals (New York, NY). The specific activities for antiviral activity of these preparations established in this laboratory were 6.5×10^7 U/mg and 5×10^8 U/mg, respectively assayed by inhibition of cytopathic effects of vesicular stomatitis virus (VSV) on L cells (16).

Vesicular Stomatitis Virus: The Indiana (HRC) serotype of VSV was prepared in chicken embryo fibroblasts, clarified by centrifugation (1,000 *g*, 10 min), and frozen at -70°C. This stock contained 5×10^7 pfu/ml of VSV. For use, this stock was diluted in Dulbecco's modified Eagle's medium with 2% fetal calf serum.

Selection of IFN-resistant Variants: Cells were cloned in soft agar in 60-mm tissue culture dishes (Falcon A 3002; Falcon Labware, Oxnard, CA) above E_3 or rat embryo fibroblasts feeder layers (14, 17). A solution of 0.5% agarose (wt/vol) (Sea Plaque, Marine Celloids, Inc., Rockland, MD) in macrophage growth medium was layered onto the feeder layer and solidified at 4°C for 10 min. The cells to be cloned (2,000 cells/plate) were suspended in 1 ml of 0.43% agarose in medium. The dishes were again cooled at 4°C for 10 min and then placed in a 37°C incubator.

For selection of IFN-resistant variants, each independently derived clone was recloned 4–5 times in increasing concentrations of IFN, ranging from 500 U/ml to 5,000 U/ml over 6 mo period. All variants remained stable when grown for 3 mo in the absence of IFN.

Growth Curves: Cells to be tested ($0.5–1 \times 10^4$ were seeded in 1 ml of medium in 2.0 cm^2 tissue culture wells (Linbro) in the presence or absence of IFN, 8Br-cAMP, or cholera toxin (Schwarz Mann, Orangeburg, NY). At appropriate times, cells were harvested and cells excluding trypan blue were counted. On day 3 each well was fed with 0.5 ml of media containing the appropriate drug.

Preparation of Cell Membrane Extracts: Plasma membranes were prepared according to the procedures devised by Neville (18). Cells ($4–5 \times 10^7$) grown to confluence were collected, washed in phosphate buffered saline, pH 7.4, and sedimented at 200 *g* for 10 min. The cells (1.5×10^6/ml) were then resuspended in a dounce buffer containing 5 mM Tris-HCl, pH 7.8, 5 mM NaCl, 0.1 mM EGTA, and 1 mM dithiothreitol and incubated in ice for 3 min. $MgCl_2$ was added to a final concentration of 5 mM and the cells incubated for 2 min. They were then dounced with 45 strokes in a tight homogenizer, centrifuged at 400 *g* and the supernatant fluids were collected. The pellets were dounced with additional 25 strokes in a small volume of buffer and the supernatant fluids combined, centrifuged at 40,000 *g* for 15 min and, washed with dounce buffer (dithiothreitol was excluded) under the same conditions. Protein was determined by the method of Lowry et al. (19) using bovine serum albumin as a standard. Membranes were stored at 1.2 mg/ml at -70°C and thawed only once.

Adenylate Cyclase Determinations: Reaction mixtures consisted of cell membrane protein, 25 mM Tris-HCl buffer, pH 7.5, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 10 mM creatine phosphate, 0.5 U of creatine phosphokinase, 1 mM cAMP, 0.5 mM α-[^{32}P] ATP (4.2–10.2 cpm/pmol) in a volume of 55 ul. Guanyl-5'-imidodiphosphate (GppNHp) (100 μM), NaF (20 mM), or isoproterenol (60 uM) were added as indicated. The reaction was carried out at 37°C for 15 min and stopped by addition of 100 μl of a solution containing 2% sodium dodecyl sulfate and, 40 mM ATP, pH 7.5, (Amersham Corp., Arlington Heights, IL). ^{3}H-cAMP (approximately 20,000 cpm) was added to monitor recovery. cAMP was isolated according to the method of Salomon et al. (20).

Intracellular cAMP Determinations: cAMP was measured by displacement of ^{3}H-cAMP from a preparation of cAMP binding protein as described in (21). Cells (1×10^7) were washed with saline and extracted (1 ml/100-mm dish) with 50% glacial acetic acid. The cAMP binding assay was carried out for 1.5 h in ice, stopped by adding ammonium sulfate (3.8 M, final concentration) and the reaction mixture was filtered (Schleicher and Schell GF/C filters) and counted as described (21). The assay was calibrated for 0.25 to 3.0 pmol cAMP. Cholera toxin (100 ng/ml or 300 ng/ml) was added to the medium 2 h and IFN (1,000 or 2,000 U/ml) 20 h before the cells were harvested.

Infection with VSV: Cells (2×10^5) were plated in 2-cm^2 wells (Linbro) for 24 h in 1 ml of medium, and IFN was then added for an additional 24 h. VSV, at multiplicities of infection of 0.12 and 0.012 was added and the cells cultured for 48 h. Supernatants were collected and stored frozen at −70°C. Cells were fixed with formol-saline for 10 min and stained with crystal violet for 20 min. Cytopathic effects were expressed as percentage of each stained cell monolayer destroyed.

Viral Yields: Serial 10-fold dilutions (0.2 ml) of the above supernatant fluids were added to confluent Vero cell monolayers in 2-cm^2 wells. After 1 h adsorption at 37°C, 1 ml of warm medium containing 1% agar was added, and wells were incubated for an additional 48 h. The monolayers were then fixed, stained with crystal violet, and virus plaques were counted.

Assay of 2',5'-oligoadenylate Synthetase Activity: 2',5'-oligoadenylate synthetase activity was assayed by the method described by Gupta et al. (22). The cells were cultured for 10 h with or without 500 U of IFN. The cells were extracted with 0.5% NP40, clarified, and the supernatant fluids frozen at −70°C. ^{3}H-ATP (1 mCi/ml, sp act 52.6 Ci/mmol) was repurified on DEAE cellulose and adjusted to a final concentration of 8 mM with cold ATP.

Cell extracts were applied to 50 μl of poly(I):poly(C) agarose columns (P. L. Biochemicals, Inc., Milwaukee, WI), and washed with 25 ml of 10 mM HEPES, pH 7.5, 90 mM KCl, 1.5 mM magnesium acetate, 7 mM β-mercaptoethanol, 20% glycerol, and three times with 100 μl of cold reaction mixture (containing the same buffer and 5 mM ATP, 0.2% creatine phosphate, and 0.03% creatine phosphokinase) followed by the same mixture containing purified ^{3}H-ATP. The column was incubated at 30°C for 16 h and 2',5'-A was eluted and boiled for 3 min. The 2',5'-A was separated from unconverted ATP on DEAE columns by elution with buffer containing 350 mM KCl at pH 7.5.

RESULTS

Isolation of IFN-Variants

In initial experiments it was established that the parental macrophage clone, J774.2, was sensitive to growth inhibition by IFN. Growth was inhibited at 500 U/ml, and the culture was killed over a 5-d period by 2,000-4,000 U/ml of IFN (Fig. 1). To select for IFN-resistant variants, cells were cloned in medium containing 5,000 IFN without prior mutagenesis. Fewer than 10 clones appeared per 2,000 cells plated. Each clone was isolated and tested for cell growth in the presence of various concentrations of IFN, along with the parental J774.2 clone. Those primary clones that were resistant to IFN were picked and grown in sucessively increasing concentrations of IFN, 1,000, 3,000, and ultimately 5,000 U/ml over 6 mo. From this selection, 15 independent resistant (IFN^r) clones were obtained. The growth characteristics of some of these variants in the presence of IFN are shown in Fig. 1. All grew in medium containing 4,000 U/ml of IFN.

To test the prediction that some IFN^r clones would be defective in adenylate cyclase or cAMP-dependent protein kinase activity, the growth of the IFN^r clones in the presence of cholera toxin was examined. Two classes of IFN^r variants were found, cholera toxin resistant (CT^r) and cholera toxin sensitive clones (CT^s). Clones 68, 82, 92, and 103 were cholera toxin resistant. Clones 95, 97, 99, and 100 were as sensitive to cholera toxin as the parental J774.2 clone.

To explore the possibility that some IFN^r/CT^r clones were defective in cAMP-dependent protein kinase, the effect of 8Br-cAMP on the growth of these clones was examined. All were as growth inhibited by 50–100 uM 8Br-cAMP (in the presence or absence of 0.05 mM of 1-methyl-3-isobutylxanthine) as the parental cell line (J774.2). These results suggested that some IFN^r/CT^r clones might be defective in adenylate cyclase, but not in cAMP-dependent protein kinase.

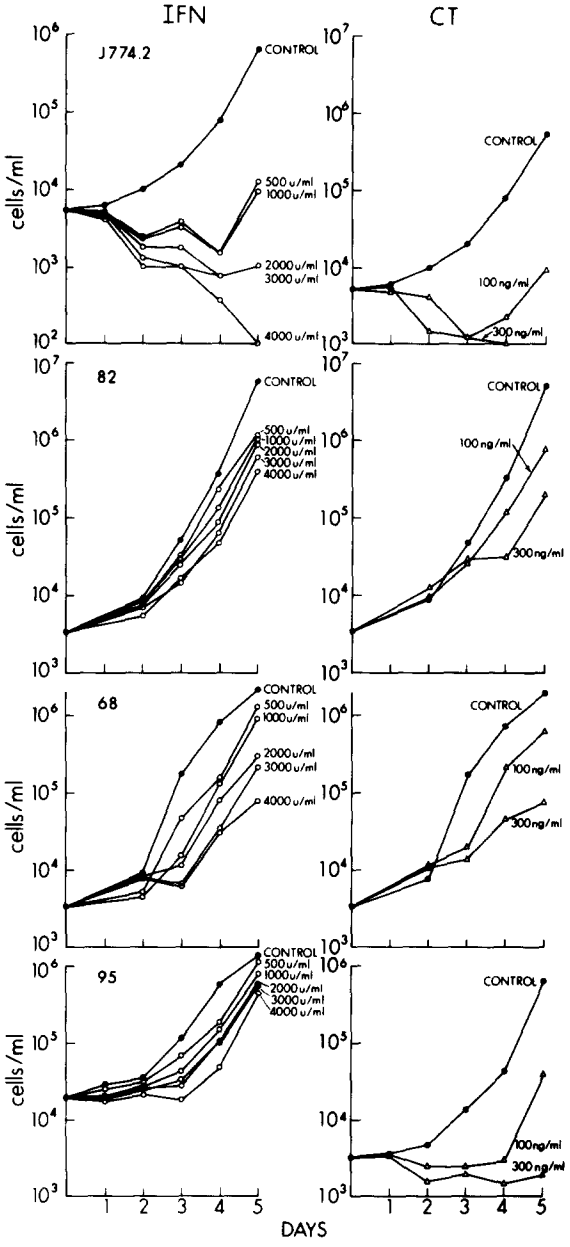

FIGURE 1 Effect of IFN and cholera toxin on growth of wild type J744.2 and IFN^r clones. Cells were plated at 0.5–1 × 10^4/1 ml in medium alone (●) or in the presence of varying concentrations of either IFN (○) or cholera toxin (△). Fresh IFN was added on day 3.

Adenylate Cyclase Activity in IFN^r Variants

Adenylate cyclase activity in crude membranes of the IFN^r clones was measured following stimulation with GppNHp, fluoride, or isoproterenol (Table I). Basal adenylate cyclase activity of the parental clone (J774.2) was stimulated 15–18-fold by GppNHp or fluoride and 6-fold by isoproterenol. In contrast, all of the IFN^r/CT^r variants had reduced basal and stimulated adenylate cyclase activity. The IFN^r/CT^s variants showed similar activities to the parental clone. The addition of IFN for 2 h directly to the membrane preparations did not affect adenylate cyclase activity (data not shown).

The adenylate cyclase activity of both J774.2 and clone 82, an IFN^r/CT^r variant, was proportional to the amount of membrane protein added, up to 50 μg (Fig. 2). When membranes prepared from a mixture of J774.2 and clone 82 were analyzed, the adenylate cyclase activity was additive, indicating that the diminished activity of clone 82 could not be attributed to a diffusible inhibitor of adenylate cyclase (data not shown).

TABLE I

Adenylate Cyclase Activity in Wild Type and IFN^r Clones

		cAMP (pmol/15 min/50 μg protein)			
Type	Clone	No addn	GppNHp	F^-	Isoproterenol
Wild type	J774.2	2.65	41.22	47.40	15.75
IFN^r/CT^r	82	0.73	7.94	29.08	0.82
	68	0.26	1.33	3.87	1.82
	92	0.04	10.01	12.31	1.53
IFN^r/CT^s	95	2.10	24.90	45.77	21.68
	97	14.76	108.42	78.87	60.88
	99	4.12	56.06	29.81	10.70

GppNHp, fluoride, or isoproterenol were added to the reaction mixture at the final concentrations of 100, 20, and 60 μM, respectively.

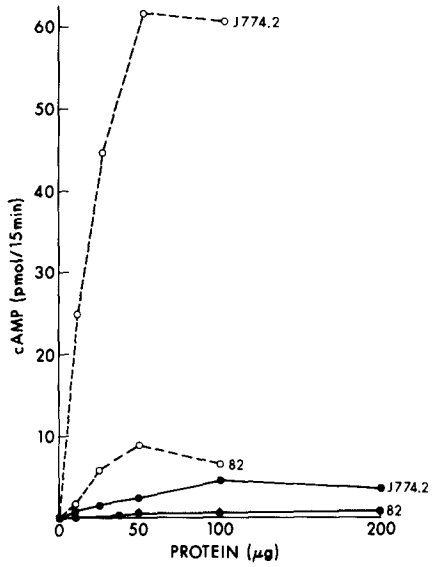

FIGURE 2 Adenylate cyclase activities in wild type J774.2 and IFN^r/CT^r variant 82. Various concentrations of membrane protein were added with (○) or without (●) 100 mM GppNHp in the reaction mixture.

Intracellular cAMP Levels in Variant Cells

To obtain an indication of the nature of the defect in adenylate cyclase activity in the IFN^r/CT^r clones, intracellular cAMP levels were measured in cell extracts (Table II). Exposure to 100 ng/ml of CT augmented cAMP levels significantly in J774.2 and in the IFN^r/CT^s clones, 95, 99, and 100. However, the IFN^r/CT^r resistant clones 82, 68, and 103 failed to show a response to cholera toxin, although the basal cellular cAMP levels were not strikingly different from the other clones. When intracellular levels of the parental and mutant clones were measured following esposure to 1,000 U of IFN, as expected there was a 2–3-fold stimulation in J774.2 as reported previously (12), with essentially no stimulation in the IFN^r/CT^r or IFN^r/CT^s mutants (Table III). The results from the assays of adenylate cyclase in vitro combined with the measurements of cellular cAMP content suggest a lesion

TABLE II

Stimulation of Intracellular cAMP by Cholera Toxin

Clone	Phenotype	Basal	Cholera toxin 100 ng/ml	Cholera toxin 300 ng/ml
J774.2	wt	11.0	16.6	21.8
82	IFN^r/CT^r	8.0	4.5	7.1
68		6.8	5.4	9.6
103		7.4	6.3	5.5
95	IFN^r/CT^s	9.4	11.6	20.3
99		7.0	56.9	43.0
100		9.2	42.2	29.8
CTRM1	ad cyc−	9.2	14.5	13.6

Cholera toxin was added at 100 and 300 ng/ml for 2 h before cell harvest. The results depicted are the average of 2 or 3 (J774.2) independent experiments, each performed in triplicate. cAMP (pmol/mg protein).

TABLE III

Stimulation of Intracellular cAMP by Interferon

Phenotype	Clone	cAMP (pmol/mg protein) Basal	cAMP (pmol/mg protein) IFN*
Wild type	J774.2	7.8	20.1
IFN^r/CT^r	82	6.3	4.4
	68	4.9	4.9
IFN^r/CT^s	95	5.6	7.5
	99	5.5	8.9
	100	4.6	6.3

IFN was added at 1,000 U/ml 20 h prior to cell harvest. The results represent the average of two experiments, each carried out in triplicate, except for clones 95 and 99 which represent a single experiment performed in triplicate. In one experiment with IFN present at 2,000 U/ml the level of cAMP in the parental cells was increased to 23.6 pmol/mg protein, similar to that observed with 1,000 U/ml (20.1 pmol/ml protein). In contrast 2,000 U/ml of IFN failed to increase the level of cAMP in any of the mutants.
* 1,000 U/ml.

in the regulatory, guanine nucleotide-binding component of the adenylate cyclase, rather than in the catalytic moiety of the enzyme.

Antiviral Effects of IFN on IFN^r Variant Clones

The most obvious mechanisms for resistance of these clones to the growth inhibitory effects of IFN are a lack of receptors for IFN, defective adenylate cyclase activity, or both. The simplest available method to discriminate between these possibilities was to test for antiviral effects of IFN. This effect is receptor-mediated and we have shown it to be cAMP independent in these cells (12). The antiviral activity of IFN was determined both by protection against the cytopathic effects of VSV and by measuring viral yields of IFN treated clones (Table IV). IFN (10 U/ml) protected the parental clone, J774.2, and IFN^r/CT^r variants 82 and 68 against the cytopathic effects of VSV. In contrast, IFN^r/CT^s clones, 95, 99, 97, and 100 were resistant to the antiviral effects of IFN.

This distinction was confirmed in more quantitative terms when viral yields were examined (Table IV). Infection of the Vero cells with VSV at multiplicities of ~0.12 and 0.012 revealed that the IFN^r/CT^r variants were as, or more resistant to the antiviral effects of IFN as the parental clone. Their responsiveness formally established the conclusion that these cells expressed receptors for IFNα. In contrast, the IFN^r/CT^s clones failed to demonstrate antiviral activity produced by IFN. The simplest interpretation is that these clones are growth resistant to IFN because they lack receptors for IFN. While chemically pure murine IFNα was not available for receptor binding studies, this conclusion was supported by examining 2′,5′-oligoadenylate synthetase activity following IFN treatment. As shown in Table V, IFN caused a marked increase in 2′,5′A synthetase activity in the parental cells and in the IFN^r/CT^r clones, but failed to stimulate 2′,5′A synthetase activity in the IFN^r/CT^s clones 95 and 99.

TABLE IV

CPE and Virus Yields in Wild Type and IFN^r Clones

Clone	Phenotype	IFN treatment	CPE 0.12	CPE 0.012	Yields 0.12	Yields 0.012
		IU/ml	%		*pfu*	
J774.2	Wild type	None	100	100	3.1×10^6	0.7×10^6
		10	75	60	90×10^3	6.5×10^3
		10^2	25	10	1.1×10^3	0.05×10^3
		10^3	0	0	0.75×10^3	0.04×10^3
82	IFN^r/CT^r	None	30	5	3.5×10^6	0.9×10^6
		10	0	0	6.5×10^3	5.0×10^3
		10^2	0	0	5.0×10^3	0.15×10^3
		10^3	0	0	3.5×10^3	0.35×10^3
68	IFN^r/CT^r	None	75	15	3.0×10^6	0.3×10^6
		10	55	0	7.0×10^3	5.0×10^3
		10^2	30	0	6.5×10^3	10
		10^3	0	0	5.5×10^3	10
95	IFN^r/CT^s	None	90	90	5.5×10^6	1.7×10^6
		10	90	75	1.5×10^6	0.3×10^6
		10^2	80	60	1.2×10^6	0.8×10^6
		10^3	80	60	1.0×10^6	0.4×10^6
99	IFN^r/CT^s	None	95	60	1.4×10^6	0.5×10^6
		10	95	60	1.2×10^6	0.2×10^6
		10^2	90	50	1.6×10^6	0.3×10^6
		10^3	90	60	1.7×10^6	0.6×10^6

Cells (2×10^5/ml) were incubated for 48 h with the concentrations of IFN indicated. They were then infected with VSV and cultured for an additional 48 h. After the supernatants were collected, cells were fixed, stained, and cytopathic effects were assessed. For assay of virus yields, 0.2 ml of diluted 48-h supernatants were titered on confluent Vero cell monolayers. After 1 h adsorption at 37°C, 1 ml of medium containing 1% agar was overlayed and the plates incubated for 48 h. The cells were fixed, stained, and VSV plaques were counted.

TABLE V

Induction of 2′,5′-Oligoadenylate Synthetase Activity after IFN Treatment

Clone	Phenotype	2′,5′-Oligoadenylate synthetase activity* —	2′,5′-Oligoadenylate synthetase activity* IFN‡
J774.2	Wild type	21.8	201.3
68	IFN^r/CT^r	17.5	148.5
82	IFN^r/CT^r	12.5	128.1
95	IFN^r/CT^s	16.5	22.0
99	IFN^r/CT^s	22.5	28.0

* Nanomoles ATP converted per milligram protein.
‡ 5,000 μ/ml.

DISCUSSION

Because of the multiplicity of cellular and biochemical effects of interferons on cultured cells, it has been very difficult to delineate those involved in the direct growth inhibitory activity of interferons. We have previously observed that although IFN augmented Fc-receptor mediated phagocytosis in the J774.2 macrophage line used for these studies, it failed to do so in variants defective in adenylate cyclase or cAMP-dependent protein kinase derived from this clone (12). Of more general interest was the finding that these variants in cAMP metabolism were at least 50-fold less sensitive to the growth inhibitory activity of IFN than the parental clone. In contrast, both the parental and variant clones were fully sensitive to the antiviral effects of IFN. These experiments suggested that the inhibitory effects of IFN on cell growth might be mediated by cAMP.

The present experiments were undertaken to test that hypothesis using a genetic approach. We argued that if cAMP mediated the growth inhibition produced by IFN, among variants selected for resistance to this growth inhibition would be clones defective in either cAMP synthesis or function. Accordingly, J774.2 cells resistant to the growth inhibitory effects of IFN were selected, and 15 independent clones, capable of growing in concentrations of 4,000 U/ml of IFNα were characterized. One third (5/15) of these clones were also resistant to growth inhibition produced by cholera toxin. None of the clones was resistant to growth inhibition by 8Br-cAMP. Since growth inhibition by 8Br-cAMP is mediated by cAMP-dependent protein kinase (23), this enzyme activity must be both present and normally sensitive to cAMP in the CT^r/CT^s resistant cells. This suggested a defect in either the receptor for cholera toxin or the adenylate cyclase.

When the adenylate cyclase activity of the IFN^r/CT^r clones was examined in vitro, the variants had reduced basal activity as well as marked reduction in GppNHp, F^-, or isoproterenol-stimulated activities. Although these cells had normal basal levels of cAMP, they failed to show an increase in response to cholera toxin or IFN. Thus many of the clones selected for resistance to the growth inhibitory activity of IFN (IFN^r/CT^r) have a defect in adenylate cylcase activity, most likely in the guanine nucleotide-binding component. This appears to be the same kind of defect found in cells selected for cholera toxin resistance in these (14, 24) or other cell lines (25, 26).

The remaining IFN^r clones were susceptible to the growth inhibitory effects of cholera toxin and possessed adenylate cylcase activity similar to that of the parental clone. The most likely explanation for their resistance is a loss of IFN receptors, a defect reported previously for other IFN-resistant cells (27).

Since pure murine IFNα was not available for receptor binding analysis, we assessed receptor function by assaying for IFN-induced protection against virus infection and stimulation of 2′,5′-oligoadenylate synthetase activity. IFN protected all of the IFN^r/CT^r clones against infection by VSV and markedly stimulated 2′,5′-oligoadenylate synthetase activity, establishing both the integrity of their IFN receptors and the independence of the antiviral and cell growth inhibitory effects of IFN. Not unexpectedly, IFN afforded little antiviral protection for the IFN^r/CT^s clones, supporting the interpretation that these cells have defective or deficient IFN receptors. This conclusion was strengthened by showing that IFN failed to stimulate 2′,5′-oligoadenylate synthetase, an enzyme known to be closely coupled to IFN receptors in all cells studied (6). The possibility, however, that they may have lesions in other metabolic pathways critically involved in both antiviral and growth inhibitory activities of IFN cannot be excluded.

There are a variety of mechanisms unrelated to cAMP by which IFN could conceivably inhibit cell growth. For example, IFN significantly inhibits thymidine transport in L1210 cells. Indeed, it is for that reason that all the assays of cell growth in this report utilized actual cell counts rather than incorporation of ^{3}H-thymidine. IFN can also induce synthesis of two cellular enzymes, 2′,5′-oligoadenylate synthetase and a cAMP-independent protein kinase (eIF2 kinase), which may affect cellular RNA and protein synthesis (6, 7). A number of cell lines including human fibroblastic tumor cells (28), murine leukemia (29), mouse embryonal carcinoma cells (30), the Daudi human lymphoblastoid cell (31) and HEC-1 cells (32) have been selected for resistance to the growth inhibitory effects of IFN. While measurements of adenylate cyclase and cAMP dependent protein kinase activity have not been reported in these cells, induction of 2′,5′-oligoadenylate synthetase and a cAMP independent (eIF2) protein activity kinase have. In some of the cell lines, e.g., $L1210^r$, IFN has neither antiviral activity nor the capacity to induce these enzymes (29, 33). This cell has been shown to lack receptors for IFN (27). In other IFN resistant cell lines, e.g., the IFN^r variant of RSa cells, embryonal carcinoma cells, Daudi cells, 2′,5′-oligoadenylate synthetase activity may be normal (31, 34, 35). In some, e.g., RSa cells, Daudi cells, and HEC-1 cells, the cAMP-independent (eIF2) protein kinase actively may be present as well (28, 31, 32). Thus far there is no consistent association between the levels of these two enzyme activities and responsiveness to growth inhibitory activities of IFN.

The results presented here provide clear genetic and biochemical evidence that the growth inhibitory effects of IFN, at least in a murine macrophage cell line, are mediated by cAMP. It will be of interest to examine adenylate cyclase and cAMP dependent protein kinase activities in other IFN resistant cell lines known to have IFN receptors to establish the generality of this phenomenon. Because the cell receptors for IFNα and γ appear to be distinct and independent, it will also be important to learn whether the growth inhibitory activity of IFNγ, which on an antiviral unit basis may be more profound than that of IFNβ or α (36, 37), are mediated through cAMP or by a different mechanism.

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REFERENCES

1. Paucker, K., K. Cantell, and W. Henle. 1962. Quantitative studies on viral interference in suspended L cells. III. Effect of interfering viruses and interferon on the growth rate of cells. *Virology.* 17:324–334.
2. Gresser, I., D. Brouty-Boye, M.-T. Thomas, and A. Macieira-Coelho. 1970. Interferon and cell division. I. Inhibition of the multiplication of mouse leukemia L 1210 cells *in vitro* by interferon preparations. *Proc. Natl. Acad. Sci. USA.* 66:1052–1058.
3. Knight, E., Jr. 1976. Antiviral and cell growth inhibitory activities reside in the same glycoprotein of human fibroblast interferon. *Nature (Lond.).* 262:302–303.

4. Taylor-Papadimitriou, J. 1980. Interferon. Academic Press, Inc. New York. 5:2–13.
5. Tovey, M., and D. Brouty-Boye. 1979. The use of the chemostat to study the relationship between cell growth rate, viability, and the effect of interferon on L 1210 cells. *Exp. Cell Res.* 118:383–388.
6. Kerr, I. M., and R. E. Brown. 1978. pppA2′p5′A2′p5′A: an inhibitor of protein synthesis synthesized with an enzyme fraction from interferon-treated cells. *Proc. Natl. Acad. Sci. USA.* 75:256–260.
7. Farell, P. J., G. C. Sen, M. F. Dubois, L. Ratner, E. Slattery, and P. Lengyel. 1978. Interferon action: Two distinct pathways for inhibition of protein synthesis by double-stranded RNA. *Proc. Natl. Acad. Sci. USA.* 75:5893–5897.
8. Gresser, I. 1961. Metamorphosis of human amnion cells induced by preparations of interferon. *Proc. Natl. Acad. Sci. USA.* 47:1817–1822.
9. Tovey, M. G., C. Rochette-Egly, and M. Castagna. 1979. Effect of interferon on concentrations of cyclic nucleotides in cultured cells. *Proc. Natl. Acad. Sci. USA.* 76:3890–3893.
10. Hamburg, S. I., H. B. Fleit, J. C. Unkeless, and M. Rabinovitch. 1980. Mononuclear phagocytes: responders to and producers of interferon. *Ann. NY Acad. Sci.* 350:72–90.
11. Vogel, S. N., L. L. Weedon, R. N. Moore, and D. L. Rosenstreich. 1982. Correction of defective macrophage differentiation in C3H/HcJ mice by an interferon-like molecule. *J. Immunol.* 128:380–387.
12. Schneck, J., B. Rager-Zisman, O. M. Rosen, and B. R. Bloom. 1982. Genetic analysis of the role of cAMP in mediating effects of interferon. *Proc. Natl. Acad. Sci. USA.* 79:1879–1883.
13. Ralph, P., M. A. S. Moore, and K. Nilsson. 1976. Reticulum cell sacrcoma: an effector cell in antibody-dependent cell-mediated immunity. *J. Immunol.* 114:898–905.
14. Muschel, R. J., N. Rosen, and B. R. Bloom. 1977. Isolation of variants in phagocytosis of a macrophage-like continuous cell line. *J. Exp. Med.* 145:175–186.
15. Bloom, B. R., B. Diamond, R. Muschel, N. Rosen, J. Schneck, G. Damiani, O. Rosen, and M. Scharff. 1978. Genetic approaches to the mechanism of macrophage functions. *Fed. Proc.* 37:2765–2771.
16. Vassef, A., G. Beaud, K. Paucker, and P. Lengyel. 1973. Interferon assay based on the inhibition of double-stranded reovirus RNA accumulation in mouse L cells. *J. Gen. Virol.* 19:81–87.
17. Rosen, N., J. Piscitello, J. Schneck, R. J. Muschel, B. R. Bloom, and O. M. Rosen. 1979. Properties of protein kinase and adenylate cyclase-deficient variants of a macrophage-like cell line. *J. Cell. Physiol.* 98:125–136.
18. Neville, D. M., Jr. 1968. Isolation of an organ specific protein antigen from cell-surface membrane of rat liver. *Biochim. Biophys. Acta.* 154:540–552.
19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–276.
20. Salomon, Y., C. Londos, and M. Rodbell. 1974. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* 54:541–548.
21. Gilman, A. G. 1970. A protein binding assay for adenosine 3′:5′ cyclic monophosphate. *Proc. Natl. Acad. Sci. USA.* 67:305–312.
22. Gupta, S. L., B. Y. Rubin, and S. L. Holmes. 1981. Regulation of interferon action in human fibroblasts: transient induction of specific proteins and amplification of the antiviral response by antinomycin D. *Virology.* 111:331–340.
23. Kuo, J. F., and P. Greengard. 1969. Cyclic nucleotide-dependent protein kinase. IV. Widespread occurrence of adenosine 3′:5′ monophosphate-dependent protein kinase in various tissues and phyla of the animal kingdom. *Proc. Natl. Acad. Sci. USA.* 64:1349–1355.
24. Rosen, N., J. Schneck, B. R. Bloom, and O. M. Rosen. 1978. Inhibition of plasminogen activator secretion by cyclic AMP in a macrophage-like cell line. *J. Cyclic Nucleotide Res.* 5:345–358.
25. Bourne, H. R., P. Coffino, and G. M. Tomkins. 1975. Somatic genetic analysis of cyclic AMP action: characterization of unresponsive mutants *J. Cell. Physiol.* 85:611.
26. Evain, D., M. Gottesman, I. Pastan, and W. B. Anderson. 1979. A mutation affecting the catalytic subunit of cyclic AMP-dependent protein kinase in CHO cells *J. Biol. Chem.* 254:6931–6937.
27. Aguet, M. 1980. High-affinity binding of ^{125}I-labelled mouse interferon to a specific cell surface receptor. *Nature (Lond.).* 284:459–461.
28. Kuwata, T., A. Fuse, and N. Morinaga. 1976. Effects of interferon on cell and virus growth in transformed human cell lines. *J. Gen. Virol.* 33:7–15.
29. Gresser, I., M. T. Bandu, and D. Brouty-Boye. 1974. Interferon and cell division. IX. Interferon-resistant L1210 cells: characteristics and origin. *J. Natl. Cancer Inst.* 52:553–559.
30. Burke, D. C., C. F. Graham, and J. M. Lehman. 1978. Appearance of interferon inducibility and sensitivity during differentiation of murine teratocarcinoma cells *in vitro. Cell.* 13:243–248.
31. Silverman, R. H., D. Watling, F. R. Balkwill, J. Trowdale, and I. M. Kerr. 1982. The ppp (A 2′p)nA and protein kinase systems in wild-type and interferon-resistant Dandi cells. *Eur. J. Biochem.* 126:333–341.
32. Verhaegen, M., M. Divizia, P. Vandenbusshe, T. Kuwata, and J. Content. 1980. Normal behavior of interferon-induced enzymatic activities in an interferon-resistant cell line. *Proc. Natl. Acad. Sci. USA.* 77:4479–4483.
33. Vandenbussche, P., J. Content, B. Leblew, and J. Werenne. 1978. Comparison of interferon action in interferon resistant and sensitive L1210 cells. *J. Gen. Virol.* 41:161–166.
34. Vandenbussche, P., M. Divizia, M. Verhaegen, A. Fuse, T. Kuwata, E. Clercg, and J. Content. 1981. Enzyme activities induced by interferon in human fibroblast cell lines differing in their sensitivity to the anti cellular activity of interferon. *Virology.* 111:11–22.
35. Wood, J. N., and A. G. Hovanessian. 1979. Interferon enhances 2-5A synthetase in embryonal carcinoma cells. *Nature (Lond.).* 282:74–76.
36. Blalock, J. E., J. A. Georgiades, M. P. Langford, and H. M. Johnson. 1980. Purified human immune interferon has more potent anticellular activity than fibroblast or leukocyte interferon. *Cell Immun.* 49:390–394.
37. Rubin, B. Y., and S. L. Gupta. 1980. Differential efficacies of human type I and type II interferons as antiviral and antiproliferative agents. *Proc. Natl. Acad. Sci. USA.* 77:5928–5932.