Early Effect of Interferon on Mouse Leukemia Cells Cultivated in a Chemostat*

(cell division/chemotherapeutic substances)

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ABSTRACT

Mouse interferon preparations inhibited the multiplication of mouse leukemia L 1210 cells cultivated under steady-state conditions in a chemostat. The use of this sensitive and controlled system led to the detection of a rapid inhibition in the incorporation of [3H]thymidine into cellular acid-precipitable material 2 hr after the addition of interferon, whereas an effect on cell multiplication was not detected until 22 hr later. Interferon exerted only a transitory effect on the incorporation of [3H]uridine into acid-precipitable material and no effect on the incorporation of 14C-amino acids into cellular protein. It is suggested that the chemostat offers many advantages for the investigation of those physiologic factors or chemotherapeutic substances that modify cell division.

Interferon preparations have been shown to inhibit the multiplication of tumor and normal cells in vitro (1–3). Understanding of the mechanisms of inhibition of cell division in conventional cell culture is often difficult, since the environmental conditions change continuously with cell multiplication. However, in the chemostat cell multiplication occurs at a constant rate and in a constant environment. The steady-state cell concentration is controlled by the concentration of a single growth-limiting component of the medium, and the cell growth rate is controlled by the rate of supply of this component to the culture. Although the chemostat has been used extensively in the study of bacterial physiology (4, 5), most attempts to cultivate animal cells in a chemostat have met with only partial success (6–9), and thus the chemostat has not been used heretofore in studies of animal cell multiplication. Based on the previous work of one of us (10), we have cultivated mouse leukemia L 1210 cells under glucose limitation in a chemostat. (A detailed account of this work will be presented elsewhere.) We describe herein the use of this sensitive and controlled system for the study of the effect of interferon on cell multiplication.

MATERIALS AND METHODS

Tissue Culture. Mouse leukemia L 1210 cells were cultivated in Eagle's minimal essential medium (Eurobio, France) supplemented with 10% heat-inactivated horse serum (Gibco, New York). Glucose-limited medium was prepared by reducing the concentration of glucose in Eagle's medium from 2.0 to 1.0 mg/ml. The resulting change in osmolality was compensated by adjusting the NaCl concentration. Cells were counted in a hemocytometer using the trypan blue dye exclusion test to determine viability. The L 1210 cells were repeatedly tested and shown to be free of mycoplasma.

Chemostat Culture. The apparatus used for the continuous culture of mouse L 1210 cells was based on the small chemostat previously described (8, 10). Two cultures vessels of 145 ml and 300 ml working volume were used in this investigation. The dilution rate (D) is defined as the quotient: medium flow rate (ml/day)/culture volume (ml).

\[
\text{In the steady-state } \mu = \frac{\ln 2}{td} = D \text{ (day}^{-1})
\]

where

\[
\mu = \text{specific growth rate and }
\]

\[
\text{td (day)} = \text{doubling time of cells.}
\]

Samples (1–2% of culture volume) were removed directly from the culture vessel via a sampling port.

Determination of Macromolecular Synthesis. Samples withdrawn from the chemostat were immediately distributed into tubes and the rates of DNA, RNA, and protein synthesis at 37°C were determined under the following pulse labeling conditions: [3H]thymidine, 5 \(\mu\)Ci/ml for 30 min (CEA, France, 25 Ci/mmol); [3H]uridine 5 \(\mu\)Ci/ml for 30 min (25 Ci/mmol); 14C-amino-acid lysate, 1 \(\mu\)Ci/ml for 20 min (0.473 Ci/g), as previously described (11, 12). Incorporation of labeled precursors into cellular material was determined by measuring the radioactivity in the trichloroacetic-acid-insoluble material retained on a Millipore filter, and uptake of labeled precursors was determined by the radioactivity of the filtrate. Both the precipitate and the filtrate were dissolved in Instagel (Packard Instruments, U.S.A.) and the radioactivity was measured in a liquid scintillation counter (Intertechnique, France).

Autoradiography. Cells were labeled for 20 min at 37°C with 1 \(\mu\)Ci/ml of [3H]dT (25 Ci/mmol), fixed, and processed for autoradiography (11).

Interferon Production and Assay. Interferon was prepared from suspension cultures of mouse sarcoma C243-3 cells (13) inoculated with Newcastle disease virus (NDV). The methods of production and partial purification of the interferon have previously been described (14). Mock interferon was prepared by omitting the interferon inducer NDV, and was concentrated and purified in a manner identical to that used in the preparation of interferon. Interferon was assayed on L-cells inoculated with vesicular stomatitis virus (15). One unit of interferon as expressed in the text is the equivalent of four mouse reference units.

Analytical Methods. d-Glucose and l-lactate were determined enzymatically using Biochemica test combinations (Boehringer, Germany).
RESULTS

Cultivation of L 1210 cells in the chemostat

Steady-state cultures of mouse leukemia L 1210 cells were obtained in the chemostat under conditions of glucose-limited growth at dilution rates of 0.30 days⁻¹ (cell doubling time 55.4 hr) (Fig. 1) and 0.693 days⁻¹ (cell doubling time 24 hr) (Fig. 2). Stable steady-states were obtained only after extensive periods of adjustment ranging from 200 to 300 hr of continuous operation of the chemostat. The steady-states were characterized by: a constant cell number, a constant concentration of D-glucose (< 5 μg/ml) and L-lactate† in the culture supernatant, a constant percentage of cells labeled by autoradiography, and constant rates of incorporation of [³H]thymidine, [³H]uridine, and ¹⁴C-labeled amino acids into cellular acid-precipitable material (Fig. 1). The standard deviation of the steady-state means of these parameters were all of the same magnitude as the standard deviation of their respective assays. Cultures of mouse L 1210 cells were regularly maintained in the chemostat continuously for periods in excess of 1000 hr.

Effect of interferon on total cell concentration

Interferon preparations (specific activity 10⁷ reference units/mg of protein) were introduced into the chemostat both by direct injection into the culture vessel and by simultaneous addition to the inflowing medium to maintain a concentration of 6000 units/ml for the duration of the experiment. When the steady-state cell concentration was established at a dilution rate of 0.30 days⁻¹, the first significant decrease in cell concentration was observed 24 hr after addition of interferon (Fig. 1), whereas an effect was observed 12 hr after addition of interferon to steady-state cell cultures established at a dilution rate of 0.693 days⁻¹ (Fig. 2). There was no increase in the number of dead cells until 50–72 hr after contact with interferon. The total cell concentration decreased progressively in the ensuing 9 days. Towards the tenth day the total cell concentration increased despite the continued presence of interferon in the medium. These cells were found to be resistant to interferon and eventually became established at a new steady-state. [The emergence of interferon-resistant L 1210 cells in batch cultures of interferon-treated cells has previously been reported (2, 16).]

Effect of interferon on macromolecular synthesis

A significant decrease in the incorporation of [³H]thymidine into cellular acid-precipitable material was observed 2–3 hr in all four experiments after the addition of interferon at both dilution rates (Figs. 1 and 2) (Table 1). In two experiments in which an early sample was taken (D = 0.693 days⁻¹) a slight but questionably significant effect was observed at 15 or 20 min after addition of interferon. In each experiment a maximum inhibition of 50–70% was observed 8–12 hr after the addition of interferon (Table 1). The duration of maximum

† Lactate concentration: 500 ± 17 μg/ml at a dilution rate of 0.30 days⁻¹; 700 ± 65 μg/ml at a dilution rate of 0.693 days⁻¹.
Interferon inhibits cell division in a chemostat

**Table 1. Interferon decreases the uptake of [3H]thymidine in a steady-state chemostat culture of L 1210 cells**

(D = 0.693 days⁻¹)

<table>
<thead>
<tr>
<th>Time after the addition of interferon (hr)</th>
<th>cpm/5 × 10⁶ cells + % Deviation from steady-state mean value</th>
<th>cpm/5 × 10⁶ cells + % Deviation from steady-state mean value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steady-state mean value</td>
<td>[3H]Thymidine incorporation into acid-precipitable fraction</td>
<td>[3H]Thymidine uptake into acid-soluble fraction</td>
</tr>
<tr>
<td>0.25</td>
<td>35,060 ± 5,629</td>
<td>3,481 ± 173</td>
</tr>
<tr>
<td>2</td>
<td>29,342 ± 4,049</td>
<td>3,756 ± 232</td>
</tr>
<tr>
<td>5</td>
<td>24,518 ± 1,906</td>
<td>2,803 ± 280</td>
</tr>
<tr>
<td>8</td>
<td>16,661 ± 1,150</td>
<td>2,743 ± 210</td>
</tr>
<tr>
<td>11</td>
<td>19,373 ± 766</td>
<td>2,544 ± 107</td>
</tr>
<tr>
<td>24</td>
<td>15,304 ± 922</td>
<td>2,095 ± 443</td>
</tr>
<tr>
<td></td>
<td>18,792 ± 910</td>
<td>2,290 ± 85</td>
</tr>
</tbody>
</table>

NS = not significant.

Inhibition varied from 30 to 100 hr. Both the duration and degree of inhibition were similar at the two dilution rates. The results of autoradiography showed that the number of labeled cells decreased in parallel with the inhibition of incorporation of [3H]thymidine into acid-precipitable material (Fig. 1).

Interferon treatment also resulted in some inhibition in the uptake of [3H]thymidine into the acid-soluble pool. The inhibition was first observed 2–8 hr after the addition of interferon. A maximum inhibition of 20–40% of the steady-state mean value occurred 8–24 hr after the addition of interferon (Table 1).

A similar but less pronounced inhibition in the incorporation of [3H]uridine into cellular acid-precipitable material was first observed 5–12 hr after addition of interferon. The inhibition was maximal (30–40%) by 5–12 hr and of short duration, ranging from 8 to 24 hr (Figs. 1 and 2). No effect was observed at either dilution rate on the incorporation of [3H]uridine into the acid-soluble fraction.

Interferon treatment did not affect the incorporation of 14C-labeled amino acids into cellular protein at either dilution rate (Figs. 1 and 2).

**Evidence that interferon is the factor responsible for the effects observed**

Confidence that the effects observed were due to interferon stems from (1) the use of interferon preparations of high specific activity (10⁶ reference units/mg of protein), (2) mock interferon, prepared and purified in the same manner as the interferon, exerted no effect on steady-state cultures of L 1210 cells, (3) interferon had no detectable effect on steady-state cultures of a clone of L 1210 cells (2, 16) selected for resistance to interferon (Fig. 3).

**DISCUSSION**

We have previously reported that interferon inhibits the multiplication of L 1210 cells in conventional batch culture (1–3). It is difficult, however, in the continuously changing environment of batch culture (i.e., depletion of nutrients, production of metabolites, pH changes, etc.), to distinguish between those events that lead to inhibition of cell multiplication and events that accompany the decreased rate of cell division as cells enter the "stationary phase." Use of the chemostat obviates these difficulties, since under steady-state conditions cell concentration is maintained constant, while cell multiplication proceeds exponentially for as long as desired.

The results of the experiments presented herein show that interferon does inhibit the multiplication of mouse leukemia L 1210 cells cultivated in the chemostat under the controlled conditions of steady-state growth. Moreover, use of the chemostat has enabled us to detect an inhibition in the incor-

**Fig. 2.** The effect of interferon on the multiplication of L 1210 cells cultivated under steady-state conditions in the chemostat at a dilution rate of 0.693 days⁻¹. ---, steady-state mean. ↓, time of addition of interferon. ○, concentration of viable L 1210 cells. Steady-state mean (4.39 × 10⁶ cells/ml, standard deviation 0.21 × 10⁶ cells/ml) before the addition of interferon. □, incorporation of [3H]thymidine into acid-precipitable material. Steady-state mean (3.06 ± 5629 cpm/5 × 10⁶ cells) before the addition of interferon. △, incorporation of [3H]uridine into acid-precipitable material. Steady-state mean (17317 ± 2352 cpm/5 × 10⁶ cells) before the addition of interferon. ▲, incorporation of 14C-amino acids into acid-precipitable material. Steady-state mean (11067 ± 932 cpm/5 × 10⁶ cells) before the addition of interferon.
The effect of interferon on the multiplication of interferon-resistant L 1210 cells cultivated under steady-state conditions in the chemostat at a dilution rate of 0.693 days⁻¹.

- - - , steady-state mean. 

- - - , time of addition of interferon. •, concentration of viable L 1210 cells. Steady-state mean (3,400 ± 10⁶ cells/ml, standard deviation 0.27 ± 10⁶ cells/ml) before the addition of interferon. ○, incorporation of [³H]thymidine into acid-precipitable material. Steady-state mean (3635 ± 3347 cpm/5 × 10⁶ cells) before the addition of interferon. ●, incorporation of [³H]uridine into acid-precipitable material. Steady-state mean (2582 ± 3461 cpm/5 × 10⁶ cells) before the addition of interferon. △, incorporation of [¹⁴C]amino acids into acid-precipitable material. Steady-state mean (5535 ± 1487 cpm/5 × 10⁶ cells) before the addition of interferon.

The effect of interferon on thymidine incorporation into cellular acid-precipitable material only 2 hr after the addition of interferon, whereas an effect on cell multiplication was not detected until 22 hr later. [In our previous work with L 1210 cells in batch culture an inhibitory effect on thymidine incorporation occurred concomitantly with the effect on cell multiplication, i.e., 18 hr after addition of interferon.] O'Shaughnessy and co-workers (17) reported that interferon "delayed the peak" of [³H]thymidine incorporation after release from a double thymidine block in synchronized batch cultures of L-cells. To our knowledge the rapid inhibitory effect on [³H]thymidine incorporation in the chemostat appears to be one of the earliest effects of interferon on cells.

It is not yet clear whether this effect on the incorporation of [³H]thymidine into cellular acid-precipitable material reflects an inhibition of DNA synthesis, changes in nucleotide precursor pools, or an inhibition of nucleotide transport. Interferon treatment did result in a small but significant inhibition in the uptake of radioactivity in the acid-soluble fraction. Even though the maximum inhibition was only 20–40% of the steady-state mean value, in accord with the observations of others (18–20) we found that the thymidine pool was small and equilibrated rapidly so that small changes in pool size could result in a relatively large change in the synthesis of DNA (18–20).

Our results suggest that the effect of interferon on thymidine incorporation may be a selective effect (rather than a non-specific action of interferon, for example, on the cell membrane), since interferon exerted only a transitory effect on the incorporation of [³H]uridine (no effect was seen on the uptake of [³H]uridine into the acid-soluble pool), and no effect was observed on the incorporation of [¹⁴C]amino acids into cellular protein. The absence of an effect on protein synthesis in L 1210 cells cultivated in the chemostat is in contrast to our previous findings (12), and those of others (21), using conventional cell cultures. However, as we have pointed out previously, inhibition of some macromolecular synthesis in batch culture may occur pari passu with the decreased rate of cell division and then would not be observed under the steady-state conditions of the chemostat.

We have used the chemostat to study the effect of interferon on cell multiplication, but we believe that this sensitive and controlled system offers many advantages in the investigation of those physiologic factors or chemotherapeutic substances that modify cell division.

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