Effect of Interferon on Deoxyribonuclease Induction in Chick Fibroblast Cultures Infected with Cowpox Virus

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An exonuclease which degrades native deoxyribonucleic acid at pH 9.2 was induced in chick embryo fibroblast cultures and in human amnion cells by infection with cowpox virus. Highly purified chick embryo interferon suppressed the induction of the enzyme in the homologous cell system but not in the human amnion cell cultures. "Mock" interferon prepared from uninfected chicken eggs and purified in the same manner as biologically active interferon preparations had no effect on the induction of the enzyme.

In interferon-treated cells, the messenger ribonucleic acid (mRNA) of vaccinia virus cannot combine with ribosomes to form polysomes (9). This effect is probably mediated by a protein which is not interferon, but which is produced by the host cell after exposure to interferon (11, 13, 25, 26). Results obtained with Sindbis virus by Marcus and Salb (15) suggest that this protein exerts its effect by binding to ribosomes, thereby specifically inhibiting the process of translation of viral mRNA. Either of the two mechanisms of interferon action would result in the prevention of the synthesis of virus-specific proteins.

Interferon has been found to prevent the induction of certain "early" enzymes such as deoxyribonucleic acid (DNA) polymerase, thymidinekinase, and probably viral RNA polymerase (4-6, 9, 20). The purpose of this paper is to show that interferon inhibits the formation of another early enzyme, which is induced in chick embryo fibroblasts after infection with cowpox virus, namely, "alkaline" deoxyribonuclease (7, 18).

MATERIALS AND METHODS

Cells. Chick embryo fibroblasts were prepared by the standard trypsin procedure. Cells were grown in either Carrel flasks for interferon assays or in milk dilution bottles for deoxyribonuclease induction experiments. Parker's medium 199 with 10% heat inactivated calf serum was used for cell growth. Maintenance medium consisted of Parker's medium without serum.

The U line of human amnion cells was grown in milk dilution bottles for deoxyribonuclease induction experiments or in Roux bottles for the preparation of radioactive DNA (22, 24). Growth medium consisted

of Eagle's minimal essential medium, supplemented with 15% inactivated calf serum and 0.3% Tryptose Phosphate Broth (Difco). For the maintenance of cells, the serum content was reduced from 15 to 2%.

Preparation of ^{14}C -labeled DNA. To one Roux bottle containing a growing culture of human U cells, $10\ \mu\text{C}$ of ^{14}C -labeled thymidine was added (The Radiochemical Centre, Amersham, England; specific activity, 40 mc/mmole) and was left in contact with cells for 48 hr. DNA was prepared from the cells by the method of Marmur (16). The specific activity of the DNA preparation was between 1,000 and 2,000 counts per min per μg of DNA.

Viruses. The LEE strain of influenza B (ATCC) and cowpox virus strain Brighton were propagated in 10- to 12-day-old chick embryos.

Cowpox virus was used after purification by the method of Joklik (8). No deoxyribonuclease activity was detectable when preparations containing 4×10^7 plaque-forming units (PFU) of virus were investigated under the conditions described below for deoxyribonuclease assay.

Preparation of chick interferon. Interferon was prepared in 11-day-old chick embryos with influenza B Lee virus (1). The crude allantoic fluid was purified by treatment with perchloric acid and by precipitation of interferon with zinc acetate at pH 7.2, according to the method of Lampson et al. (10), followed by chromatography on CM-Sephadex C-25 (19). Interferon was eluted by raising the pH from 6.0 to 8.0 with 0.1 M K phosphate buffer. Chromatography on CM-Sephadex C-25 was repeated on a smaller column; this time the interferon was eluted between pH 6.0 and 7.0. This repeated CM-Sephadex chromatography gave higher yields of interferon than the one-step gradient elution employed by Merigan et al. (19). Final purification was effected by molecular sieve chromatography on a column (150 by 2 cm) of Biogel P-200 (Bio-Rad Laboratories, Richmond, Calif.), by

use of a buffer containing 0.25 M boric acid and 0.038 M NaOH with a pH of 8.1 (Bodo, *in preparation*). Samples were concentrated by precipitating the interferon with zinc acetate at pH 7.2. Finally, concentrated interferon samples were dialyzed against phosphate-buffered saline and were stored in polypropylene tubes at 4 C. The interferon preparation for the deoxyribonuclease experiments had a titer of 110,000 units/ml and exhibited a specific activity of 0.010 μ g of protein per unit of activity. Disc electrophoresis in polyacrylamide at pH 4.3 by the method of Reisfeld et al. (23) and at pH 8.9 by the method of Ornstein and Davies (21, 21a) showed that the purified interferon preparations could be resolved into several protein bands.

Preparation of "mock" chick interferon. "Mock" interferon was prepared from the allantoic fluid of uninoculated chick embryos and was purified exactly in the same manner as active interferon preparations. Disc electrophoresis in polyacrylamide at pH 4.3 and 8.9 (*see above*) gave protein patterns exhibiting the same protein bands as the active interferon preparations (Bodo, *in preparation*).

Interferon assay. Growth medium was replaced by 5 ml of Parker medium 199 5 to 6 hr before the test. In the interferon test, each Carrel flask received 2.0 ml of Parker's medium, 0.3 ml of interferon dilution in Parker's medium containing 0.05% bovine serum albumin, and 0.2 ml of gelatin saline (2) containing about 200 PFU of cowpox virus strain Brighton (12). Plaques were counted after 44 to 48 hr of incubation at 37 C.

One unit of interferon was defined as the amount of the material that inhibited 50% of the plaques found in the controls.

Protein determination. Protein determinations were done by the method of Lowry et al. (14).

Induction of deoxyribonuclease. For infection, growth medium was withdrawn from confluent monolayers, and the cells were infected with cowpox virus at a multiplicity of approximately 5 to 10 PFU per cell. Uninfected controls received gelatin saline only. Adsorption was allowed to proceed for 90 min at 37 C. Cells were then washed with the appropriate maintenance medium and were incubated for various amounts of time at 37 C with 7.5 ml of maintenance medium per flask.

To study the effect of interferon on enzyme induction, each flask received 1.0 ml of interferon diluted in Parker medium 199 containing 0.05% bovine serum albumin and 6.5 ml of the appropriate maintenance medium (controls received dilution medium only). After 24 hr at 37 C, the overlay medium was withdrawn, and the adsorption of virus was carried out as described above. After infection, interferon was again added to the cell cultures. For harvest, cells were scraped from the glass, washed twice in cold 0.01 M tris(hydroxymethyl)aminomethane buffer (pH 8.0) containing 0.15 M KCl and 0.003 M β mercaptoethanol, suspended in 1 ml of the same buffer, and kept frozen at -80 C until use.

Deoxyribonuclease assay. Frozen cells were disrupted by three cycles of freezing and thawing, followed by sonic treatment for 2 min with an MSE 60-w

ultrasonic disintegrator; care was taken to avoid warming of the samples above 10 C. Extracts were centrifuged at 30,000 $\times$ G for 1 hr. Deoxyribonuclease activity in the supernatant fluid was measured as described by McAuslan (17) within 1 day after preparing the extract. Of each reaction mixture, 300 μ liters contained 20 μ liters of 0.5 M glycine-NaOH buffer (pH 9.2), 20 μ liters of 0.1 M $MgCl_2$, 50 μ liters of ^{14}C -labeled native DNA (equivalent to about 60,000 counts per min and 30 to 60 μ g of DNA), and different volumes of enzyme extract (usually 25 to 200 μ liters). After incubation at 37 C for 40 min, the reaction was stopped by chilling in an ice bath and adding 50 μ liters of 40% trichloroacetic acid solution. After centrifugation, acid-soluble radioactivity of the supernatant fluid was determined in a Tri-Carb liquid scintillation spectrometer.

RESULTS

Induction of a deoxyribonuclease in chick embryo fibroblast cultures by cowpox virus. Extracts prepared from uninfected chick embryo fibroblast cells exhibited very little activity when tested for "alkaline" deoxyribonuclease with ^{14}C -labeled native DNA as substrate (Fig. 1, 2, and 3). In some extracts from uninfected cells, the existence of the enzyme could not be demonstrated unambiguously.

Induction of deoxyribonuclease activity by cowpox virus became detectable at about 3 hr after infection and increased rapidly thereafter. The specific activity was very high even as late as 22 hr postinfection (Fig. 1). At 22 hr postinfection, severe destruction of the infected cells was observed, and, consequently, no further samples were collected.

As a rule, each extract was tested at different dilutions to assure a linear relationship between activity and protein content up to about 100 μ g of protein per test sample. There was, however, some variation in the extent of this linear region among different sets of experiments.

Suppression of deoxyribonuclease induction by interferon. Figure 2 shows the effect of interferon on the induction of deoxyribonuclease at two different times after infection. Interferon was used in three different concentrations. The highest dose of interferon (1,000 units per flask) completely suppressed the induction of "alkaline" deoxyribonuclease at 5 hr postinfection. At 22 hr, the suppression was still considerable. The lowest dose of interferon (10 units per flask) caused only a very slight inhibition of deoxyribonuclease induction. Comparison of the effect of interferon at 5 and at 22 hr postinfection indicated that suppression of enzyme induction was more pronounced at 5 hr postinfection. This is best shown with the intermediate interferon concentration (100 units per flask).

To compare the effect of interferon on deoxyribonuclease induction with its protection against viral cytopathogenic effects, monolayers were inspected microscopically for cell destruction at

FIG. 1. Time course of increase in "alkaline" deoxyribonuclease activity in chick embryo fibroblasts infected with cowpox virus. Specific activity of deoxyribonuclease plotted against time after infection. Abscissa, hours postinfection; ordinate, enzyme activity expressed as counts per minute rendered acid-soluble per 50 μ g of protein under the assay conditions used. Symbols: Δ , uninfected; $\bullet$, infected.

regular intervals. Interferon concentrations of 1,000 units per flask completely protected the monolayers up to 22 hr after infection. In contrast, interferon concentrations of 10 units per flask had no detectable protective effect. Interferon doses of 100 units per flask afforded good protection at 5 hr, but very little protection at 22 hr postinfection. The extent of cell protection observed microscopically was, therefore, paralleled by the suppression of deoxyribonuclease induction.

Since interferon was present in the overlay medium until cells were harvested, the possibility could not be excluded that a small amount of interferon was introduced into the reaction mixture of the enzymatic test. To rule out any effect of interferon on deoxyribonuclease activity, interferon was added directly to the deoxyribonuclease assay mixture. No significant influence of purified chick interferon on the estimation of deoxyribonuclease activity could be detected, even at interferon concentrations of 12,000 units per test.

Specificity of interferon action. Since the highly purified interferon preparations were not homogeneous, experiments were conducted to demonstrate that the observed suppression of deoxyribonuclease induction was due to the interferon component in our preparation and not to a nonspecific inhibitor.

One control was used to study the effect of "mock" interferon on the induction of the enzyme. There was no evidence that "mock"

FIG. 2. Suppression of deoxyribonuclease induction in chick embryo fibroblasts by interferon, 5 and 22 hr post-infection. Enzyme activities plotted against protein content of cell extracts. Abscissa, amounts of protein used per assay mixture; ordinate, deoxyribonuclease activity expressed as counts per minute rendered acid soluble. Symbols: Δ , uninfected; $\bullet$, infected; and $\circ$, infected, interferon treated. Interferon doses were 10, 100, and 1,000 interferon units per flask.

interferon had any influence on deoxyribonuclease induction, even at a protein level five times as high as that used in the experiments with the highest active interferon concentrations (Fig. 3).

A second control made use of the known species specificity of interferon. Thus, chick interferon does not protect against viral infection in heterologous cells and, consequently, should not suppress the induction of deoxyribonuclease

by cowpox virus in human cells. The interferon-sensitive U cell line derived from human amnion (22, 24) was used to test this prediction. It can be seen from Fig. 4 that cowpox virus induced high activities of "alkaline" deoxyribonuclease in U cells also. Incubation of these cells with purified chick interferon did not suppress the induction of the enzyme.

It is evident from these experiments that the

FIG. 3. Effect of "mock"-interferon on deoxyribonuclease induction in chick embryo fibroblasts 5 and 22 hr post-infection. Enzyme activities plotted against protein content of cell extracts. Abscissa, amounts of protein used per assay mixture; ordinate, deoxyribonuclease activity expressed as counts per minute rendered acid soluble. Symbols: ▲, uninfected; ●, infected; and △, infected, treated with "mock"-interferon (50 μ g of protein per flask).

FIG. 4. Effect of chick interferon on deoxyribonuclease induction in human U cells, 5 and 21 hr postinfection. Enzyme activities plotted against protein content of cell extracts. Abscissa, protein used per assay mixture; ordinate, deoxyribonuclease activity expressed as counts per minute rendered acid-soluble. Symbols: ▲, uninfected; ●, infected; and ○, infected, treated with chick embryo interferon (1,000 units per flask).

observed suppression of deoxyribonuclease induction by interferon was not caused by impurities in the interferon preparations, but must have been due to the interferon component itself.

DISCUSSION

Our experiments confirm data on the induction of an "alkaline" deoxyribonuclease in chick embryo fibroblasts by viruses of the vaccinia group (18). They show, in addition, that an enzyme with similar properties is induced in human amnion cells after infection with cowpox virus.

The reason for choosing chick embryo fibroblast cultures for our experiments is that interferon from chicken eggs can be highly purified and is by far the chemically best characterized interferon preparation available.

The amount of interferon required for inhibition of deoxyribonuclease induction was found to be considerably higher than the amount needed for complete suppression of plaque formation by cowpox virus. However, this does not necessarily reflect a higher sensitivity of infectious virus formation to the action of interferon; rather, it may be explained by the different multiplicities of infection used in the two different types of experiments. The suppression of viral cytopathogenic effect under the conditions of enzyme induction was in fact approximately as strong as the inhibition by interferon of "alkaline" deoxyribonuclease induction. In spite of the presence of interferon in the medium throughout the experiments, the inhibitory effect of interferon on deoxyribonuclease induction was usually more marked at 5 hr than at 22 hr postinfection. This suggests that the observed inhibition by interferon is partially overcome during prolonged incubation.

The suppression of deoxyribonuclease induction by interferon was species specific, as shown by the failure of chick interferon to inhibit deoxyribonuclease induction in human U cells. In addition, biologically inactive "mock" interferon from uninfected chicken eggs had no effect on the induction of viral deoxyribonuclease in chick embryo fibroblasts. These experiments strongly support the notion that it is the interferon component rather than an unspecific impurity contained in our interferon preparation which is responsible for the inhibition of deoxyribonuclease induction.

The "alkaline" deoxyribonuclease induced by vaccinia virus in HeLa cells differs in various properties from the enzyme found in uninfected cells (17). Also, its induction is inhibited by the addition of puromycin (18). Therefore, the "alkaline" deoxyribonuclease induced in HeLa

cells is probably a new protein formed in response to vaccinia infection. Whether this new protein is coded by the genome of the virus or the host cell cannot be decided at this time. Our observation that interferon inhibits the induction of this early enzyme cannot be taken as unequivocal evidence that "alkaline" deoxyribonuclease is a viral enzyme.

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