ENHANCEMENT OF HUMAN INTERFERON PRODUCTION BY NEUTRAL RED AND CHLOROQUINE: ANALYSIS OF INHIBITION OF PROTEIN DEGRADATION AND MACROMOLECULAR SYNTHESIS *

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Synthetic double-stranded RNA polymers such as polynosinic:polycytidylic acid (poly I:C)\(^1\) can induce interferon production in cells in vitro (1). Interferon, a glycoprotein (2), is synthesized and secreted into the culture medium. It has been observed that an increase in intracellular interferon content precedes the increase in extracellular interferon (3–5), that most of intracellular interferon present in a postnuclear supernate is associated with membrane vesicles (6), and that vinblastine sulfate inhibits interferon secretion with a concomitant increase in the concentration of intracellular interferon (7). These observations, together with cell fractionation studies carried out recently,\(^2\) strongly suggest that the synthesis and secretion of interferon follows the pathway for secretory proteins outlined by Palade and his colleagues (8–10) for the pancreatic exocrine cell. It is thus likely that interferon is synthesized in the rough endoplasmic reticulum and is processed through the smooth endoplasmic reticulum and the Golgi apparatus, undergoing glycosylation (11) en route to the extracellular space.

The rate of interferon production by human or rabbit cells induced with poly I:C peaks 3–4 h after induction and is rapidly shut off by 6–8 h (4). Interferon production can be enhanced 10- to 1,000-fold by the judicious use of inhibitors of RNA or protein synthesis (4,12). This enhancement is mainly a result of a prolongation of the production of interferon (13). It has therefore been suggested that inhibitors of RNA and protein synthesis interfere with the process which normally shuts off interferon production (4,14).

A paradoxical increase in the amount of a protein or enzyme activity which follows the exposure of a cell system to inhibitors of RNA or protein synthesis was first observed by McAuslan (15), who reported that thymidine kinase activity in HeLa cells infected with cowpox virus increased further when these cells were treated with actinomycin D. This

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1 Abbreviations used in this paper: DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; PBS, phosphate-buffered saline; poly I:C, polynosinic:polycytidylic acid polymer.

2 Falloff, E., E. Havell, J. Lewis, M. Lande, R. Falcoff, D. Sabatini, and J. Vilček. Manuscript in preparation.
phenomenon, termed "superinduction," has now been observed in numerous other systems (16). At least three different hypotheses have been proposed to explain superinduction. The first, initially outlined by McAuslan (15) and later extended by Tomkins and his colleagues (16), proposes that inhibitors of macromolecular synthesis interfere with the transcription or translation of the messenger RNA (mRNA) for a repressor protein which normally limits the translation of the mRNA for the protein under study. This hypothesis requires that the repressor mRNA and repressor protein be relatively unstable. The second hypothesis (17) suggests that the "repressor" protein is not a translational inhibitor but participates in the degradation of the protein which is measured. This hypothesis, in addition to the requirement that the repressor mRNA and repressor protein be relatively unstable, also requires that the protein being measured have a rapid turnover. The third hypothesis is particularly applicable to relatively stable mRNAs and their protein products and states that, under conditions of inhibition of RNA synthesis, the relatively unstable fraction of cytoplasmic mRNA decays and thus the stable mRNA has more of the cellular protein synthetic apparatus available for its translation (18). This could then lead to an increase in the amount of protein products synthesized from the stable mRNA. These hypotheses are not mutually exclusive (19). The shutoff of interferon production could thus be explained by, among other possibilities, a translational repressor (4,14), intracellular degradation of interferon, or an inhibition of the translation of interferon mRNA due to competition by newly synthesized mRNA.

In the present investigation we have attempted to analyze the possible contribution of inhibition of protein degradation to interferon superinduction. It has been observed recently that neutral red and chloroquine inhibit intracellular degradation of protein probably by inhibiting intralysosomal proteases (20). We have therefore used these drugs to investigate the question whether inhibition of intralysosomal degradation of interferon could explain interferon superinduction.

We report that neutral red and chloroquine enhance human interferon production. However, a detailed analysis indicates that the superinduction seen correlates with inhibition of macromolecular synthesis. Furthermore, inhibition of intralysosomal proteolysis does not appear to contribute to or affect this phenomenon. The results are compatible with the view that interferon is an "export" protein.

Materials and Methods

Cell Cultures. A human diploid fibroblast strain (FS-4) which has been identified as a high interferon producer (5,21), has been used throughout. The cells were grown to confluency in 35 or 60 mm Falcon plastic Petri dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) in Eagle's minimal essential medium (22) supplemented with 10% heated (56°C for 0.5 h) fetal bovine serum (Grand Island Biological Co., Grand Island, N. Y.). Incubation was in a humidified CO2 incubator at 37°C. The medium in 60-mm cultures was changed 6-8 days after plating. The cultures were usually used between 9 and 15 days after plating.

Chemicals and Radiochemicals. Poly I:C was obtained from the Antiviral Substances Program, Infectious Disease Branch, National Institute of Allergy and Infectious Diseases, Bethesda, Md. Actinomycin D and cycloheximide were purchased from Merck, Sharp and Dohme Research Laboratories, Rahway, N. J. and Polysciences, Inc., Warrington, Pa., respectively. Neutral red (analytical reagent, Koch-Light Laboratories, Ltd., Bucks, England) was a gift from Dr. Brian Poole of The Rockefeller University, New York, while chloroquine diphosphate was purchased from Sigma Chemical Co., St. Louis, Mo. Appropriate concentrations of actinomycin D, cycloheximide, and chloroquine were made up in maintenance medium (Eagle's medium supplemented with 2% heated fetal bovine serum) just before use. Neutral red was stored as a 1% sterile solution.
in distilled water and was diluted to the appropriate concentration in Eagle's medium without
serum within 60 s of use. [5-3H]uridine (21 Ci/mmol), L-[carboxyl-14C]leucine (57 mCi/mmol), and
L-[4,5-3H]leucine (44 Ci/mmol) were purchased from Schwarz/Mann Div., Becton, Dickinson & Co.,
Orangeburg, N. Y.

Interferon Induction. Interferon was induced by exposing confluent 35- or 60-mm cultures,
washed once with warm phosphate-buffered saline (PBS) (23), to 1 or 2 ml, respectively, of a
solution of poly I:C (100 µg/ml in Eagle's medium) for 1 h at 37°C. The cultures were then washed
four times with warm PBS and incubated with 1 or 2 ml of maintenance medium at 37°C. The
beginning of poly I:C treatment is designated as "0 h" in all experiments.

Interferon Titrations. Interferon released into culture medium was titrated using the semimi-
cromethod (24) modified by Havell and Vilcek (21). Briefly, wells of a plastic tissue culture plate
(Multi-dish Disposo-Tray, model IS-FB-96-TC; Linbro Chemical Co., New Haven, Conn.) were
filled with 100 µl of Eagle's medium containing 5% heated fetal bovine serum and duplicate serial
twofold dilutions of each sample to be assayed were prepared using an automatic micropipette. A
suspension of FS-4 cells in 100 µl of the same medium was then added to each well (30,000
cells/well). After 18–20 h of incubation, approximately 1,000 plaque-forming units of vesicular
stomatitis virus (Indiana strain) were added to each well in 50 µl of the same medium. The highest
dilution of the assay sample, which protected at least 50% of the cell sheet from the cytopathic
action of the virus as scored 36–48 h after addition of the virus, was taken as the end point dilution.
The geometric mean titer for each sample is expressed in terms of the 69119 reference standard for
human interferon (obtained from the Antiviral Substances Program, Infectious Disease Branch,
National Institute of Allergy and Infectious Diseases, Bethesda, Md.).

Rates of Precursor Transport and RNA and Protein Synthesis. RNA and protein synthesis was
measured in duplicate 35-mm cultures as follows: the medium was decanted and 0.5 ml of Eagle's
medium containing 50 µCi/ml of [3H]uridine and 1 µCi/ml of [14C]leucine was added to each culture
dish. After mixing, the cultures were incubated at 37°C for 10.25 min, rapidly washed 10 times
with cold PBS (4°C), and solubilized in 1 ml of 1% sodium-dodecyl-sulfate. 1 ml of 20% TCA was
then added and the mixture kept at 4°C for at least 0.5 h. Samples of 10 µl in duplicate were spotted
on Whatman GF/C glass fiber filters, dried, and the radioactivity counted in a liquid scintillation
spectrometer. This gave a measurement of the precursor transport into cells. The remaining
sample was filtered through Whatman GF/C filters, and the precipitate was washed thrice with
5% TCA and twice with ethanol. The radioactivity on the dried filters was measured as above.
TCA-precipitable [3H]uridine and [14C]leucine counts were corrected for inhibition of precursor
uptake into drug-treated cells in order to determine the rate of RNA or protein synthesis. It is
assumed that there was no alteration in the processing of the precursors after transport into drug-
treated cells.

Measurement of Protein Degradation. The rate of protein degradation was determined using
the procedure of Poole and Wibo (25). Rapidly turning over protein was monitored by labeling cells
in 60 mm dishes with [14C]leucine, 10 µCi/ml in 2 ml maintenance medium for 1 h, washing the
cultures with warm PBS, and incubating for a further 30 min in 5 ml maintenance medium to
allow rapid efflux of intracellular unincorporated precursor. The cells were then washed four
times with warm PBS and the cultures were incubated in 2 ml of maintenance medium containing
the appropriate drug. With neutral red, Eagle's medium was used. After 1 or 5 h of incubation the
medium was harvested, the cells washed four times with cold PBS, and the washed cells dissolved
in 0.1 M NaOH-0.4% sodium deoxycholate. Total and 8% TCA-soluble radioactivity was measured
on each sample of cells and medium as described by Poole and Wibo (25). TCA-soluble radioactivity
in the medium expressed as a fraction of the total recovered in dissolved cells and medium,
represents the extent of protein degradation.

Degradation of slowly turning over protein was followed by labeling cells in 60-mm dishes with
10 µCi/ml [14C]leucine for 24 h in 2 ml maintenance medium and then incubating for 16–24 h in 5 ml
of unlabeled maintenance medium. The cells were then washed four times with PBS and protein
solubilization over subsequent time intervals determined as described above. In some experiments
the degradation of rapidly and slowly turning over proteins was measured in the same culture by
first labeling the cells for 24 h with [14C]leucine (1 µCi/ml) followed by incubation in unlabeled
medium for 16–20 h and then labeling again for 1 h with [3H]leucine (10 µCi/ml). Solubilization of
14C and 3H radioactivity in the subsequent time interval provides a measure of the rate of
degradation of the slow and rapid components, respectively.
Normalized Dose-Effect Plots. These were constructed by determining the inhibitory effects of a drug at varying concentrations on RNA and protein synthesis and on the rate of degradation of the rapidly turning over protein, and the stimulatory effect on interferon production. The extent of inhibition observed at a particular drug concentration was normalized with respect to the maximum effect observed in the experiment. The increase in interferon titer over the drug-free control was expressed as a percentage of the maximum increase obtained in the experiment.

Results

The Enhancing Effect of Neutral Red. Exposure of FS-4 cells to poly I:C leads to a prompt induction of interferon (5,13). Production begins by 1.5–2 h after poly I:C treatment, peaks at 3–4 h, and is shut off by 6–8 h. If such cells are treated with actinomycin D between 2.5 and 3.5 h after poly I:C induction, there is a moderate increase in the rate of production and a marked prolongation of the period of interferon production (P. B. Sehgal, unpublished observations). The increase in cumulative interferon yield subsequent to actinomycin D treatment is referred to as "superinduction." We have carried out similar experiments and compared the effect of neutral red with that of actinomycin D.

FS-4 cells in 60 mm dishes in duplicate were induced with poly I:C and treated with neutral red (80 μg/ml in Eagle's medium) or actinomycin D (1 μg/ml) for 1 h between 2.5 and 3.5 h. The cultures were then washed four times with PBS and one dish was incubated, with repeated medium changes, to monitor the rate of interferon production. The second dish was incubated to 24 h to measure cumulative interferon yield. Control cultures without any drug treatment were run in parallel.

Fig. 1 shows that in untreated control cultures interferon production is shut off rapidly while actinomycin D-treated cultures continue to produce interferon at a high rate and for a long period. Neutral red-treated cultures also showed an increase in the rate of production and a prolongation of the period of production of interferon. The resulting cumulative yields in neutral red- and actinomycin D-treated cultures were 16- and 48-fold higher, respectively, than in the untreated controls. In different experiments, the increase in interferon yield caused by neutral red has ranged between 16- and 64-fold.

Dose-Response Analysis of the Effects of Neutral Red. Preliminary experiments revealed that neutral red, in addition to inhibiting proteolysis, could also inhibit cellular RNA and protein synthesis. It was therefore necessary to determine whether interferon superinduction by this drug is causally linked to the inhibition of proteolysis or the inhibition of macromolecular synthesis. To distinguish between these two alternatives, normalized dose-effect plots of the responses involved were constructed. In principle, two effects of a drug that are causally linked should appear at the same concentration of the drug and the normalized dose-effect plots should be closely similar. Deviations from this ideal would be expected from a threshold requirement of one effect in order to produce the second effect, and from considerations of drug toxicity. The process of normalizing the effect observed at a given concentration of the drug to the maximum obtained in the system eliminates the absolute magnitude of an effect from this analysis.

Replicate cultures in 35 mm dishes were induced with poly I:C and treated with neutral red at varying concentrations between 2.5 and 3.5 h. Four cultures were used at each concentration. At the end of the neutral red treatment two cultures at each concentration were washed thoroughly
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FIG. 1. Enhancement of interferon production by neutral red and actinomycin D. Poly I:C-induced cultures of FS-4 cells were treated with neutral red (○) or actinomycin D (▲) between 2.5 and 3.5 h and the rate of subsequent interferon production monitored in these and untreated controls (●). The numbers in the figure indicate the cumulative interferon yield from parallel cultures.

with warm PBS and 1 ml of maintenance medium was added to each dish. The cells were then incubated until 24 h at which time interferon was assayed in the medium separately for each culture. The other two cultures were used to measure the rate of RNA and protein synthesis by pulsing the cells with \(^{3}H\)uridine and \(^{14}C\)leucine for 10.25 min in 0.5 ml Eagle's medium at 3.5 h (see Materials and Methods). The inhibition of degradation of the rapidly turning over protein caused by 1 h of treatment of FS-4 cells with neutral red was determined separately using the procedure of Poole and Wibo (25) as outlined in the Materials and Methods.

Fig. 2A shows the effects of neutral red at varying concentrations on several processes expressed as percent of control rates in the absence of the drug. Fig. 2B gives the normalized dose-effect curves for the various effects of the drug. It can be seen in Fig. 2A that neutral red strongly inhibits the transport of \(^{3}H\)uridine into cells. This inhibition can be detected at a drug concentration as low as 1 µg/ml (data not shown). It is therefore necessary to correct TCA-precipitable \(^{3}H\)uridine radioactivity for the inhibition of uridine transport to arrive at the rate of RNA synthesis. Inhibition of \(^{14}C\)leucine transport is also seen but at concentrations of neutral red (150–200 µg/ml) that are cytotoxic as judged by light microscopy 20 h after drug treatment. The rate of RNA synthesis is reduced by 60% and that of protein synthesis by more than 90% at the highest neutral red concentrations used. In contrast Table IA and Fig. 2A show that neutral red inhibits the rate of protein degradation only moderately; the maximum inhibition was approximately 25%.

Fig. 2B presents the normalized data derived from values in Fig. 2A and Table IA. In addition, a normalized dose-effect plot for interferon superinduction is also depicted. It can be seen that superinduction of interferon occurs in the same concentration range of neutral red as inhibition of RNA and protein
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Fig. 2. Relation between concentration of neutral red and effects on RNA and protein synthesis, protein degradation, and interferon superinduction. (A) RNA and protein synthesis and the extent of protein degradation (rapid component) measured 1 h after the beginning of neutral red treatment. Uptake of \[^{3}H\]uridine (○), uptake of \[^{14}C\]leucine (△), RNA synthesis (●), protein synthesis (▲), and protein degradation (┄┄┄) were measured. The control (100%) values were \(6.33 \times 10^4\) cpm for cellular uptake of \[^{3}H\]uridine, \(2.04 \times 10^4\) cpm for TCA-precipitable \[^{3}H\]uridine radioactivity, \(5.6 \times 10^3\) cpm for \[^{14}C\]leucine uptake, and \(7.9 \times 10^1\) cpm for TCA-precipitable \[^{14}C\]leucine radioactivity. The protein degradation curve is based on data in Table IA. (B) Normalized dose-effect curves of inhibition of RNA (●) and protein (▲) synthesis, of inhibition of protein degradation (○), and of interferon superinduction (■) are shown.

synthesis. Maximum superinduction occurs at a neutral red concentration of 80 μg/ml. This concentration is only slightly lower than the concentrations at which near maximal inhibition of RNA and protein synthesis is observed. In contrast, the inhibition of proteolysis occurs in a lower concentration range and reaches a maximum at 60 μg/ml of neutral red. Thus it is well resolved from interferon superinduction.

The dose-effect curves for inhibition of RNA and protein synthesis cannot be clearly resolved from each other or from the ascending limb of the interferon superinduction curve. The interferon superinduction curve deviates from the inhibition curves for RNA and protein synthesis in two regions. Interferon superinduction shows a marked lag as neutral red concentration is increased. This could reflect a "threshold" requirement for superinduction, as macromolecular syntheses may have to be inhibited beyond a minimal extent before inter-
Neutral red and chloroquine on the degradation of rapidly turning over protein

Table 1

| Drug concentration (μg/ml) | TCA-soluble radioactivity* in medium after 1 h of drug treatment | % of total radioactivity in system† (±SE) | % of control |
|---------------------------|---------------------------------------------------------------|-----------------------------------------|-------------|
|                           |                                                               |                                        |             |
| A. Neutral red            |                                                               |                                        |             |
| 0                         | 10.84 ± 0.45                                                  | 100                                     |             |
| 5                         | 10.53 ± 0.10                                                  | 97.1                                    |             |
| 10                        | 10.24 ± 0.42                                                  | 94.5                                    |             |
| 30                        | 8.80 ± 0.06                                                   | 81.2                                    |             |
| 60                        | 8.20 ± 0.09                                                   | 75.7                                    |             |
| 100                       | 8.35 ± 0.09                                                   | 77.0                                    |             |
| 200                       | 8.21 ± 0.04                                                   | 75.8                                    |             |
| B. Chloroquine            |                                                               |                                        |             |
| 0                         | 15.90 ± 0.13                                                  | 100                                     |             |
| 25                        | 14.31 ± 0.03                                                  | 90.0                                    |             |
| 50                        | 13.07 ± 0.05                                                  | 82.2                                    |             |
| 125                       | 12.97 ± 0.51                                                  | 81.5                                    |             |
| 250                       | 13.27 ± 0.97                                                  | 83.4                                    |             |
| 375                       | 12.78 ± 0.01                                                  | 80.4                                    |             |

* [3H]leucine radioactivity determined in duplicate at each concentration as described in the Materials and Methods.
† The system contained 35,000 cpm in the neutral red experiment and 80,000 cpm in the chloroquine experiment.

Interferon superinduction becomes detectable. Interferon superinduction falls off at the higher neutral red concentrations, which is probably due to a cytotoxic effect of the drug. That this is very likely is indicated by the fact that there is microscopic evidence of cytotoxicity in cultures treated with neutral red at a concentration of 100 μg/ml or higher.

Taken together, these observations suggest that interferon superinduction by neutral red is the result of inhibition of RNA or protein synthesis or both. The inhibition of proteolysis by neutral red, though significant, is small. Furthermore, the clear separation between the dose-response curves of interferon superinduction and the inhibition of protein degradation (Fig. 2 B) suggests that inhibition of proteolysis plays a very minor role, if any, in the enhancement of interferon production by neutral red.

Dose-Response Analysis of the Effects of Chloroquine. Chloroquine was examined for a possible enhancing effect on interferon production in a manner similar to that used with neutral red. Poly I:C-induced cultures were treated with chloroquine (50 μg/ml to 1,000 μg/ml) in maintenance medium between 2.5 and 3.5 h after induction and the interferon yield was measured between 3.5 and 24 h. Such a treatment enhanced interferon production and the maximum effect, seen at 375 μg/ml chloroquine, was a 4- to 16-fold increase in interferon yield (data not shown). Chloroquine concentrations of 500 μg/ml or higher led to
microscopic cytotoxicity. Preliminary experiments also revealed that at these concentrations of the drug, there was marked inhibition of RNA and protein synthesis in addition to inhibition of protein degradation.

An investigation of dose-effect relationships with chloroquine gave results which are summarized in Fig. 3 and Table I B. Fig. 3 A shows that the transport of [3H]uridine into cells was moderately inhibited at the higher chloroquine concentrations while that of [14C]leucine was unaffected, except at very high concentrations of the drug (not shown). Chloroquine, like neutral red, had a greater inhibitory effect on protein synthesis than on RNA synthesis. At the highest concentrations used, chloroquine reduced the rate of RNA synthesis by 80% and that of protein synthesis by 99%. The rate of degradation of rapidly turning over protein (see Materials and Methods) was inhibited to a significant but small extent; at a concentration of 375 μg/ml, chloroquine caused 20% inhibition (Table I B and Fig. 3 A).
The normalized dose-effect plots derived from these data are shown in Fig. 3 B together with a plot for interferon superinduction obtained from the same experiment. Interferon superinduction was clearly detectable at 50 µg/ml chloroquine, reached a maximum at 375 µg/ml, and declined thereafter. In contrast, the inhibition of proteolysis was 51% of its maximum at 25 µg/ml and was near maximal at 50-125 µg/ml of the drug. Most of the increase in interferon superinduction was observed after the inhibition of proteolysis had reached its maximum. This feature, together with the large difference in the middle portions of the two plots, strongly suggests that inhibition of protein degradation cannot be a major contributing factor to interferon superinduction by this drug.

As would be expected from data in Fig 3 A, the normalized plots of the inhibitions of macromolecular syntheses show a partial resolution between the inhibitions of RNA and protein synthesis (Fig. 3 B). A comparison of these curves with the interferon superinduction plot reveals a considerable lag in interferon superinduction at the lower chloroquine concentrations. However, because interferon superinduction reaches a peak at a concentration which is close to the concentrations at which near maximal inhibition of protein and RNA synthesis is observed, it is possible that interferon superinduction is causally related to inhibition of macromolecular synthesis.

The marked discrepancy between interferon superinduction and RNA synthesis inhibition curves at the lower drug concentrations could be the result of several modifying factors. First, as indicated before for neutral red, the detection of interferon superinduction could require the inhibition of RNA synthesis to exceed a threshold level. Second, since interferon production itself requires protein synthesis, the severe inhibition of protein synthesis produced by chloroquine could partially prevent potential enhancement of interferon yield through the superinduction mechanism. This could serve to raise the apparent threshold level for the inhibition of RNA synthesis for interferon superinduction.

Since interferon yield was determined between 3.5 and 24 h, the rapidity with which an inhibition of protein synthesis might reverse on removal of the drug at 3.5 h would also affect the superinduction curve. A slower reversal would reduce the interferon yield. That inhibition of protein synthesis is indeed modifying interferon superinduction by chloroquine is suggested by the observation that the maximal superinducing effect of this drug has ranged between a 4- and 16-fold increase over the inhibitor-free control whereas a 16- to 64-fold increase was seen with neutral red. Therefore, despite the discrepancy noted above, it appears possible that interferon superinduction by chloroquine is causally related to an inhibition of macromolecular synthesis.

Effects of Actinomycin D and Cycloheximide on Protein Degradation. The effects of actinomycin D and cycloheximide on the rate of protein degradation were determined under conditions which are known to enhance interferon production (4). The rates of degradation of rapidly and slowly turning over protein were measured simultaneously during a 1 h treatment with 1 µg/ml of actinomycin D or a 5 h treatment with 5 or 50 µg/ml of cycloheximide using the double label method of Poole and Wibo (25).

The data presented in Table II A show that actinomycin D treatment did not affect the rate of degradation of either the rapidly or the slowly turning over
TABLE II
Effect of Actinomycin D and Cycloheximide on the Degradation of Rapidly and Slowly Turning Over Protein

| Treatment          | [\textsuperscript{3}H]leucine | [\textsuperscript{14}C]leucine |
|--------------------|------------------------------|-----------------------------|
|                    | % of total radioactivity in system\(\%\) \(\text{SE}\) | % of control \(\%\) | % of total radioactivity in system\(\%\) \(\text{SE}\) | % of control \(\%\) |
| A. Actinomycin D, 1 \(\mu\)g/ml, 1 h | 13.40 ± 0.51 | 103 | 1.59 ± 0.10 | 96.7 |
| Control            | 12.99 ± 0.26 | 1.65 ± 0.16 |
| B. Cycloheximide, 50 \(\mu\)g/ml, 5 h | 27.71 ± 0.92 | 90.8 | 3.67 ± 0.46 | 64 |
| Control            | 30.57 ± 0.39 | 5.74 ± 0.65 |

* Duplicate cultures were labeled with both [\textsuperscript{3}H]leucine and [\textsuperscript{14}C]leucine in order to follow the degradation of rapidly and slowly turning over protein, respectively, as described in the Materials and Methods.

† Approximately 85,000 cpm.

§ Approximately 37,000 cpm.

protein. These observations clearly indicate that interferon superinduction by actinomycin D cannot be attributed to inhibition of proteolysis. Treatment of cells with cycloheximide, 50 \(\mu\)g/ml, for 5 h caused a significant reduction in the rate of protein degradation (Table II B). The slow component was inhibited by 30–40%, but the rapid component by only 10%. Similar effects were also seen at 5 \(\mu\)g/ml of the drug. A dissociation between the turnover of these two components has been noted earlier by Poole and Wibo (25), who observed that the addition of conditioned medium to rat embryo fibroblasts in culture increased the degradation of the slow component without affecting that of the rapid component. These observations suggest that the two components turn over by distinct cellular mechanisms.

Treatment of Cells for a Prolonged Period with Chloroquine at Low Concentration. A drawback in the above discussion of the possible role of inhibition of protein degradation in interferon superinduction is that the magnitude of the inhibitory effect of drugs on the rate of proteolysis was small in the experiments described, amounting to approximately 20–25% reduction in the rate. While the results obtained indicate that inhibition of proteolysis does not play a major role in interferon superinduction, they leave open the question as to whether such an inhibition could contribute to superinduction produced through another mechanism. It was therefore necessary to set up conditions under which considerable inhibition of protein degradation could be obtained. The procedure we have used is based on the idea that chloroquine is concentrated in lysosomes as a result of facilitated diffusion (20). It has been suggested that uncharged chloroquine molecules diffusing through the lysosomal membrane are protonated at intraly-
sosomal pH and hence cannot readily diffuse back into the cytosol. This would then lead to a progressive increase in intralysosomal chloroquine concentration which, in turn, would result in a greater inhibition of protein degradation (20).

We therefore exposed FS-4 cells to chloroquine at low concentration for a prolonged period of time with the view of obtaining a considerable inhibition of proteolysis without significant cytotoxicity.

Preliminary experiments revealed that an exposure for 24–36 h to 50 μg/ml of chloroquine was toxic to confluent FS-4 cells. Hence chloroquine concentrations below 50 μg/ml and treatment periods up to but not exceeding 24 h were explored further. Turnover of rapidly turning over protein could not be measured under these conditions using the procedure outlined in the Materials and Methods. Sequestration in the dilated vacuolar system of chloroquine-treated cells of [3H]leucine radioactivity taken up during the pulse probably led to subsequent release of large amounts of TCA-soluble [3H] radioactivity into the culture medium. This interfered with the determination of [3H] radioactivity released as a result of protein degradation. Therefore, in the initial experiments, we measured the rate of turnover of the slow component. For this, the cells were labeled before drug treatment and chloroquine was present only during the 16–24 h chase with unlabeled medium (see Materials and Methods).

Fig. 4A presents the results of an experiment in which the rate of degradation of the slow component was measured after a 16 h exposure to chloroquine at varying concentrations. Increasing the chloroquine concentration up to 25 μg/ml increased the inhibitory effect. There was little further increase in inhibition between 25 and 50 μg/ml at which concentrations the rate of protein degradation was decreased by 35–40%. These two concentrations were used to determine the effect of increasing the period of drug treatment. Fig. 4B shows that increasing the duration of drug treatment increased the inhibitory effect. Again there was only a small difference between the effects of 25 and 50 μg/ml of chloroquine. Chloroquine treatment for 24 h at a concentration of 25 μg/ml caused a 35–40% reduction in the rate of proteolysis. Since this treatment was minimally toxic to the fibroblasts it was adopted for subsequent experiments.

The effect of a 24 h treatment with 25 μg/ml of chloroquine on the degradation of the rapidly turning over protein was determined using a procedure that permits such a measurement, but is less accurate than the method used in the experiments reported above. Chloroquine-treated and control cultures in 35 mm dishes were pulse labeled with [3H]leucine (10 μCi/ml) in 1 ml of maintenance medium for 1 h. At the end of this time the cultures were washed 10 times with warm PBS and then incubated in 2 ml of maintenance medium. Beginning at the termination of the pulse and at varying times thereafter, cultures in triplicate were processed for the determination of residual TCA-precipitable radioactivity using the procedure described in the Materials and Methods for the determination of the rates of macromolecular syntheses.

Fig. 5 shows that prolonged treatment with chloroquine at low concentration inhibits solubilization of rapidly turning over protein as determined by residual TCA-precipitable radioactivity. In three separate experiments the inhibition in the rate of protein degradation was estimated to be 31, 45, and 72%. The variation in these results probably reflects the error involved in calculating the rate of protein degradation from curves of TCA-precipitable radioactivity. The results, however, serve to indicate that chloroquine treatment does considerably decrease the rate of proteolysis of rapidly turning over protein.

The rates of RNA and protein synthesis after a 24 h exposure to 25 μg/ml chloroquine were also determined using the procedure described in the Materials and Methods. It was found that the rate of protein synthesis was inhibited.
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FIG. 4. Effect of a prolonged treatment with chloroquine on protein degradation (slow component). (A) Effect of varying chloroquine concentration. The control (100%) rate was 3.62% protein solubilization in 2 h, equivalent to 25,000 cpm. (B) Effect of varying duration of treatment. The control (100%) rate was 3.80% protein solubilization in 2 h, equivalent to 15,000 cpm.

FIG. 5. Protein degradation in cells treated with chloroquine at a concentration of 25 μg/ml for 24 h (●) and in untreated controls (○). TCA-precipitable radioactivity at "0" h was $7.6 \times 10^6$ cpm/dish in both instances.

by 40–45%. The rate of $[^3]H$urdine transport into cells was reduced by 15–20% but the rate of accumulation of TCA-precipitable uridine radioactivity was increased to almost 200% of that seen in chloroquine-free controls. This result could be due to a reduction in the size of the intracellular precursor pool, an actual increase in the rate of RNA synthesis, or an inhibition of the degradation of very rapidly turning over RNA. We cannot distinguish among these or other possibilities at the present time.

Effect of Prolonged Chloroquine Treatment on the Shutoff of Interferon Production. As indicated earlier, a major characteristic of interferon superinduction is an inhibition of the rapid shutoff of interferon production. A slowing of the kinetics of this shutoff after chloroquine treatment would provide a sensitive indication of the participation of protein degradation in the shutoff.
Fig. 6. Kinetics of interferon production in cells treated with chloroquine at a concentration of 25 \( \mu \text{g/ml} \) for 24 h (\( \bigcirc \)) and in untreated controls (\( \bullet \)).

Since it has already been reported that chloroquine can inhibit the production of interferon induced in chick embryo fibroblasts by ultraviolet-irradiated Newcastle disease virus (26), the appropriate test for its effect on superinduction could not be based on the amount of interferon produced, but could be based on the shape of the curve describing the kinetics of interferon production.

Cultures in 60 mm dishes were treated with 25 \( \mu \text{g/ml} \) chloroquine in maintenance medium for 24 h. Untreated control cultures and drug-treated cultures were washed four times with warm PBS and induced with poly I:C for 1 h. The cultures were again washed thoroughly with warm PBS and the rate of interferon production followed by repeated collection of drug-free maintenance medium and assay for interferon.

Fig. 6 shows that pretreatment of cultures with chloroquine did inhibit interferon production. Furthermore, the peak in the rate of interferon production was delayed in chloroquine-treated cultures. However, the shutoff of production occurred just as promptly in drug-treated cultures as in the control culture. Preliminary experiments (data not shown) on the kinetics of intracellular interferon accumulation, also showed an inhibition of interferon synthesis and a shutoff of synthesis in drug-treated cultures similar to that seen in controls. The lag in the peak rate of interferon production in chloroquine-pretreated cells was also observed when intracellular interferon was measured. We have also determined that incubation of drug-treated cultures in chloroquine-free maintenance medium leads to a very slow reversal of the inhibition of protein degradation. The inhibition of the rate of proteolysis 6 h after chloroquine removal was approximately 60% of that seen immediately after 24 h chloroquine pretreatment (data not shown). Further, treatment of cells with poly I:C did not alter the rate of proteolysis. We therefore suggest that intralyso-
INTERFERON SUPERINDUCTION BY NEUTRAL RED AND CHLOROQUINE

Table III

Interferon Superinduction by Actinomycin D in Chloroquine-Pretreated Cultures

| Drug treatment | Interferon yield†, ref. units/ml | Fold increase with actinomycin D |
|----------------|----------------------------------|---------------------------------|
|                | Chloroquine*, 25 μg/ml, 24 h     |                                 |
| A              | Actinomycin D, 1 μg/ml, 0.5 h    |                                 |
|                |                                 | 16                              |
|                |                                 | -                               |
|                |                                 | -                               |
|                |                                 |                                 |
| B              |                                 | 7                               |
|                |                                 | +                               |
|                |                                 | +§                              |
|                |                                 | 91                              |
|                |                                 |                                 |
| C              |                                 | 5                               |
|                |                                 | +                               |
|                |                                 | +$                              |
|                |                                 | 91                              |
|                |                                 |                                 |

* Cultures were treated with chloroquine for 24 h before poly I:C induction.
† Interferon yield per milliliter was measured for the period from removal of actinomycin D to 24 h. Control cultures not treated with actinomycin D were run in parallel.
§ From 3.0 to 3.5 h with respect to beginning of poly I:C induction.
| From 3.5 to 4.0 h.

somal degradation does not play a significant role in the shutoff of interferon production.

Interferon Superinduction by Actinomycin D in Chloroquine-Treated Cells. If inhibition of protein degradation contributes significantly to interferon superinduction, the effectiveness of actinomycin D in enhancing interferon yields should be greater in chloroquine-treated cultures. Table III summarizes an experiment in which the superinducing effect of actinomycin D in chloroquine-treated cultures (25 μg/ml for 24 h) was compared to that in cultures which did not receive chloroquine. It is seen that exposure of cultures not treated with chloroquine to actinomycin D between 3 and 3.5 h after poly I:C induction led to a 16-fold increase in interferon produced between 3.5 and 24 h. Treatment of cultures with chloroquine reduced the amount of interferon produced in both actinomycin D-treated and -untreated cultures. However, the fold increase in interferon yield on actinomycin D treatment was similar in cultures treated or not treated with chloroquine. This result strongly suggests that an inhibition of intralysosomal protein degradation does not play a significant role in the superinduction of interferon production.

Discussion

We have shown that neutral red and chloroquine enhance poly I:C-induced interferon production by FS-4 cells in vitro. The two drugs inhibit the rate of cellular protein degradation in these cells (Table I). This observation confirms an earlier report that neutral red and chloroquine reduce the rate of proteolysis in cells cultured in vitro (20). In addition, however, the two drugs were found to inhibit RNA and protein synthesis (Figs. 2 A and 3 A). Normalized dose-effect curves were used to relate the inhibition of macromolecular synthesis and that
of cellular protein degradation to the enhancement of interferon production. Interferon superinduction curves were clearly resolved from those of inhibition of protein degradation, but were generally close to those describing inhibition of macromolecular synthesis (Figs. 2 B and 3 B). We therefore suggest that the enhancement of interferon production observed with neutral red and chloroquine is causally linked only to the inhibition of macromolecular synthesis. This suggestion is further strengthened by the observations that the kinetics of shut-off of interferon production (Fig. 6) and the effectiveness of actinomycin D superinduction (Table III) are unaltered in chloroquine-treated cultures under conditions in which the rate of protein degradation was reduced by approximately 40%.

Vilcek and his colleagues have proposed that superinduction of interferon production by inhibitors of macromolecular synthesis is the result of an interference with the function of a post-transcriptional control element which exerts an inhibitory effect on interferon production and hence leads to its normal rapid shut off (4,5,14). Since interferon can be superinduced by inhibitors of both RNA and protein synthesis, it has been suggested that this control element consists of a repressor mRNA and its protein product which presumably inhibits the translation of interferon mRNA. The hypothesis that inhibition of RNA synthesis and interferon superinduction are causally linked has received strong support from our recent findings that 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), a reversible and selective inhibitor of nuclear heterogeneous RNA synthesis, markedly enhances poly I:C-induced interferon production in FS-4 cells (13). With DRB, a normalized dose-effect plot shows a close correlation between the superinducing effect of the drug and inhibition of RNA synthesis. Drug-containing cultures continue to produce interferon for up to 4 days. Removal of DRB at any time during this period leads to a prompt shut off of interferon production. The present conclusion that the enhancing effect of neutral red and chloroquine are closely linked to inhibition of macromolecular synthesis is entirely consistent with the repressor hypothesis.

It is of interest to note that, in addition to known inhibitors of RNA or protein synthesis, several other agents such as diethylaminoethyl-dextran (27), ultraviolet irradiation of cells (28,29), ascorbic acid (30), tilorone (31), vinblastine (7), and osmotic and ionic (orthophosphate) shock (P. B. Sehgal, unpublished observations) have been found to prevent the shut off of interferon production or to increase its yield or both. The superinducing effect of most of these agents has been best seen under conditions which just fail to produce microscopic cytotoxicity. In the light of our results with neutral red and chloroquine we suggest that these agents may also enhance interferon production as a result of inhibition of macromolecular synthesis. We further suggest that any agent used at just below cytotoxic levels and under appropriate experimental conditions may lead to an inhibition of the shut off of interferon production, if it has an inhibitory effect on RNA or protein synthesis.

Electron microscope studies of Fedorko et al. (32,33) have suggested that chloroquine might block the cellular secretory pathway at the level of the Golgi apparatus. We have therefore attempted to determine whether the delay in the peak rate of interferon production seen in chloroquine-treated cells (Fig. 6) is the
result of such a block. Preliminary results (data not shown) have indicated that a corresponding delay can be observed in intracellular interferon accumulation. This suggests that the secretory pathway in such cells is still largely intact.

An important corollary of our unsuccessful attempts to link intralysosomal protein degradation to the shutoff of or the superinduction of interferon production is that significant amounts of interferon do not pass into the lysosomal compartment. It therefore appears that interferon is a cellular product destined essentially for secretion into the extracellular milieu.

Summary

Two lysosomotropic drugs, neutral red and chloroquine, enhance polyinosinic-polycytidyllic acid-induced interferon production by a strain of diploid human fibroblasts (FS-4). Treatment of cells with neutral red or chloroquine between 2.5 and 3.5 h after induction increases interferon yields 16- to 64- and 4- to 16-fold, respectively, in the subsequent 20.5 h. The two drugs inhibit the rates of protein degradation and of RNA and protein synthesis. In addition, neutral red is a very potent inhibitor of uridine transport into cells. Normalized dose-effect curves show that interferon superinduction is correlated with the inhibition of macromolecular synthesis, but not with that of protein degradation.

Treatment of cells with chloroquine at low concentration (25 μg/ml) for a prolonged period of time (24 h) caused approximately 40% reduction in the rate of protein degradation. The usual rapid shutoff of interferon production and the effectiveness of actinomycin D superinduction are not altered by this treatment. This strongly suggests that inhibition of intralysosomal protein degradation does not significantly contribute to interferon superinduction.

Degradation of the rapidly and the slowly turning over proteins was unaffected by actinomycin D under conditions of treatment known to enhance interferon production. Treatment with cycloheximide (5 or 50 μg/ml for 5 h) inhibited the rate of degradation of the rapidly turning over component by 10% and the slow component by 30-40%, which suggests that the two components turn over by distinct cellular mechanisms.

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