The Effects of an Acid pH upon the Induction of Antiviral Resistance *in Vitro* by Interferon (37207)

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(Introduced by J. A. Morris)

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With the aid of metabolic inhibitors and cell hybrids the antiviral action of interferon has been shown to be mediated by a second cellular protein, termed the antiviral protein (1, 2). It has also been shown that when the pH of the tissue culture fluid in which cells are grown is lowered to 6, interferon no longer induces a state of antiviral resistance (3). Exposure of cells to an acid pH is followed by a rapid disaggregation of polyribosomes and up to a 90% diminution of macromolecular synthesis, and these effects have been proposed as the probable cause of interferon inactivity (3, 4).

The following experiments were undertaken (1) to investigate cellular regulatory mechanisms involved in the induction of antiviral resistance, using an acid pH in place of the usual metabolic inhibitors as a tool for blocking its development, and (2) to clarify the manner in which an acid pH exerts its inhibitory effect.

Material and Methods. Tissue culture and media. Mouse fibroblasts (L cells) maintained in continuous culture were planted in vertical hemolysis tubes at a concentration of 150,000 cells/0.5 ml/tube in Eagle's medium containing 10% calf serum and antibiotics. Cell confluency was attained in 24 hr, at which time the medium was changed to the nonvolatile buffer HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid), at a 0.028 *M* concentration, containing 2% calf serum and antibiotics, and adjusted to the desired pH with 1/*N* NaOH or HCl. The stability of the pH was repeatedly monitored during the test and did not vary by more than 0.15 unit over a period of 24 hr in the cell culture tubes. Before addition of the challenge virus, HEPES buffer was replaced by Eagle's medium containing 2% calf serum and antibiotics. The pH of the Eagle's medium during the initial 24 hr of cell growth and during the period of subsequent viral replication was maintained at 7 by incubation of the tubes in a sealed box containing air and 5% CO_2.

Interferon. Mouse interferon was induced in transformed Balb/c cells (MSV-IA strain) by infection with Newcastle disease virus (5). The supernatant was held at pH 2 for 5 days, centrifuged, readjusted to pH 7, and dialyzed to one-tenth its original volume. The preparation was assayed by the virus yield inhibition method; 1 unit of interferon was defined as the dilution which reduced by 90% the yield of vesicular stomatitis virus in L cells infected at a multiplicity of infection (m.o.i.) of 0.1.

Virus. The Indiana strain of vesicular stomatitis virus (VSV) grown in L cells, and with a titer of 2×10^7 plaque forming units (PFU)/ml, was used to challenge L cells at an m.o.i. of 0.1.

General methods. Sets of 4 tubes were used for each experimental point, and cells were incubated with interferon at a dose of 50 units/0.5 ml medium/tube. Cytopathic effect was read 18 hr after VSV challenge (2 growth cycles) and the tubes were frozen at −80°. Virus yields of the centrifuged cell–supernatant fluids were determined in duplicate by a routine plaque method using a final concentration 0.8% agarose-medium overlay on L cells in plastic petri dishes. Cell, interferon, and virus controls were included in all tests. Specific protocols are included for each

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experiment in the results section.

Experiments on cellular incorporation of radioactive precursors were performed using L cells cultivated in plastic petri dishes. ^{3}H-Uridine (sp act, 20 Ci/mmole) or a mixture of ^{3}H amino acids were diluted in pH 7.2 phosphate-buffered saline to a final concentration of 1 μCi/ml. After 15 min of incubation at 37° for the labeled amino acid mixture, and 30 min for the labeled uridine, incorporation was arrested by addition of PBS chilled to 4°, and the petri dishes were placed in ice. Cells were scraped, disrupted, and the suspension precipitated with chilled 5% trichloroacetic acid. Acid-insoluble material was collected under vacuum on Whatman GFB filters, dried, and the radioactivity measured in a Tricarb liquid scintillation counter. Duplicate petri dishes were pooled for each measurement, which was calculated as counts per minute per 1000 cells (chamber counts of trypsinized cell sheets were made from a parallel series of petri dishes exposed to identical conditions).

Results. Effect of varying pH on the action of interferon. A series of pH levels between 6 and 7.3 was tested to determine the pH which would most completely inhibit the action of interferon without also producing cytotoxicity. Cultures were incubated with HEPES medium containing interferon for 4 hr at 37°. They were then washed and incubated with fresh HEPES medium for a further 24 hr at 37° before challenge with VSV. The results are shown in Fig. 1. The state of antiviral resistance diminished progressively as the pH was lowered from 7.3 to 6 where it virtually disappeared. At pH 6 a slight toxic effect on cells was observed, which did not, however, interfere with subsequent viral replication.

Development of antiviral resistance as a function of time before exposure of interferon-treated cells to pH 6. As in the previous experiment, cultures were incubated with HEPES medium containing interferon for 4 hr at 37°, followed by a further 24 hr incubation in HEPES medium without interferon before VSV challenge. However, at varying times during and after incubation of the cells with interferon, medium at pH 7.2 was replaced in successive sets of tubes by medium

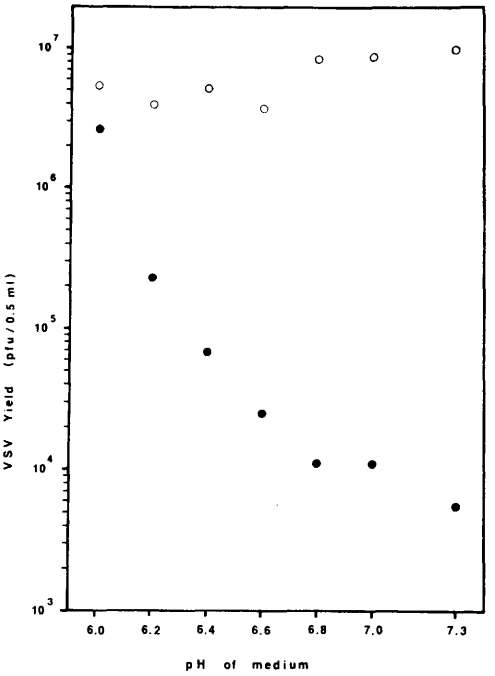

FIG. 1. Effect of varying pH on the action of interferon to inhibit the replication of VSV. (●) Cells treated with interferon; (○) control cells not treated with interferon.

at pH 6. Thus, series of tubes were incubated for 1, 2, 3 hr and so on at pH 7.2 before being changed to pH 6 for the remainder of the test. The final series of tubes was held at pH 7.2 for the entire experiment.

The results of 2 such tests are presented in Fig. 2, expressed in terms of inhibition of viral yield in interferon-treated cells as compared to untreated cells under identical conditions. The state of antiviral resistance develops slowly over the first 3 hr, quickly accelerates during the next hour, when a plateau or even diminution of resistance occurs for 3 hr, succeeded by a final progressive augmentation to attain by 24 hr the same level as that seen in cells maintained at pH 7.2 during the entire experiment.

Reversibility of the effect of pH 6 on the action of interferon. In order to dissociate the initial stage of interferon adsorption onto cells from subsequent events, cultures were incubated with medium at pH 7 containing interferon for 4 hr at 4°. Under these conditions it is known that adsorption of interferon occurs normally, but that antiviral re-

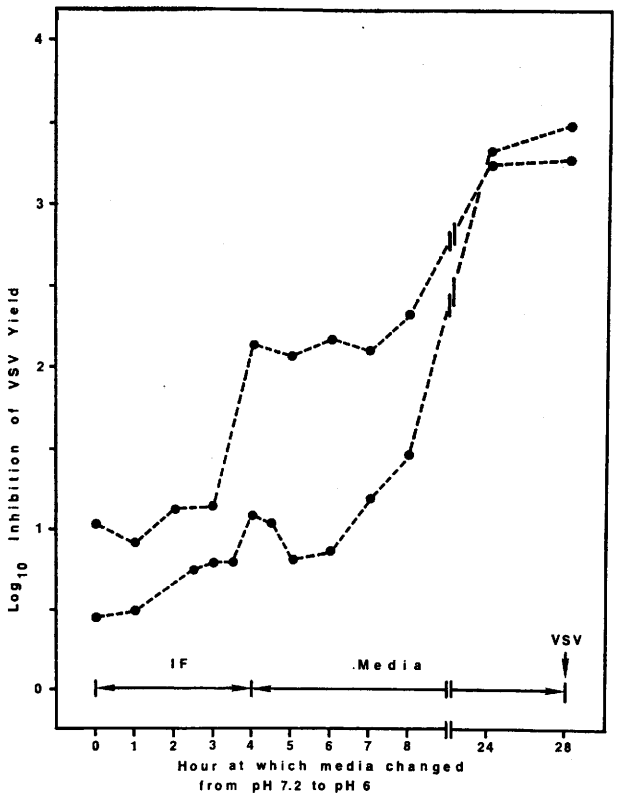

FIG. 2. The development of increasing antiviral resistance induced by interferon. During the first 4 hr, all media contained interferon, whether at pH 7.2 or 6; after this time all media was interferon-free. Interferon activity is expressed as the $\log_{10}$ inhibition of VSV yield compared to cells not treated with interferon. Two different experiments are shown.

sistance nevertheless does not develop (6). The remainder of the test was performed at 37°. After thorough washing, one series of tubes was then exposed for 12 hr to HEPES medium at pH 7.2 and another to HEPES medium at pH 6. During the next 12 hr, those cells at pH 7.2 were changed to pH 6, and those at pH 6 changed to pH 7.2. Parallel series of tubes were held at pH 6 and 7.2 during the entire 24 hr.

The results are presented in Table I. The effect of interferon in cells exposed to pH 6 after 12 hr at pH 7.2 was equal to that of cells kept at pH 7.2 during the entire experiment, showing that the antiviral state established by the end of this 12 hr period was not thereafter affected by pH 6. In cells exposed to pH 6 during the first 12 hr, only a small degree of antiviral resistance developed during the next 12 hr at pH 7.2. Thus, the inhibiting effect of pH 6 on the establishment of antiviral resistance was not readily reversible.

Mechanism of the effect of pH 6 on the action of interferon. The following experiment was conducted in parallel with the preceding tests. Cells exposed for 12 hr to pH 6 were then incubated at pH 7.2 and labeled at varying times with either ^{3}H-uridine or a mixture of ^{3}H amino acids. In a control series pH 7.2 was maintained throughout the test. The results are presented in Fig. 3, which shows that between 6 and 9 hr after removing the cells from an acid medium both protein and nucleic acid synthesis had not only fully recovered but had surpassed the level of synthesis shown by cells maintained continuously at pH 7.2.

A second parallel experiment was undertaken to test the possibility that the failure

TABLE I. Study of the Reversibility of the Effect of pH 6 on the Action of Interferon.[a]

pH of medium		Log_{10} inhibition of VSV (mean of 4 experiments)
0–12 hr	12–24 hr	
7.2	7.2	1.40
7.2	6.0	1.39
6.0	7.2	0.64
6.0	6.0	0.31

[a] Results are expressed as log_{10} inhibition of VSV compared to control tubes subjected to same pH sequences but not incubated with interferon.

of interferon to recover its activity following exposure to an acid pH was simply due to detachment of superficially bound interferon on the cell membrane in an acid medium, with subsequent loss of the material in the course of cell washing before reestablishment of pH 7.2.

Cells were incubated for 4 hr at 4° with medium at pH 7 containing 50 units of interferon, and the tubes were washed thoroughly before addition of fresh medium at pH 6 or 7.2. After 12 hr of further incubation at 37° the amount of interferon present in the supernatant fluids was measured. Supernates from both the pH 6 and pH 7.2 tubes contained approximately 5 units of interferon.

Discussion. The studies of Hallum, Youngner and Arnold (3) have shown that an acid medium of pH 6 has no direct effect on interferon itself, nor on its initial binding to cell membranes, and that once established, the state of antiviral resistance induced by interferon is equally unaffected. Additional studies showing the profound effects of an acid pH on cellular polyribosome structure and macromolecular metabolism suggested these as the likely cause for the observed failure of interferon to induce cellular antiviral protein (3, 4).

We have confirmed the inhibition by an acid pH of the induction of antiviral resistance by interferon (Fig. 1) and the fact that resistance once established is no longer affected (Table I). Using an acid pH as a tool to block the progression of antiviral resistance induced by interferon, the pattern of evolving resistance was then determined (Fig. 2). The plateau observed between 4

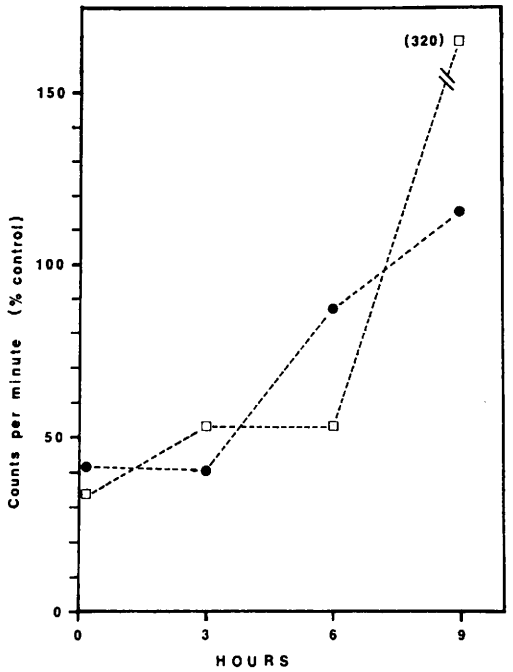

FIG. 3. The recovery of cellular nucleic acid and protein synthesis at pH 7.2 following a 12 hr exposure to pH 6. Zero hour marks the point at which pH was changed to pH 7.2. Results are expressed as the percentage of radioactivity in cells exposed to pH 6 compared to cells held at pH 7.2 during the preceding 12 hr. Measurements of radioactivity following pulse labeling (□) by a mixture of ^3H amino acids; and (●) by ^3H-uridine.

and 7 hr after the beginning of incubation with interferon, followed by a further augmentation of resistance, was unexpected. It could be the result of several interacting events, such as the removal of interferon from the culture medium after the initial 4 hr incubation period, the effects of pH on the rate of decay of messenger RNA and antiviral protein, and the possible partial recovery of those cultures exposed to an acid pH toward the end of the test, during the period of virus challenge at pH 7. But it is intriguing to consider also the possible appearance during this period of a cellular antagonist to the activity of the antiviral protein. The time sequence of this antagonistic effect is very similar to that seen in earlier experiments using the metabolic inhibitor antinomycin D, suggesting the concept of a cellular "control" protein (7, 8).

Having thus established the limits of an acid pH as a method of blocking the induction of antiviral resistance by interferon, we wished to know to what extent this effect could be reversed. The results in Table I indicate that a 12 hr block is practically irreversible for at least another 12 hr following reestablishment of pH 7.2. This failure to recover from an acid pH would appear not wholly due to inhibition of intracellular macromolecular metabolism, since the rate of nucleic acid and protein synthesis was shown to have achieved and even surpassed that of normal cells within 6 to 9 hr at pH 7.2 (Fig. 3). Nor was it due to detachment of membrane-bound interferon in an acid medium. It is thus possible that an important and as yet relatively unexplored effect of an acid pH on the activity of interferon may be upon cellular receptors involved in its transport across the cell membrane (9).

Summary. The inhibiting effect of an acid pH upon the induction of antiviral resistance by interferon was confirmed. Using this effect to block progression of antiviral resistance, a curve is described showing a transient arrest in the development of resistance 4 to 7 hr following contact of cells with interferon. The blocking effect of an acid pH is practically irreversible over a period of at least 12 hr following removal of the cells from the acid medium. This irreversibility appears not to be explained solely by inhibition of intracellular metabolism, and could relate to inhibition of a membrane-transport system by which externally bound interferon is received within the cell.

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