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

YUMIKO NAGATA, ORA M. ROSEN, MAYNARD H. MAKMAN, and BARRY R. BLOOM
Departmentsof Microbiology and Immunology, Molecular Pharmacology and Biochemistry, Albert
Einstein College of Medicine, Bronx, New York 10461

ABSTRACT While a multiplicity of cellular and biochemical effects are mediated by interferons 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'/CT') clones against infection by vesicular stomatitis virus and markedly stimulated 2', 5'-oligoadenylate synthetase activity. These IFN'/CT' 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'/cholera-toxin sensitive clones against infection by vesicular stomatitis virus and failed to stimulate 2', 5-oligoadenylate synthetase, suggesting that they have defective or deficient IFN receptors. In addition, IFN failed to increase intracellular cAMP levels in both IFN'/CT' and IFN'/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) 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', cholera-toxin resistant clones; CT', cholera-toxin sensitive clones; GppNHp, guanyl-5'-imidodiphosphate; IFN, interferons; IFN', interferon-resistant clones; VSV, vesicular stomatitis virus.
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) or 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% CO2.

The mouse hepatoma cell line, E6, was incubated in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco Laboratories), 10% NCTC 109, 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-a + b, specific activity 1.3 x 10^5 IU/mg protein) was purchased from Lee Biomolecular Research Laboratories (San Diego, CA) and IFN a from Enzo Biochemicals (New York, NY). The specificity for antiviral activity of these preparations established in this laboratory was 6.5 x 10^7 UI/mg and 5 x 10^7 IU/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, centrifuged at 1,000 g, 10 min, and frozen at -70°C. This stock contained 5 x 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 E6 or rat embryo fibroblasts feeder layers (14, 17). A solution of 0.5% agarose (wt/vol) (Sea Plaque, Marine Cellolds, 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 then incubated 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.

Preparation of Cell Membrane Extracts: Plasma membranes were prepared according to the procedures devised by Neville (18). Cells (4-5 x 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 x 10^9)/ml) were then resuspended in a dounce buffer containing 5 mM Tris-HCl, pH 7.8, 5 mM MgCl2, 1 mM dithiothreitol, and 1 mM EDTA (final concentration) and resuspended on ice in a total volume of 3 ml. MgCl2 was added to a final concentration of 5 mM and the membranes 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 centrifuged, centrifuged at 40,000 g for 15 min and, washed with dounce buffer (dithiothreitol was excluded) under the same conditions. Protein content 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 MgCl2, 0.5 mM dithiothreitol, 10 mM creatine phosphate, 0.5 U of creatine phosphokinase, 1 mM cAMP, 0.5 mM ATP (4.2-10.2 cpmp/pmol) and 2',5'-oligoadenylate synthetase activity was assayed by the method described by Schleicher and Schei (21).

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. No 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 successively increasing concentrations of IFN, 1,000, 3,000, and ultimately 5,000 U/ml over 6 mo. From this selection, 15 independent resistant (IFN-) 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 possibility that some IFN-variant clones would be defective in adenylate cyclase or cAMP-dependent protein kinase activity, the growth of the IFN clones in the presence of cholera toxin was examined. Two classes of IFN variants were found, cholera toxin resistant (CT+) and cholera toxin sensitive clones (CT-). 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-variant clones were defective in adenylate cyclase or 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 U/ml 8 Br-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-variant clones might be defective in adenylate cyclase, but not cAMP-dependent protein kinase.
Adenylate Cyclase Activity in IFN' Variants

Adenylate cyclase activity in crude membranes of the IFN' 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'/CT' variants had reduced basal and stimulated adenylate cyclase activity. The IFN'/CT' 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'/CT' 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).

Intracellular cAMP Levels in Variant Cells

To obtain an indication of the nature of the defect in adenylate cyclase activity in the IFN'/CT' 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'/CT' clones, 95, 99, and 100. However, the IFN'/CT' 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 exposure 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'/CT' or IFN'/CT' mutants (Table III). The results from the assays of adenylate cyclase in vitro combined with the measurements of cellular cAMP content suggest a lesion.
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/CT' variants 82 and 68 against the cytopathic effects of VSV. In contrast, IFN/CT' 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/CT' 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/CT' 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/CT' clones, but failed to stimulate 2',5'-A synthetase activity in the IFN/CT' clones 95 and 99.
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'/CT' 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'CT' 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'CT') have a defect in adenylate cyclase 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' clones were susceptible to the growth inhibitory effects of cholera toxin and possessed adenylate cyclase 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'/CT' 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'/CT' 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 3H-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', 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' 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 activity 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.

We are most grateful to Dr. Sohan Gupta, Sloan-Kettering Institute for Cancer Research, for generously helping us to carry out the assays for 2',5'-oligoadenylate synthetase.

This research was supported by United States Public Health Service grants AI 07118, AI 09807, AI 10702, AG 00374, AI 17816, P30CA-13330, 2R01GM-29042, and 1R01-EY 04633.

Received for publication 26 July 1983, and in revised form 14, November 1983.

REFERENCES

1. Paucker, K. K., Cantrell, and W. Heake. 1962. Quantitative studies on viral interference in suspeptible L cells. III. Effect of interfering viruses and interferon on the growth rate of cells. Virology. 17:324–334.

2. Genauer, J., D. Broudy-Bove, M.-T. Thomas, and A. Macieira-Coelho. 1970. Interference and cell division. 1. 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.
