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**REVERSIBLE INHIBITION OF INTERFERON SYNTHESIS BY
PUROMYCIN: EVIDENCE FOR AN INTERFERON-SPECIFIC
MESSENGER RNA***

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The capacity to synthesize interferon appears to be a latent cellular function which is induced by viral infection or other stimuli. The sequence of events can be conveniently studied in suspended cultures of Krebs-2 mouse ascites cells infected with Newcastle disease virus (NDV). Interferon synthesis in this model system begins 3–4 hr after infection and reaches maximal levels within 20 hr after viral infection.^{1, 2} Previous studies have shown that interferon formation requires unimpaired cellular RNA synthesis during the first 4 hr after viral induction.^{1, 2} Following this time, interferon continues to be formed even if RNA synthesis is inhibited by actinomycin. The present experiments provide further support for the hypothesis that a virus can switch on the cellular interferon gene by inducing the transcription of a messenger RNA that specifies the synthesis of the interferon protein. These two events in the cycle of interferon production can be dissected by suppression of protein synthesis with puromycin and of RNA synthesis with actinomycin.

Materials and Methods.—Cells and media: As previously described,² interferon production was studied in suspensions of Krebs-2 carcinoma cells maintained in the ascitic form by weekly intraperitoneal passage at 10^{-3} to 10^{-6} dilutions in adult Swiss Webster mice. Washed and packed cells were prepared from ascitic fluid harvested just prior to each experiment. Cells were suspended in medium 199 supplemented with 2% calf serum to give a final concentration of 2×10^6 cells/ml. Interferon content of cell-free media was assayed on monolayer cultures of the yL line³ of L-929 mouse fibroblasts which were grown in medium 199 with 20% calf serum. Interferon-treated and infected monolayers of yL cells were overlaid with medium 199 containing 1% agar and no serum.

Viruses: Interferon synthesis was induced by infecting packed Krebs-2 cells with a vaccine strain of Newcastle disease virus (NDV).^{1, 2} The virus stock consisted of chick-embryo allantoic fluid with a hemagglutinin titer of 128 and an infectivity titer of approximately 10^8 plaque-forming units/ml when plated on chick embryo cells. In all experiments 1 ml of NDV stock was mixed with 2×10^7 packed Krebs-2 cells at 37° for 30 min with constant shaking. This procedure resulted in greater than 90% attachment of virus and virtually complete penetration as determined by lack of elution after addition of neuraminidase (Behringwerke, Marburg, Germany). Pretreatment of cells with puromycin or actinomycin did not prevent NDV attachment and penetration, provided that the reaction was carried out at 37°. Virus attached at 4° but did not penetrate.

Encephalomyocarditis (EMC) virus was used as the test virus for interferon assays. The plaque-inhibition method for measuring interferon content has been described in detail.^{1, 2} All preparations tested for interferon were subjected to three 1-hr cycles of centrifugation at 100,000 *g* and dialyzed overnight against 0.1 *M* HCl. Neutralized samples were diluted serially and 0.2-ml aliquots adsorbed on duplicate L-cell monolayers for 2 hr prior to challenge with 100 plaque-forming units (PFU) of EMC virus. Interferon titers were recorded as the reciprocal of the dilution that reduced the number of EMC plaques to 50% of control counts.

Chemicals: Puromycin-dihydrochloride and the aminonucleoside of puromycin were purchased from Nutritional Biochemicals Corp., Cleveland. Actinomycin D (Cl) was a gift of Dr. F. D. Lawrason, Merck, Sharpe and Dohme, West Point, Pennsylvania. H³-uridine (specific activity, 21.5 c/mmole) and H³-dl-leucine (specific activity, 10.9 c/mmole) were obtained from Nuclear-Chicago Corp., Des Plaines, Illinois.

Determinations of RNA and protein synthesis: The procedure for measuring uptake of H³-uridine into Krebs-2 cells by a modification of the Baltimore-Franklin method⁴ has been described elsewhere.² After a 30-min pulse of H³-uridine, cells were chilled, washed with unlabeled uridine, disrupted by ultrasonic vibration, and their contents precipitated with 0.5 *M* cold perchloric acid (PCA). The results were comparable² to those obtained by the procedure of Scherrer and Darnell⁵ for extracting RNA with phenol and sodium lauryl sulfate. Protein synthesis was studied by incubating Krebs-2 cells for 1 hr at 37° with H³-leucine. The cells were then washed with excess unlabeled leucine, disrupted by sonication, their contents precipitated with 0.5 *M* PCA at 4°, and the mixtures heated to 90° for 15 min. The precipitates were washed twice with PCA and once with ethanol-ether (1:1), dissolved in hyamine, and radioactivity was measured in a Nuclear-Chicago scintillation counter. Cellular incorporation of both H³-uridine and H³-leucine was expressed as cpm/ μ g of protein as determined by the Lowry method. The values for H³-leucine incorporation were corrected for considerable nonspecific absorption.

Results and Discussion.—Inhibition by puromycin of cellular RNA, protein, and interferon synthesis: Nathans⁶ has recently reviewed current concepts of the site and mechanism of puromycin action. This antibiotic is a structural analogue of the adenosine end of aminoacyl-sRNA and is incorporated into growing peptide chains. It also inhibits the transfer of amino acids from sRNA into acid-precipitable polypeptides and causes premature release from polyribosomes of incomplete protein molecules. Biologically inactive proteins may be formed in the presence of low doses of puromycin which may not appreciably influence total amino acid incorporation. Ho and Breinig⁷ have demonstrated slight inhibition of interferon formation in chick embryo cells exposed to 10 μ g/ml of puromycin, a dose that was found to reduce amino acid incorporation into protein by about 67 per cent. We have repeated these studies with Krebs-2 cells in order to compare their susceptibility and to establish a baseline for later experiments.

Figure 1 shows the effect of various concentrations of puromycin on protein, RNA, and interferon synthesis by Krebs-2 cells. Inhibition of H³-leucine incorporation into acid-precipitable protein of uninfected cells required more than 20 μ g/ml of puromycin. Much smaller doses caused marked inhibition of interferon formation. As little as 1 μ g/ml (1.84×10^{-6} *M*) reduced the interferon yield to 50 per cent and 6 μ g/ml to 2 per cent of controls.

We were surprised to find that relatively low doses of puromycin inhibited total RNA synthesis in Krebs-2 ascites cells (Fig. 1). Rabinovitz and Fisher⁸ reported that puromycin does not impair incorporation of adenine into ribosomal or sRNA of Ehrlich ascites cells; doses of puromycin similar to those used in our experiments were required for 90 per cent inhibition of protein synthesis. On the other hand, Holland⁹ has demonstrated considerable reduction in ribosomal RNA synthesis in HeLa cells exposed to 100 μ g/ml of puromycin. It seems evident that different

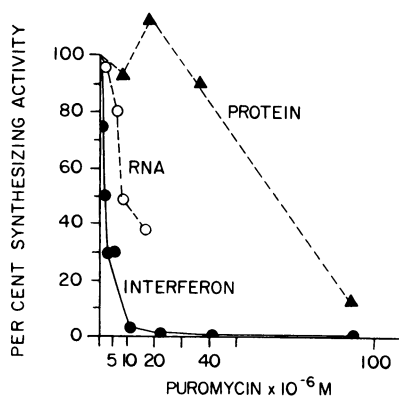


FIG. 1.—Inhibition of interferon, total protein, and RNA synthesis by varying concentrations of puromycin. For the interferon studies Krebs-2 cells were infected with NDV (multiplicity $\cong 5$), washed, suspended in medium-199 to a concentration of 2×10^6 cells/ml, and distributed in 10-ml volumes in siliconized flasks containing puromycin at the concentrations shown. The medium was withdrawn after incubation at 37° for 8 hr, processed to remove cells, residual virus, and puromycin, and assayed by the plaque-reduction method. The interferon titers of two control cultures were each 320 units/ml. Protein synthesis was determined by measuring 1-hr incorporation of H^3 -leucine ($10 \mu\text{c}/\text{ml}$) into the hot PCA-precipitable fraction of 5×10^6 uninfected Krebs-2 cells incubated in puromycin-containing Eagle's medium free of unlabeled leucine. The results are recorded as the % specific activity of puromycin-treated cells based on the specific activity ($14.9 \text{ cpm}/\mu\text{g}$ of protein and 2393

cpm/ml) of control cells incubated in the absence of puromycin. RNA synthesis was determined by measuring 30-min incorporation of H^3 -uridine ($1.29 \mu\text{c}/\text{ml}$) into the cold PCA-precipitable fraction of Krebs-2 cells that had been incubated for 3 hr in puromycin-containing MM-S medium. The RNA results are recorded as % uridine uptake based on control levels in cells incubated without puromycin of $60.7 \text{ cpm}/\mu\text{g}$ of protein and $4632 \text{ cpm}/\text{ml}$. In a separate experiment, $9.2 \times 10^{-5} M$ puromycin reduced RNA synthesis in Krebs-2 cells by 78%.

vertebrate cells vary considerably in their susceptibility to inhibition by puromycin of protein, RNA, and interferon synthesis. Further studies with Krebs-2 cells were performed with puromycin doses of $50 \mu\text{g}/\text{ml}$, $9.2 \times 10^{-5} M$.

Effect of aminonucleoside of puromycin: One possible explanation for the results of the foregoing experiments is that puromycin inhibits interferon synthesis by blocking transcription of cellular RNA in a manner similar to that of actinomycin. Such an effect might be anticipated if a cellular enzyme can hydrolyze off the 3' amino-linked L-O-methyltyrosine group of puromycin leaving the aminonucleoside residue. Farnham¹⁰ has shown that the aminonucleoside of puromycin inhibits RNA synthesis in L cells, although the effect is primarily on the ribosomal fraction rather than on messenger RNA. Total RNA synthesis in Krebs-2 cell was reduced by about 45 per cent after incubation for 3 hr with 0.5 – $2.0 \times 10^{-4} M$ aminonucleoside. To test the possibility that the aminonucleoside is the active puromycin component that inhibits interferon synthesis, NDV-infected Krebs-2 cells were incubated for 8 hr in the presence of $10^{-4} M$ concentrations of either the aminonucleoside or of intact puromycin. No interferon was produced in the presence of puromycin, whereas the aminonucleoside-treated cells produced 80 units/ml as did control cells. It seems unlikely, therefore, that the effect of puromycin on RNA synthesis in Krebs-2 cells is responsible for its capacity to inhibit interferon formation.

Comparative effects of puromycin and actinomycin on interferon synthesis: If puromycin inhibits interferon synthesis primarily by virtue of its capacity to interfere with completion of polypeptide chains, it should be active throughout the cycle of interferon formation. If, on the other hand, the action of puromycin resembles that of actinomycin, it should be less effective in the late stages of interferon formation. To test these alternatives Krebs-2 cells were exposed to puromycin ($50 \mu\text{g}/\text{ml}$) or actinomycin ($2 \mu\text{g}/\text{ml}$) at intervals after induction of interferon synthesis with NDV. This dose of actinomycin reduces total cellular RNA synthesis to about 1 per cent of that in control cells.²

Figure 2 shows the previously described² delay in appearance of interferon and the linear increase in titer in control cultures not exposed to puromycin or actinomycin. Interferon synthesis was shut off when either antibiotic was added to infected cells at 2 or 4 hr. Introduction of actinomycin at 6 or 8 hr did not appreciably reduce the final yields of interferon. However, puromycin inhibited continuing interferon synthesis regardless of when it was added to the cells. The final interferon titers in puromycin-treated cultures were identical to the titers at the time that the antibiotic was introduced.

These data indicate that puromycin acts in this system primarily as an inhibitor of protein synthesis, although multiple metabolic effects cannot be ruled out by these experiments. As shown by the following studies, the interferon-inhibiting action of puromycin is reversible, whereas the effect of actinomycin is not.

Reversal of puromycin inhibition: Previous studies² and the preceding experiments (Fig. 2) suggest that the interferon-specific messenger RNA is stable as indi-

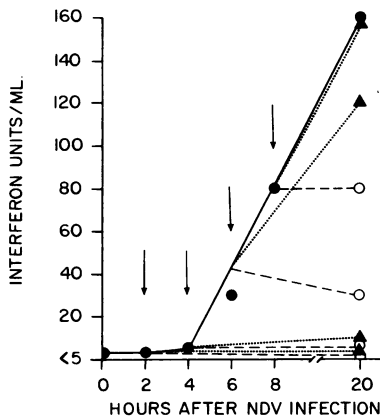


FIG. 2.—Effect on final interferon yields of puromycin (---○) and actinomycin (...▲) added to cultures at varying times after viral induction. The fluids from untreated control cultures (●—●) of 2×10^7 Krebs-2 cells were withdrawn at intervals after NDV infection and assayed for interferon content. At times indicated by the arrows, puromycin ($50 \mu\text{g}/\text{ml}$) or actinomycin ($2 \mu\text{g}/\text{ml}$) was introduced into a series of replica te flasks and their interferon content determined at 20 hr.

cated by continuing synthesis of interferon when actinomycin is added to cultures 6–8 hr after viral induction. If, as has been assumed, puromycin does not affect transcription of the messenger, the prediction can be made that interferon synthesis will resume promptly after puromycin is washed out of inhibited cells.

Figure 3 shows the rate of interferon synthesis by NDV-induced Krebs-2 cells that had been incubated for 8 hr in media containing $50 \mu\text{g}/\text{ml}$ of puromycin, then washed and resuspended in puromycin-free media. After a lag period of about 1 hr, interferon could be detected in the media, and increased in titer to a peak at 6 hr. Moreover, cells incubated with puromycin in the same way and resuspended in media containing $2 \mu\text{g}/\text{ml}$ of actinomycin exhibited equal capacity to resume interferon synthesis on being released from puromycin inhibition. These data indicate that messenger RNA specific for interferon synthesis accumulates in puromycin-blocked cells and is expressed when the puromycin is removed. The actinomycin studies also show that most of the messenger was made during incubation with puromycin rather than after the puromycin is washed out.

It is of additional interest that the lag period for resumption of interferon synthesis is shorter for cells released from puromycin inhibition than is the lag period after primary viral induction (Fig. 3). This finding may mean that more copies of

the messenger are available for interferon synthesis in cells previously exposed to puromycin. However, although there is a 3-hr delay, the rate of interferon synthesis after primary viral induction parallels that of cells released from puromycin inhibition.

No explanation readily comes to mind for the lag period of 1 hr before onset of interferon synthesis after puromycin is washed out of viral-induced cells. The

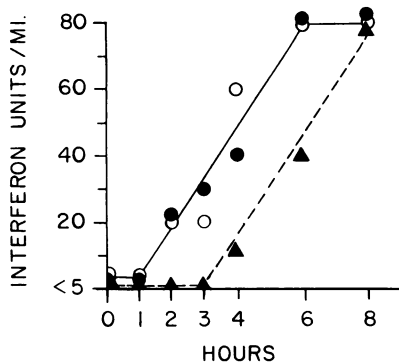


FIG. 3.—Three experiments on onset and rate of interferon synthesis: (1) after reversal of puromycin inhibition (●—●), (2) puromycin inhibition reversed and synthesis proceeding in presence of actinomycin (○—○), and (3) directly after NDV infection in the absence of puromycin (▲---▲). In expt. (1), Krebs-2 cells infected with NDV were suspended in medium containing 50 $\mu\text{g}/\text{ml}$ of puromycin and preincubated at 37° for 8 hr, at which time no interferon could be detected in the medium (0 hr). The cells were then sedimented, washed three times, resuspended in puromycin-free medium, and interferon titers determined at intervals during incubation for an additional 8 hr. In expt. (2), NDV-induced cells preincubated in puromycin for 8 hr were washed and resuspended in puromycin-free medium containing 2 $\mu\text{g}/\text{ml}$ of actinomycin. In expt. (3), interferon synthesis was measured after primary viral induction as in the controls shown in Fig. 2.

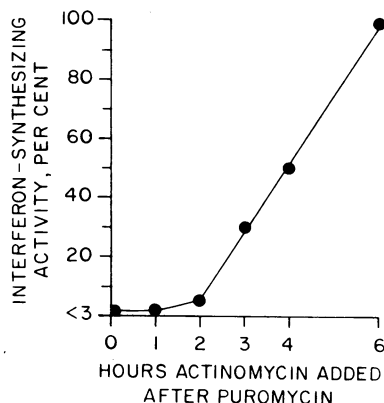


FIG. 4.—Time course of development of interferon-synthesizing capability by puromycin-inhibited cells. Krebs-2 cells were infected with NDV, washed, and immediately suspended in medium containing 50 $\mu\text{g}/\text{ml}$ of puromycin (time 0). At intervals thereafter, actinomycin was added to the media in sets of replicate flasks to give a final concentration of 2 $\mu\text{g}/\text{ml}$. Eight hr after viral induction, the cells in each flask were sedimented by centrifugation, washed three times, suspended in puromycin-free media, and incubated for an additional 12 hr. The fluids were then withdrawn (20 hr after infection) and their interferon titers measured. The results are expressed as the % interferon-synthesizing activity of actinomycin-treated cells based on a titer of 320 units/ml of interferon produced in 12 hr by virus-infected cells previously exposed to puromycin alone for 8 hr.

possibility that puromycin acts on the inducing virus by preventing some early expression of viral function was excluded by delaying exposure to puromycin for 1 hr after NDV infection. When this experiment was done, interferon could be detected once again at 2 hr after washing out the puromycin but not at 30 or 60 min. It also seems unlikely that the 1-hr lag period is due to insensitivity of the interferon assay, because all the requisite messenger RNA should have been available to make a readily detectable amount of interferon in 1 hr. The most plausible explanation for the lag period is incomplete removal of puromycin by the washing procedure, thus delaying resumption of normal protein synthesis.

Despite these difficulties, reversal of puromycin inhibition provides some indica-

tion of the rate of interferon synthesis by cells with optimal levels of messenger RNA. Although the assay method for interferon is relatively crude, a reasonable estimate of the efficiency of the system, once it is primed, is that 2×10^6 Krebs-2 cells can produce interferon at the rate of 16 units/hr.

Kinetics of interferon-specific mRNA synthesis: The reversible effect of puromycin on protein synthesis and the irreversible effect of actinomycin on RNA synthesis permit an indirect analysis of the rate of transcription of the hypothetical mRNA that codes for interferon formation. Advantage can be taken of the fact that non-functioning, stable mRNA should accumulate in the presence of puromycin, and transcription can be stopped by actinomycin added at intervals after viral induction. When puromycin is washed out of the cells, the amount of interferon formed in a given period of time should reflect the relative amount of mRNA transcribed prior to introduction of actinomycin. Such an assay procedure is equivalent, in a sense, to measuring the synthesis of an enzyme by determining its specific activity. The data are expressed as percentages of the 12-hr interferon yields from cells previously exposed to puromycin alone.

Figure 4 shows the rate at which the interferon-synthesizing capacity of puromycin-treated Krebs-2 cells became resistant to actinomycin. No interferon could be detected in cultures exposed to actinomycin 1 hr after viral induction. When actinomycin was added at 2 hr, 15 units/ml of interferon were formed after puromycin reversal. With increasing delay of actinomycin treatment, the interferon-synthesizing capacity of the cells increased linearly, reaching a maximal potential of 320 units/ml at 6 hr after viral induction.

This plot of developing resistance to actinomycin can be taken as a first approximation of the rate of synthesis of interferon-specific mRNA.

Summary.—Puromycin was found to inhibit interferon synthesis in NDV-infected Krebs-2 cells, presumably by preventing completion of polypeptide chains rather than by its concurrent effect on RNA synthesis. Unlike actinomycin, puromycin inhibited interferon synthesis at all stages and the inhibition could be completely reversed by washing out the puromycin. The usual 3-hr lag period before onset of interferon synthesis after primary viral induction could be shortened to 1 hr under conditions of reversal of puromycin inhibition. However, the rates of interferon synthesis were comparable in the two systems. Actinomycin interrupts the development of latent interferon-synthesizing activity in puromycin-blocked cells. These data are interpreted as evidence for induction by NDV of a cellular interferon-specific messenger RNA that accumulates in puromycin-inhibited cells. These experiments also provided a means for estimating the rate of synthesis of the interferon-specific messenger RNA.

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THE MULTIPLE MOLECULAR STRUCTURE OF THE M PROTEINS OF GROUP A STREPTOCOCCI*

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The M proteins are the antigens conferring serological specificity to the 45 or 50 types of group A streptococci. More importantly, these proteins play a predominant role in virulence by rendering the streptococci refractory to phagocytosis in the absence of type-specific antibody. Owing to unusual stability at low pH and high temperature,¹ the M proteins are best obtained from whole cells or streptococcal cell walls by solubilization at pH 2 and 100°. Recent studies have shown that the M proteins may be solubilized from cell walls with a muralytic enzyme released during bacteriophage lysis of group C streptococci.^{2, 3} In spite of these unusual physiological properties, the chemical and physical parameters of the type-specific M proteins heretofore have not been precisely defined.¹ Previous methods for the preparation of M proteins have yielded antigens not entirely pure; analytical techniques such as gel electrophoresis and immunodiffusion when applied have shown the presence of inhomogeneities.^{1, 4, 5}

This communication describes the purification of M proteins from three serotypes of group A streptococci and demonstrates a unique structure of multiple molecular forms within each serotype.

Materials and Methods.—*Streptococci and antisera:* The group A streptococci, types 12, 14, and 24, culture methods, immunodiffusion techniques, and methods for the preparation of adsorbed and unadsorbed typing sera have been previously described.^{5, 6}

Reagents: Crude lytic enzyme of the group C streptococcal bacteriophage system was prepared as previously described.⁶ The crude enzyme was concentrated from the cell lysate by precipitation at 0° in 70% ammonium sulfate. One μ g of enzyme per ml decreased the optical density of a suspension of whole streptococci from 0.20 to 0.10 in 30 min. Buffered saline containing 0.01 M potassium phosphate pH 7.0 was used as the antigen diluent for serological procedures and Sephadex chromatography.

Preparation of M proteins: Streptococci harvested after growth for 18 hr in enriched Difco Todd-Hewitt broth⁵ were washed and ruptured with glass beads in a Gifford-Wood Eppenbach Micro-Mill according to the method of Markowitz and Lange.⁷ For smaller amounts of cells the Braun MSK homogenizer was used to obtain cell walls according to the method of Bleiweis *et al.*⁸ The following is a typical protocol using 130 gm of wet cells broken in the Eppenbach mill. After removal of the glass beads with a coarse sintered glass funnel, the debris composed of cell walls and membranes was washed twice in 2-liter volumes of buffered saline by centrifugation at $9,000 \times g$ for 30 min. All operations were carried out at 0–5°. The cell walls containing the M protein were separated from the membranes by sedimenting the walls at $5,000 \times g$ for 30 min in 2 liters of buffered saline; this step was repeated three times. At each washing the walls were evenly resuspended with a Waring Blender; octyl alcohol was added to prevent foaming. After the