Cell Surface Receptor-mediated Internalization of Interferon: Its Relation to the Antiviral Activity of Interferon

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SUMMARY

The binding of [3H]leucine-labelled pure human interferon α from Namalwa cells to human FL and Daudi cells was studied, and evidence obtained to indicate receptor-mediated internalization of interferon. Cell surface-bound and internalized interferons were quantified separately as trypsin-released and unreleased radioactivities, respectively. At 37 °C, surface-bound interferon reached a maximum after 1 h and then decreased, while internalized interferon reached a maximum after 2 h. At 21 °C, in contrast, surface-bound interferon reached a maximum after 2 h and did not decrease thereafter; no internalization was observed. The same was true at 37 °C in the presence of NaF, indicating dependence of internalization on temperature and energy. In control cultures at 37 °C, internalized interferon, after reaching a maximum, decreased after prolonged incubation, and concomitantly acid-soluble radioactivity appeared in the culture medium. The decrease in internalized interferon and the emergence of degraded interferon were inhibited by the lysosomotropic agents, ammonium chloride and chloroquine. The fate of labelled interferon, bound to the cell surface of FL cells at 21 °C, was studied at 37 °C, and the results indicated that trypsin-unreleased interferon was derived from the surface-bound interferon, and was secreted in part into the culture fluid in a degraded form upon prolonged incubation. The relation of internalization of interferon to its biological activity was studied in three ways. In FL cells, the antiviral activity was not induced when internalization of interferon was entirely blocked (at 21 °C or in the presence of NaF at 37 °C). In Daudi cells, both 2'-5' oligoadenylate (2-5A) synthetase induction by interferon and internalization of interferon were inhibited completely by diethyldithiocarbamate (DDC), whereas in the case of FL cells, DDC inhibited neither 2-5A synthetase induction nor internalization of interferon. Raji cells, which have an interferon-specific binding site on the cell surface but are insensitive to interferon, were found not to internalize interferon, whereas other Burkitt's lymphoma cells, Daudi and Namalwa, which are sensitive to interferon, did internalize it. These findings suggest (but do not prove) that internalization is required for the establishment of interferon activity.

INTRODUCTION

Binding of interferon to receptors on the plasma membrane is the first important step in establishment of the antiviral state (Friedman, 1979). High affinity binding of interferon to specific cell surface receptors was reported using mouse and human interferons labelled with radioactive iodine (Aguet, 1980; Branca & Baglioni, 1981; Mogensen et al., 1981; Joshi et al., 1982), and recently, the receptor molecule was identified by cross-linking of radioactive interferon to it (Joshi et al., 1982). We have obtained [3H]leucine-labelled human interferon α from Namalwa cells in an electrophoretically pure form retaining full biological activity (Yonehara, 1982) and analysed its binding to cells of different sensitivities to interferon (Yonehara et al., 1983).
Cells internalize a variety of proteins and peptide hormones by the process of cell surface receptor-mediated endocytosis (Goldstein et al., 1979; Pastan & Willingham, 1981). To date, however, it has not been clarified whether any of the actions of the peptide hormones require the receptor-mediated internalization.

Mouse interferon bound to L1210 cells has been reported not to be internalized (Aguet & Blanchard, 1981), but internalization of human interferon α has been observed (Branca et al., 1982) and down-regulation of interferon receptor has been reported (Branca & Baglioni, 1982). In this report, we show cell surface receptor-mediated internalization of interferon and discuss its relation to the action of interferon, including the antiviral activity and the 2'-5'-oligoadenylylate (2-5A) synthetase-inducing activity.

METHODS

Materials. L-[^4,5-3H]leucine (147 Ci/mmole), [5-α^3H]uridine (28 Ci/mmole) and [8-α^3H]ATP (23 Ci/mmole) were purchased from Amersham, diethylthiocarbamate (DDC), NaF, dinitrophenol (DNP) and NH₄Cl from Nakarai Chemicals (Kyoto, Japan), chloroquine from Sigma, and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) from Calbiochem-Behring.

Cell culture and virus. An epithelial-like cell line, FL, derived from human amnion, was supplied by Dr S. Kobayashi (Basic Research Laboratory, Toray Industrial Inc.). FL cells were cultured as monolayers in Eagle's minimum essential medium (MEM) supplemented with 5% calf serum. Human Burkitt's lymphoma cells, Daudi, Namalwa and Raji, were grown as suspensions in RPMI 1640 medium supplemented with 10% foetal calf serum. Vesicular stomatitis virus (VSV), New Jersey serotype, was grown in BHK-21 cells.

Interferon assay. The antiviral activity of interferon was determined by the inhibition of [3H]uridine incorporation into VSV RNA in FL cells as described previously (Yonehara et al., 1981; Yonehara, 1982), and the titres are expressed in international reference units (IU), as calibrated against the human leukocyte reference interferon, NIH cat. no. G-093-901-527.

Preparation of interferon. Preparation of highly radioactive human interferon α from Namalwa cells has been described previously (Yonehara, 1982). In brief, interferon-producing Namalwa cells infected with Sendai virus were pulse-labelled with [3H]leucine and the labelled interferon was purified by immune precipitation with highly specific rabbit anti-Namalwa cell interferon antibody. The purity was ascertained by polyacrylamide gel electrophoresis in the presence and absence of SDS. The immune precipitate was electrophoresed in the presence of SDS, and the labelled interferon was extracted in 0.1% SDS from the polyacrylamide gel, and stored at −80 °C.

The interferon had 50 CT/min/1U.

Non-radioactive human interferon α used in this report was induced in Namalwa cells, completely purified as described previously (Yonehara et al., 1981), and stored at −80 °C in the presence of 0.1% SDS (7.6 × 10⁶ IU/mg protein).

Cellular binding of radioactive interferon. FL cells (1 × 10^⁶) were seeded in 3.5 cm plastic dishes (Lux Scientific Corp., Newbury Park, Ca., U.S.A.) at least 8 h before use, and Daudi cells were used at 1.5 × 10⁶ cells/ml. Cells were incubated with 1 ml/sample of the growth medium containing radioactive interferon for the indicated periods of time. Subsequently, the cells were washed twice quickly at 4 °C with 0.5 ml of the medium without serum and incubated with 0.5 ml of Dulbecco's phosphate-buffered saline (PBS) containing 0.5%, trypsin (1:250; Difco) and 0.02%, EDTA for 15 min at 37 °C. After centrifugation, the radioactivity in the supernatant ('trypsin-released') was measured. The pellet was dissolved in 0.4 ml of 0.5 M-NaOH at 70 °C for 30 min, 0.1 ml of 2 M-HCl was added and the radioactivity ('trypsin-unreleased') was measured. The radioactivity of all samples in this report was measured with ACS II (Amersham) by a liquid scintillation counter (Model B2450, Packard); the errors in measuring radioactivity were less than 5%.

2-5A synthetase assay. Cell extracts were prepared essentially as described previously (Krishnan & Baglioni, 1980), except that 1.5 × 10⁶ cells were lysed in 20 μl of 20 mM-HEPES-KOH pH 7.4 containing 10 mM-KCl, 1.5 mM-(CH₃COO)₂Mg and 0.5%, Nonidet P40, and then 10 μl of 50% glycerol was added. Twenty μl of the cell extract was incubated for 1 h at 30 °C in 30 μl of the incubation mixture described previously (Minks et al., 1979). The [3H]-labelled 2-5A synthesized from [3H]ATP (1 Ci/mmole) was isolated by DEAE-cellulose as described previously (Hovanessian et al., 1977; Shimizu & Sokawa, 1979). The amounts of 2-5A synthesized from [3H]ATP (1.5 × 10⁻¹⁷ mol and 9.6 × 10⁻⁶ ct/min) by the cell extract (1 × 10⁶ cells/sample) are expressed as mol equivalents of AMP incorporated into 2-5A per h (Stark et al., 1979).

Assay of VSV yield. Cells (Daudi, Namalwa and Raji) (1.5 × 10⁶) were suspended in 0.5 ml RPMI 1640 containing 200 p.f.u./cell of VSV (p.f.u. were determined in human FL cells). After incubation for 1 h at room temperature, the cells were washed with 1 ml RPMI 1640, and incubated with 1 ml RPMI 1640 containing 0.2% foetal calf serum for 24 h at 37 °C. Subsequently, the cell suspension was frozen and thawed three times. After cells and debris had been removed by centrifugation, VSV titres in the supernatant were assayed on FL cells cultured in 96-well microtitre plates (Linbro), and 50% tissue culture infectious dose (TCID₅₀)/0.1 ml was calculated.
RESULTS

Time course of binding of interferon to FL and Daudi cells

We measured the quantity of cell-associated interferon in two forms, trypsin-released and unreleased. Epidermal growth factor bound to the cell surface was reported to be dissociated from cells by acid treatment (Haigler et al., 1980) and this technique was utilized to measure the cell surface-bound interferon by Branca et al. (1982). We found that the quantity of trypsin-released radioactivity was essentially the same as that of acid-released radioactivity (data not shown).

Fig. 1 shows the time course of binding of radioactive interferon to FL and Daudi cells. The results were similar. When incubated at 37 °C, the quantity of cell surface-bound interferon, released by trypsin, reached a maximum after 1 h and decreased thereafter, dropping to one-third of the maximum after 4 h. The cell-bound radioactivity, not released by trypsin, increased more slowly with time than trypsin-released interferon, reaching a maximum after 2 h at 37 °C. Degraded interferon that was not precipitated with cold 10% trichloroacetic acid (TCA) appeared in the culture medium after incubation for 1 h and increased rapidly thereafter (Fig. 1a, c). On the other hand, when incubated at 21 °C, the bound interferon released by trypsin...
Fig. 2. Time course of binding of interferon to FL and Daudi cells in various conditions. (a to d) FL cells (1-2 × 10⁶ cells/sample) and (e to h) Daudi cells (1.5 × 10⁶ cells/sample) were incubated with 1 ml of culture medium containing 3200 ct/min (64 IU) of radioactive interferon at 37 °C in the presence of (a, e) 5 mM-NaF, (b, f) 1 mM-DNP, (c, g) 20 mM-NH₄Cl and (d, h) 0.15 mM-chloroquine after pre-incubation with the drugs for 1 h at 37 °C. Cell-associated radioactivity and TCA-soluble radioactivity in culture fluid were measured as described in the legend to Fig. 1. Non-specific binding was less than 70 ct/min in all cases.

became maximal after 2 h and did not decrease even after prolonged incubation; the bound interferon not released by trypsin was negligible, as was the TCA-soluble radioactivity in the culture medium with both FL and Daudi cells (Fig. 1 b, d). Thus, the decrease of trypsin-released interferon with time depended on temperature.

**Effect of metabolic inhibitors and lysosomotropic agents**

To analyse how the increase of trypsin-unreleased interferon with time at 37 °C is related to cellular endocytosis, the effect of two inhibitors of energy metabolism, NaF and DNP, which are known to be also indirect inhibitors of protein synthesis, were examined. NaF, an inhibitor of glycolysis, has been reported to inhibit endocytosis, whereas DNP, an inhibitor of oxidative metabolism, has been reported not to do so (Silverstein *et al.*, 1977). In the presence of NaF, trypsin-released interferon, which reached a maximum after 1 h at 37 °C, did not decrease thereafter, and neither trypsin-unreleased interferon nor degraded interferon in the culture fluid was detected (Fig. 2a, e). In contrast, in the presence of DNP, trypsin-unreleased interferon as well as degraded radioactivity in the culture fluid was observed to have kinetics similar to the control cultures (Fig. 2b, f).

To analyse the relation of degradation of interferon to lysosomes, the effects of two lysosomotropic agents, NH₄Cl and chloroquine, were examined. In the case of FL cells, NH₄Cl effectively inhibited the emergence of degraded interferon into the culture medium, and trypsin-unreleased interferon continued to increase for 4 h (Fig. 2c). In the case of Daudi cells, however, NH₄Cl had no effect (Fig. 2g). On the other hand, chloroquine partly inhibited the decrease of
Internalization of interferon

Fate of cell surface-bound interferon

The fate of interferon bound to the cell surface of FL cells was examined in the following way (Fig. 3). Cells were first incubated with labelled interferon (64 IU/ml) at 21 °C for 2.5 h (maximum surface binding); the medium was then replaced by fresh medium containing unlabelled interferon (50 IU/ml), and the culture was brought to 37 °C (time zero). As shown in Fig. 3(a), the surface-bound interferon (trypsin-released) decreased rapidly with time at 37 °C. A sizeable fraction of the initially bound interferon was immediately released into the medium in an acid-precipitable form (Fig. 3b), and another fraction was internalized as shown by an increase in trypsin-unreleased interferon (Fig. 3a) up to 40 min. After this time, trypsin-unreleased interferon decreased, and correspondingly, acid-soluble radioactivity in the medium increased markedly. These data indicate that trypsin-unreleased internalized interferon was derived from the surface-bound interferon, and, as time elapsed, was secreted into the culture medium in a degraded form.

Relation of internalization of interferon to its action

To evaluate the significance of internalization of interferon for the manifestation of its biological activity, two interferon activities, namely inhibition of virus RNA synthesis and
Table 1. Effect of temperature and inhibitors on interferon action

<table>
<thead>
<tr>
<th>Drug</th>
<th>Temperature (°C)</th>
<th>Interferon (IU/ml)</th>
<th>Inhibition of virus RNA synthesis† (% ± S.D.)§</th>
<th>Induced 2-5A synthetase activity (nmol AMP incorporated)†</th>
<th>Protein synthesis‡ (% ± S.D.)§</th>
<th>RNA synthesis‡ (% ± S.D.)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>37</td>
<td>0</td>
<td>0</td>
<td>&lt;0.01</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>-</td>
<td>21</td>
<td>0</td>
<td>0</td>
<td>&lt;0.01</td>
<td>5 ± 2</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>NaF</td>
<td>5 mM</td>
<td>37</td>
<td>0</td>
<td>&lt;0.01</td>
<td>4 ± 1</td>
<td>65 ± 5</td>
</tr>
<tr>
<td>DNP</td>
<td>1 mM</td>
<td>37</td>
<td>0</td>
<td>&lt;0.01</td>
<td>3 ± 1</td>
<td>96 ± 10</td>
</tr>
<tr>
<td>DRB</td>
<td>20 µg/ml</td>
<td>37</td>
<td>0</td>
<td>25 ± 8</td>
<td>1.16</td>
<td>11 ± 10</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>20 mM</td>
<td>37</td>
<td>0</td>
<td>&lt;0.01</td>
<td>85 ± 3</td>
<td>102 ± 10</td>
</tr>
</tbody>
</table>

* FL cells pretreated with the drugs for 1 h were incubated with human interferon α in the presence of the drug for 5 h at the indicated temperature. To protect FL cells from harmful effects of the drugs, the cells were not incubated with the drug for more than 6 h. Subsequently, the cells were washed with MEM at 4 °C and then incubated at 37 °C for 15 h with MEM containing 5% calf serum in the absence of the drug and in the presence of rabbit anti-Namalwa cell interferon serum (Yonehara et al., 1981) that could reduce 500 IU/ml interferon activity to less than 0.3 IU/ml. Induced interferon activity was then measured. After removing the drug and interferon, cells were incubated at 37 °C to produce the antiviral state in the absence of the drugs that inhibited cellular protein and RNA syntheses. Anti-interferon serum was added to inhibit the action of any interferon which was not removed by washing. In the case of NH₄Cl, cells were incubated with antiserum in the presence of NH₄Cl.

† [3H]Uridine incorporation into VSV RNA was measured as described in Methods.

‡ [3H]Leucine incorporation into total cellular proteins and RNAs insoluble in cold 5% TCA was measured. Protein and RNA syntheses in controls (100%) were 1.27 × 10⁴ ct/min and 5.18 × 10³ ct/min respectively.

§ Experiments were performed in triplicate and the standard deviation (S.D.) was calculated.

On the contrary, in the presence of DNP, which did not inhibit internalization (Fig. 2b), detectable interferon activity was observed, although weaker than in control cells. It must be noted that both protein and RNA syntheses were much lower at 21 °C, or in the presence of NaF (an inhibitor of initiation of protein synthesis), than in control cells at 37 °C (Table 1). However, this does not seem to account completely for the lack of interferon action, since DNP also inhibited protein synthesis, perhaps even more strongly than did NaF. An inhibitor of RNA synthesis, DRB, inhibited RNA synthesis more strongly than NaF, but it did allow weak but detectable interferon action to occur (Table 1); internalization of interferon was not inhibited by DRB (data not shown). NH₄Cl, which completely inhibited secretion of degraded interferon from FL cells (Fig. 2c), did not inhibit interferon action (Table 1), indicating that release of degraded interferon was not related to interferon action.

DDD, known to inhibit superoxide dismutase by chelating Cu²⁺ (Misra, 1979), has been reported to inhibit interferon action (Pottathil et al., 1981). In the case of Daudi cells, induction of 2-5A synthetase by interferon was completely inhibited by 2 × 10⁻⁹ M-DDC, but not in the case of FL cells (Table 2). As shown in Fig. 4, Daudi cells did not internalize interferon in the
Internalization of interferon

Fig. 4. Time course of binding of interferon to FL and Daudi cells in the presence of DDC. (a, b) FL cells (0.8 x 10^6 cells/sample) and (c, d) Daudi cells (1.5 x 10^6 cells/sample) were incubated with 3200 ct/min (64 IU) of radioactive interferon at 37 °C for the indicated times in the presence of (a, c) 2 x 10^{-8} M- or (b, d) 2 x 10^{-4} M-DDC, after pre-incubation with DDC for 1 h at 37 °C. Cell-associated interferon and TCA-soluble radioactivity in culture medium were measured as described in the legend to Fig. 1. Non-specific binding was less than 30 ct/min and 60 ct/min in FL and Daudi cells respectively.

Table 2. Effect of DDC on interferon action

<table>
<thead>
<tr>
<th>Cell</th>
<th>DDC (M)</th>
<th>Interferon (IU/ml)</th>
<th>Induced 2-5A synthetase activity* (nmol AMP incorporated)†</th>
<th>Protein synthesis† (% ± s.D.)§</th>
<th>RNA synthesis† (% ± s.D.)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL</td>
<td>0</td>
<td>0</td>
<td>&lt;0-10</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td></td>
<td>3.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 x 10^{-8}</td>
<td>0</td>
<td>&lt;0-10</td>
<td>87 ± 7</td>
<td>88 ± 15</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td></td>
<td>2.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 x 10^{-4}</td>
<td>0</td>
<td>&lt;0-10</td>
<td>31 ± 9</td>
<td>62 ± 10</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td></td>
<td>3.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daudi</td>
<td>0</td>
<td>0</td>
<td>&lt;0-10</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td></td>
<td>3.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 x 10^{-8}</td>
<td>0</td>
<td>&lt;0-10</td>
<td>77 ± 2</td>
<td>60 ± 10</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td></td>
<td>2.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 x 10^{-4}</td>
<td>0</td>
<td>&lt;0-10</td>
<td>47 ± 5</td>
<td>54 ± 14</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td></td>
<td>&lt;0-10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Cells were pretreated with DDC for 1 h at 37 °C and then incubated with human interferon α for 15 h at 37 °C in the presence of DDC. Subsequently, induced 2-5A synthetase activity was measured.
† Protein and RNA syntheses were measured as described in Table 1. Protein and RNA syntheses (100%) in control FL cells were 1.27 x 10^4 ct/min and 5.18 x 10^3 ct/min respectively, and in Daudi cells 5.77 x 10^3 ct/min and 2.12 x 10^3 ct/min respectively.
‡ The amounts of 2-5A synthesized are expressed as described in Methods. One nmol AMP corresponds to 640 ct/min.
§ Experiments were performed in triplicate and the standard deviation (s.D.) was calculated.
Table 3. Response of lymphoblastoid cells to interferon*

<table>
<thead>
<tr>
<th>Cell</th>
<th>Interferon (IU/ml)</th>
<th>VSV yield (log₁₀ TCID₅₀/0.1 ml)</th>
<th>Induced 2-5A synthetase activity (nmol AMP incorporated)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daudi</td>
<td>0</td>
<td>6.5</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>4.1</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>2.6</td>
<td>3.3</td>
</tr>
<tr>
<td>Namalwa</td>
<td>0</td>
<td>5.7</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>4.9</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>3.4</td>
<td>2.1</td>
</tr>
<tr>
<td>Raji</td>
<td>0</td>
<td>6.5</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5.7</td>
<td>ND‡</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>5.7</td>
<td>0.2</td>
</tr>
</tbody>
</table>

* Cells were treated with interferon α for 20 h at 37 °C before measurement of induced 2-5A synthetase activity and infection with VSV.

† The amounts of 2-5A synthesized were expressed as described in Methods. One nmol AMP corresponds to 640 ct/min.

‡ ND, Not determined.

presence of $2 \times 10^{-4}$ M-DDC, but FL cells did, to the same extent as in the presence of $2 \times 10^{-4}$ M-DDC, which was similar to controls without the drug (Fig. 1). Essentially the same results were obtained with $1 \times 10^{-3}$ M-DDC (data not shown).

Table 3 shows that a Burkitt's lymphoma cell line, Raji was insensitive to interferon as regards antiviral and 2-5A synthetase-inducing activities, compared with other Burkitt's lymphoma cell lines, Daudi and Namalwa, in agreement with previous reports (Adams et al., 1975; Tomita et al., 1982). Raji cells, however, have been reported to possess interferon-specific, high-affinity binding sites (Mogensen et al., 1981). We also found specific binding of interferon to Raji cells (Fig. 5a), although to a lower degree than to Namalwa (Fig. 5b) or Daudi cells (Fig. 1c). Interestingly, however, Raji cells showed virtually no detectable internalization (trypsin-
unreleased interferon) and degradation (acid-soluble radioactivity in the culture medium) (Fig. 5a), in contrast to Namalwa (Fig. 5b) and Daudi cells (Fig. 1c).

**DISCUSSION**

We have shown, mainly using human FL and Daudi cells, that human interferon $\alpha$ bound to a receptor on the cell surface was internalized and degraded progressively at 37 $^\circ$C. A decrease in trypsin-released interferon and an increase in trypsin-unreleased interferon with time of incubation depended on temperature and energy (Fig. 1 and 2), and degradation of interferon was inhibited by lysosomotropic agents (Fig. 2). This finding indicates that interferon was internalized by cellular endocytosis as has been reported in the case of insulin, epidermal growth factor, low density lipoprotein, $\alpha$-2-macroglobulin, and transferrin (Goldstein *et al.*, 1979; Pastan & Willingham, 1981). These results are consistent with previous results on Daudi cells (Branca *et al.*, 1982). We obtained similar results with other human cells, diploid fibroblasts FS-7, amnion-derived WISH and U, and lymphoblastoid Namalwa, all of which are sensitive to human interferon $\alpha$ (data not shown). Thus, internalization and degradation of interferon seem to be general phenomena.

In contrast to our results and those of Branca *et al.* (1982), Aguet & Blanchard (1981) did not observe internalization of mouse interferon bound to L1210 cells. The reason for this difference is not clear. In the latter system, no degradation of interferon associated with the mouse cells was observed, but it would be worthwhile determining whether degraded interferon secreted into the culture fluid could be found under those conditions.

Raji cells, which are insensitive to interferon, were found not to internalize significant amounts of interferon, although interferon was bound to specific cell surface binding sites (Fig. 5). Moreover, interferon did not exert its action on interferon-sensitive FL cells when internalization was blocked (Table 1). The action of DDC was especially interesting; in Daudi cells, the drug completely inhibited interferon action as well as internalization of interferon, whereas in FL cells it did not inhibit either the interferon action or internalization (Table 2 and Fig. 4). Previously, it has been suggested that DDC inhibits interferon action by inactivating superoxide dismutase (Pottathil *et al.*, 1981), but it seems possible that DDC may inhibit interferon action by blocking its internalization. All the findings in this study thus seem to suggest (but do not prove) that internalization of interferon is required for its biological actions. Anderson *et al.* (1982) also obtained evidence to indicate a possible role of interferon internalization and receptor recycling, from an analysis of the effects of some primary amines. On the other hand, intracellular degradation of interferon seems to be unnecessary for interferon action, because in the case of FL cells, NH$_4$Cl completely inhibited the degradation of interferon but had no effect on its action.

Previously, binding of interferon to cell surface receptors was considered to be sufficient for induction of the antiviral state, because Sepharose-bound interferon which presumably could not be internalized, was effective (Ankel *et al.*, 1973). In this type of experiment, however, the possibility cannot be excluded that part of the interferon is dissociated from the Sepharose. Recently, interferon microinjected into cells was shown not to induce an antiviral state (Higashi & Sokawa, 1982). This finding does not necessarily contradict the hypothesis that interferon action requires its internalization, because the topology and state of interferon internalized by endocytosis may well be different from those of microinjected interferon. In a study generally similar to ours, Branca *et al.* (1982) claimed that internalization of interferon might not be required for its biological activity, since cytochalasin, which inhibited internalization of interferon by 50%, had no significant effect on interferon action; also, methylamine, which has been reported to have no effect on interferon action in human fibroblast FS-7 cells (Anderson *et al.*, 1982), inhibited internalization of interferon in Daudi cells (Branca *et al.*, 1982). Their results, however, are not conclusive, because cytochalasin does not inhibit internalization of interferon as completely as NaF and DDC, and methylamine inhibits neither interferon action nor its internalization in the case of FL cells (our unpublished observations). At present, however, our evidence relating internalization of interferon to its action is not definitive, and further investigations are needed to clarify the significance of receptor-mediated internalization of interferon.
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