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# Murine interferon-β receptor-mediated endocytosis and nuclear membrane binding

(coated pits/internalization/nuclei/ferritin/colloidal gold)

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**ABSTRACT** **Radioiodinated mouse interferon-β ($^{125}$I-MuIFN-β) bound with high affinity ($K_d = 9.8 \times 10^{-10}$ M) to plasma membrane of $L_{929}$ murine fibroblasts ($4–6 \times 10^3$ receptor sites per cell). The binding was saturable and inhibited by a 100-fold excess of unlabeled MuIFN-β but not by excess mouse IFN-γ (MuIFN-γ). MuIFN-β bound at 4°C was very rapidly internalized upon warming of the cells to 37°C ($t_{1/2} = 1.5$ min). Indirect immunoferritin labeling indicated that MuIFN-β was initially located in coated pits and subsequently internalized by receptor-mediated endocytosis. Isolated $L_{929}$ cell nuclei bound $^{125}$I-MuIFN-β with a 7-fold higher affinity ($K_d = 1.4 \times 10^{-10}$ M) and higher receptor density (about $10^4$ per nucleus) than that for the plasma membrane. Binding to the nuclear membrane was inhibited by a 100-fold excess of unlabeled MuIFN-β but not by excess MuIFN-γ. Trypsin treatment of nuclei decreased IFN binding by 80%, suggesting that the putative nuclear receptors are protein. Specific binding of MuIFN-β to nuclei was also shown by fluorescence and electron microscopy. We propose that the very rapid internalization of MuIFN-β by receptor-mediated endocytosis is important in the cellular processing of IFN and that its high-affinity binding to the nuclear membrane suggests the nucleus as an intracellular site of IFN action.**

The multiple effects of interferons (IFNs) on the phenotype of vertebrate cells include the establishment of antiviral resistance, reduction in the rate of cell proliferation, and inhibition or stimulation of the expression of many differentiated cellular functions (1, 2). The primary actions of IFNs to achieve these very different effects remain uncertain, as are the loci of such actions. The first step in the action of IFN is its binding to a specific plasma membrane protein receptor (3–9) located in coated pits (4, 8), which are specialized cell membrane formations involved in a highly selective and efficient mechanism of receptor-mediated endocytosis of ligands, such as hormones, serum lipoproteins, antibodies, toxins, and viruses (10, 11). Following binding, IFN can be internalized (3, 7, 8, 12), but subsequent steps in its processing are unclear.

Treatment with IFNs at physiological temperatures stimulates the synthesis of many polypeptides (13). Although some enzymes are induced or activated that are important in the inhibition of translation of viral proteins (14, 15), the effects on host cell synthetic activities remain less clear. If IFNs act only at the plasma membrane, second messenger(s) (16, 17) or transmembrane signaling must be postulated to mediate such effects. However, if binding can be demonstrated on intracellular structures, such as nuclei, it is possible that IFN molecules can directly affect the functions of target organelles. Certain hormones, such as insulin or steroids (18–20), epidermal growth factor (21), and polyribonucleotides (22), have been shown to bind to the nuclear membrane, and some exert nuclear effects. In the present study we show by radiolabeling and immunocytochemical techniques that mouse IFN-β (MuIFN-β) is very rapidly internalized by the mechanism of receptor-mediated endocytosis and that MuIFN-β binds to isolated nuclei of mouse fibroblasts: not only are the nuclear membrane receptors of higher affinity than those on the plasma membrane, but they are also present in greater density.

## MATERIALS AND METHODS

**Cells.** Mouse $L_{929}$ fibroblasts were grown in suspension as described (4). After 3 days at 36°C, the cells were centrifuged, washed three times with Eagle's minimal essential medium (EMEM) without serum, and utilized immediately.

**IFNs and Their Assay.** Highly purified MuIFN-β of specific activity $1.3 \times 10^8$ international units (IU)/mg was obtained from Lee Biomolecular (San Diego, CA). Mouse IFN-γ (MuIFN-γ) was induced in murine T lymphocytes and purified by RNA-affinity chromatography to a specific activity of $1.4 \times 10^7$ laboratory units (LU)/mg (23). Antiviral activity of IFNs was titrated by a hemagglutination yield-reduction bioassay using GDVII virus in L cells (24), which measures the same unitage as that assigned to the MuIFN-α/β International Standard G002-904-511. MuIFN-β activity is expressed in IU and MuIFN-γ activity is expressed in LU since no homologous international standard is yet available.

**Iodination of MuIFN-β.** MuIFN-β was radioiodinated ($^{125}$I-MuIFN-β) with $^{125}$I-labeled Bolton–Hunter reagent (Amersham) to a specific activity of 60 μCi/μg (1 Ci = 37 GBq) as described (4). Iodination reduced the potency of the IFN preparation by <30%. Autoradiography after NaDodSO$_4$/PAGE (25) revealed a single band at an apparent $M_r$ of 35,000 where all the antiviral activity was also demonstrated.

**$^{125}$I-MuIFN-β Cell Binding and Uptake.** Binding of $^{125}$I-MuIFN-β to $L_{929}$ cells ($10^6$ per sample) was carried out at 0–4°C for 15 min as described (4, 26), with or without excess unlabeled MuIFN-β or MuIFN-γ. For uptake experiments 150 μl of EMEM prewarmed to 37°C was added to cells pretreated at 4°C with $^{125}$I-MuIFN-β, and the samples were transferred to a water bath at 37°C. The 0.2 M acetic acid treatment method that releases surface-bound ligands was used to distinguish uptake of $^{125}$I-MuIFN-β from that remaining on the cell surface (7, 27).

**Preparation and Treatment of Nuclei.** $L_{929}$ fibroblast nuclei were prepared by a modification of the method of Seale (28). Briefly, thoroughly washed samples of $1–5 \times 10^8$ cells were subjected to hypotonic lysis, homogenization by 35 strokes of a type-B tight-fitting Dounce pestle, and centrifugation for 4 min at $800 \times g$; the homogenate was resuspended in lysis buffer and sedimented at $1000 \times g$ for 10 min on a 15% Ficoll

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Abbreviations: IFN, interferon; MuIFN-β, mouse IFN-β; MuIFN-γ, mouse IFN-γ; IU, international unit(s); LU, laboratory unit(s).

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cushion. The nuclei were washed in lysis buffer, resuspended in EMEM, and counted in a hemocytometer. More than 65% of the nuclei appeared to be free from cytoplasmic residue. Suspensions of nuclei were capable of incorporating significant amounts of [$^{32}$P]dCTP (up to 100 pmol/40 min) in an *in vitro* DNA replication system (28), confirming their functional activity. Binding of $^{125}$I-MuIFN-$\beta$ to nuclei was carried out essentially as detailed above for cell binding.

**Ferritin Labeling.** Indirect immunoferritin labeling was performed as described (4), employing in sequence MuIFN-$\beta$ (10,000 IU/ml in EMEM), sheep anti-MuIFN-$\beta$ IgG (G024-501-568, National Institute of Allergy and Infectious Diseases, Bethesda, MD), and anti-sheep IgG–rabbit IgG conjugated with ferritin. All incubations were carried out for 15 min at 4°C with extensive washing between each step. The sheep anti-MuIFN-$\beta$ IgG and rabbit anti-sheep ferroglobulin were preabsorbed extensively with $L_{929}$ cells or nuclei, as appropriate, before the immunocytochemical experiments. Controls included (*i*) $L_{929}$ cells processed as described but without MuIFN-$\beta$ and (*ii*) $L_{929}$ cells treated with a mock MuIFN-$\beta$ preparation (4).

**Electron and Fluorescence Microscopy.** *Whole cells.* MuIFN-treated cells incubated as above with ferroglobulin at 4°C were centrifuged, and about 5 × $10^6$ cells in 0.1 ml were added to each tube containing 2 ml of EMEM prewarmed to 37°C. After appropriate intervals of incubation at 37°C, tubes were removed from the water bath, and an excess of ice-cold 4% glutaraldehyde was added to fix the cells. Cell specimens were processed for electron microscopy (4) in duplicate, and at least two series of ultrathin sections were cut from different parts of each sample. *Isolated nuclei.* Binding experiments with suspensions of nuclei were carried out as described for whole cells. For fluorescence microscopy, MuIFN-treated nuclei were incubated with sheep anti-MuIFN-$\beta$ IgG and subsequently with anti-sheep IgG–rabbit IgG conjugated with fluorescein isothiocyanate (Cappel Laboratories, Downingtown, PA) before fixation in 70% methanol at 4°C. The ferroglobulin technique as well as a method utilizing colloidal gold conjugated with staphylococcal protein A (E-Y Laboratories, San Mateo, CA) were used on replicate samples from the same batch of nuclei. Nuclei treated with MuIFN-$\beta$ and then washed were subsequently treated with anti-MuIFN-$\beta$ IgG, washed again, incubated for 1 hr at 4°C with the colloidal gold–protein A conjugate, and processed for electron microscopy as above.

**Protein Assay.** Protein was measured by the Coomassie blue/perchloric acid micromethod of Sedmak and Grossberg (29).

## RESULTS

**Cell Surface Binding.** Saturation of cell surface receptors was achieved with 12.5–25 ng of $^{125}$I-MuIFN-$\beta$ applied to $10^6$ cells (Fig. 1). The concavity of the binding curve toward the ordinate may be evidence of positive cooperativity at IFN concentrations up to 10 ng of $^{125}$I-MuIFN-$\beta$ per $10^6$ cells. Scatchard analyses (which gave rectilinear plots) provide an estimate of 4–6 × $10^3$ receptors per cell and a dissociation constant ($K_d$) of 9.8 × $10^{-10}$ M. In the presence of a 100-fold excess of unlabeled MuIFN-$\beta$, binding was reduced as much as 95%, whereas an excess of MuIFN-$\gamma$ did not affect MuIFN-$\beta$ binding (Fig. 1). Since binding was maximal by 6 min, a 15-min incubation at 4°C was used to bind MuIFN-$\beta$ in the internalization experiments described below.

**Internalization of Radiolabeled IFN.** The uptake of $^{125}$I-MuIFN-$\beta$ was very rapid (Fig. 2). After 1–2 min of warming at 37°C about 45–50% of surface-bound cpm became unavailable to release by the 0.2 M acetic acid wash ($t_{1/2} \approx 1.5$ min). Nonspecific release of $^{125}$I-MuIFN-$\beta$ from the cell surface was determined as the amount of the label removed by washing with EMEM at 4°C or 37°C at various intervals.

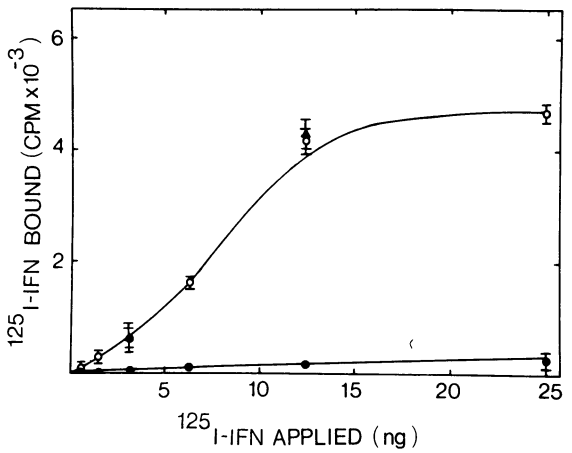


FIG. 1. Binding of $^{125}$I-MuIFN-$\beta$ to $L_{929}$ cells at 4°C. After $^{125}$I-MuIFN-$\beta$ was added to suspensions of $10^6$ cells, triplicate cell samples were taken at the times indicated, washed, and sedimented; the radioactivity in each cell pellet was measured, and the means of the cpm were plotted against the amount of $^{125}$I-MuIFN-$\beta$ applied. ○, $^{125}$I-MuIFN alone; ●, $^{125}$I-MuIFN-$\beta$ in the presence of a 100-fold excess of unlabeled MuIFN-$\beta$; ▲, $^{125}$I-MuIFN-$\beta$ in the presence of a 100-fold excess of MuIFN-$\gamma$. Vertical bars represent the range of values.

Under the conditions employed, about 0.3–0.5% of the bound label was spontaneously released at either temperature from the cells.

**Immunocytochemical Studies.** IFN receptor sites were seen almost exclusively on coated membrane areas of 85% of sections of the cells treated with MuIFN-$\beta$ for 15 min at 0°C. After 30 sec at 37°C there were no changes noted in the distribution of the ferritin label (Fig. 3*a*). However, after 60 sec many coated areas and coated pits with attached ferroglobulin label had begun to invaginate into the cytoplasm, frequently forming coated vesicles, some of which appeared to be connected with the cell membrane by a narrow channel (Fig. 3 *b–d*). By this time some of the vesicles started to lose their clathrin coating (Fig. 3*d*). At this early stage there were still many coated pits open to the external milieu such that IFN in these would be available for removal by acetic acid.

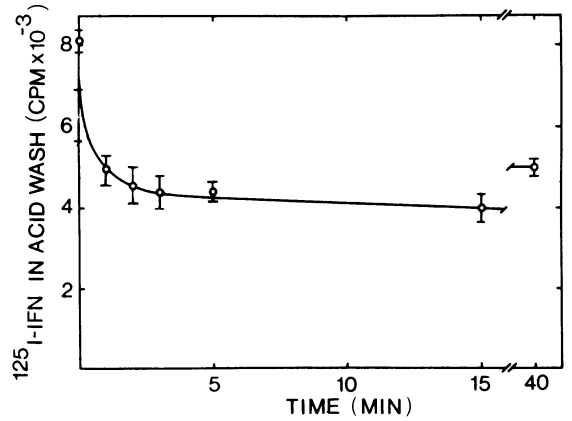


FIG. 2. Uptake of $^{125}$I-MuIFN-$\beta$ by $L_{929}$ cells. Cells were preincubated with $^{125}$I-MuIFN-$\beta$ for 15 min at 4°C and subsequently warmed at 37°C. Chilled 0.5 M acetic acid in phosphate-buffered saline was added, 150 $\mu$l per sample ($10^6$ cells per triplicate sample), to give a final concentration of 0.2 M (7, 27), and the cells were shaken gently at 0°C for 5 min; cell suspensions were then centrifuged, and the radioactivity in cell pellets and supernatant fluids was measured separately. Radioactivity in supernatant fluids was measured after treatment with acetic acid. Vertical bars represent the range of values.

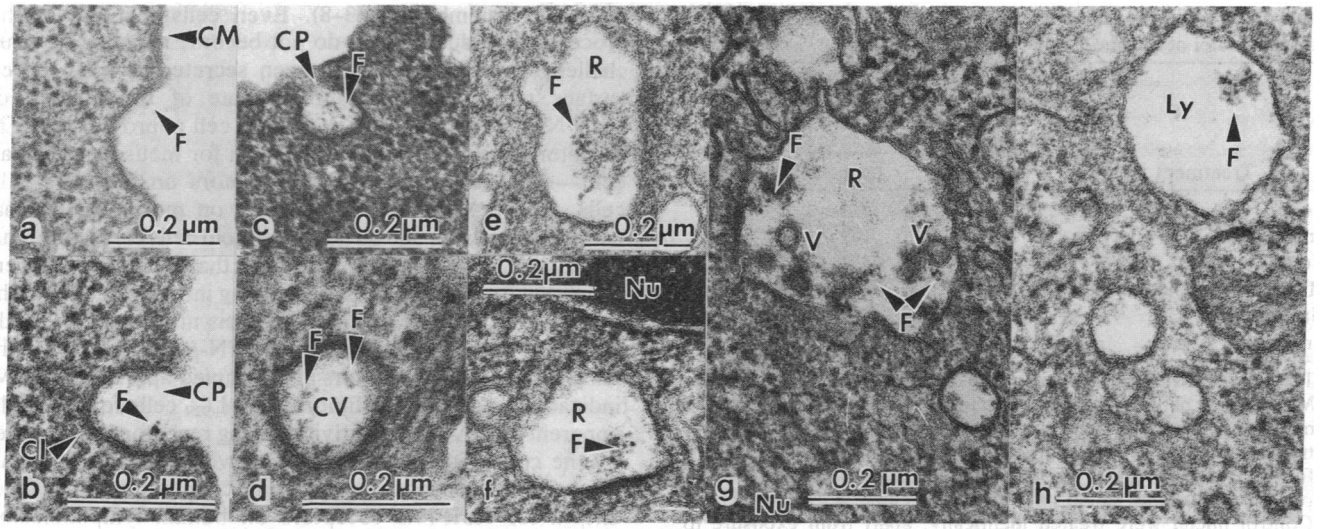


FIG. 3. Electron microscopy of $L_{929}$ cells internalizing MuIFN-$\beta$. Cells were taken from the same culture preparation used in the $^{125}$I-MuIFN-$\beta$ internalization studies represented in Fig. 2. Cells preincubated with MuIFN-$\beta$ for 15 min at 0°C and then with anti-MuIFN-$\beta$ antibody and ferroglobulin were quickly warmed to 37°C. At the times indicated they were transferred into ice-cold glutaraldehyde: 30 sec (*a*); 60 sec (*b–d*); 2–3 min (*e* and *f*); 5 min (*g*); 15 min (*h*). F, ferritin; CP, coated pit; CL, clathrin; CV, coated vesicle; R, receptosome; V, vesicle; Ly, lysosome; Nu, nucleus.

After 2–3 min of incubation at 37°C, the majority of the surface label was gone, and ferritin was observed either in coated vesicles or in receptosomes in close proximity to the limiting membrane of these organelles (Fig. 3 *e* and *f*). Many receptosomes were located in the vicinity of the Golgi apparatus or the nucleus (Fig. 3*f*). After 5 min of incubation at 37°C, only occasionally was ferritin label observed on the plasma membrane of the cell, and a significant part of the label was observed in multivesicular bodies originating from a fusion of several receptosomes (10, 11) or of receptosomes with lysosomes (Fig. 3 *g* and *h*). At this point the label observed in multivesicular bodies and lysosomes was located at a distance from membranes of the organelles; such a location of the ferritin inside these organelles may indicate dissociation of the label from ligand (10, 11). After 15 min at 37°C ferritin cores on the plasma membrane were located exclusively outside of coated membrane areas, but internalized ferritin label was not observed in any cellular compartment other than lysosomes. The subsequent fate of internalized MuIFN-$\beta$ could not be unequivocally discerned with the techniques employed in this study.

**Nuclear Binding of MuIFN.** To test the hypothesis that the nucleus might be a target for internalized IFN molecules, we determined whether IFN could bind to purified, isolated nuclei. The binding of $^{125}$I-MuIFN-$\beta$ to isolated nuclei is shown in Fig. 4*a*; saturation was achieved at 16 ng of applied $^{125}$I-MuIFN-$\beta$. In the presence of a 100-fold excess of unlabeled MuIFN-$\beta$, binding of $^{125}$I-MuIFN-$\beta$ was reduced by at least 90%. An excess of unlabeled MuIFN-$\gamma$ failed to inhibit nuclear binding of $^{125}$I-MuIFN-$\beta$ (Table 1). Scatchard analysis (Fig. 4*b*) provides estimates of 1.4 (SD ± 0.12) × $10^4$ receptors per nucleus and a $K_d$ of 1.4 × $10^{-10}$ M. Nuclear binding of $^{125}$I-MuIFN-$\beta$ was reduced by 33% in the presence of 1 M NaCl or by 70–80% following tryptic treatment of the nuclei (Table 1). Different immunocytochemical techniques showed that MuIFN-$\beta$, identified by fluorescein (Fig. 5*a*), ferritin (Fig. 5*d*), or colloidal gold label (Fig. 5*e*), was located on the nuclear membrane without the clustering of receptors. In control preparations that were treated identically but incubated in the absence of MuIFN-$\beta$, no membrane-associated label, be it ferritin, colloidal gold, or fluorescein, was detected (Fig. 5 *b* and *c*).

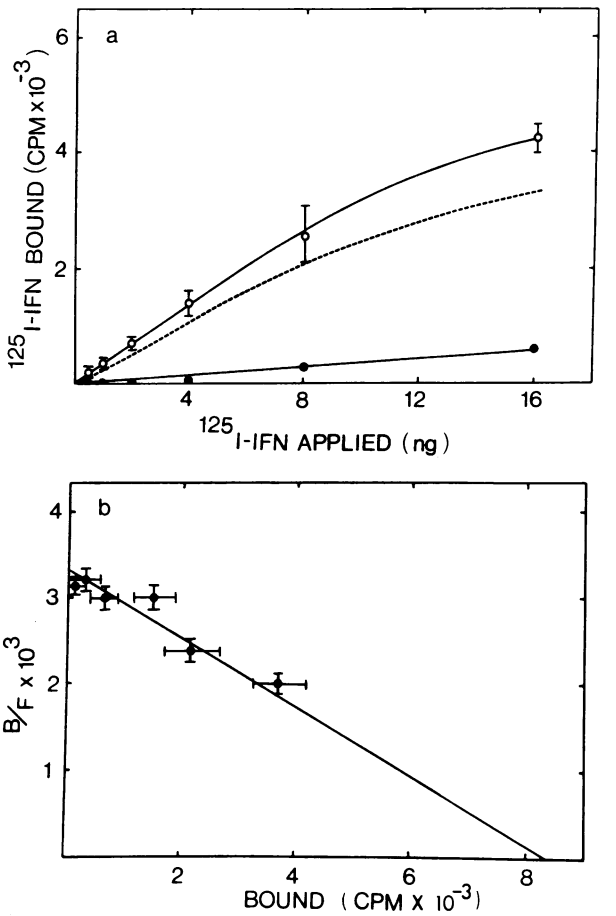


FIG. 4. Binding of $^{125}$I-MuIFN-$\beta$ to isolated nuclei of $L_{929}$ cells. (*a*) Isolated nuclei (5 × $10^5$ per triplicate sample) were incubated for 15 min at 4°C with $^{125}$I-MuIFN-$\beta$ alone (○) or $^{125}$I-MuIFN-$\beta$ in the presence of a 100-fold excess of unlabeled MuIFN-$\beta$ (●). -----, Specific binding. (*b*) Scatchard plot of the data in *a*. Linear regression analyses were performed on nuclear binding data by the method of least squares best fit of *Y* upon *X*. The vertical and horizontal bars indicate the standard error of the mean. B, bound IFN; F, free IFN.

Table 1. Conditions affecting the binding of $^{125}$I-MuIFN-$\beta$ to isolated nuclei of murine $L_{929}$ cells

| Treatment | $^{125}$I-MuIFN-$\beta$ added, ng | $^{125}$I-MuIFN-$\beta$ bound per $5 \times 10^5$ nuclei | |
|---|---|---|---|
| | | cpm | % of control |
| None | 2 | 354 | 100 |
| Trypsin* | 2 | 109 | 31 |
| None | 20 | 6414 | 100 |
| MuIFN-$\gamma$ (50-fold excess) | 20 | 6113 | 91 |
| NaCl (1 M) | 20 | 4123 | 67 |
| Trypsin | 20 | 1389 | 22 |

*Treatment was carried out with trypsin at 3 mg/ml (202 units/mg, Millipore) in phosphate-buffered saline (pH 7.2) without calcium or magnesium; trypsin digestion was stopped by adding soybean trypsin inhibitor at 12 mg/ml, after which the nuclei were centrifuged at 800 × *g* for 2 min, resuspended, washed twice in phosphate-buffered saline (pH 7.2), and incubated with MuIFN-$\beta$. Control nuclei were treated identically, apart from exposure to trypsin. After the nuclei were pelleted and washed twice, they were incubated with $^{125}$I-MuIFN-$\beta$, and the bound radioactivity was measured.

## DISCUSSION

Considerable evidence suggests that in order to express their biological activities IFNs must first bind to specific receptors on plasma membranes (3–8). Even cells that are in the process of producing IFN do not become resistant to virus challenge until the IFN has been secreted to bind to cell surface receptors (30). Our estimate of the number of MuIFN-$\beta$ receptors (4000–6000) per cell approximates IFN receptor densities reported by others for mouse and human cells—e.g., 2000 MuIFN-$\alpha/\beta$ receptors on murine L1210 cells, 20,000 MuIFN-$\alpha/\beta$ receptors on murine embryonal carcinoma cells, or 500 on human diploid cells (7, 31, 32). This range may be one magnitude lower than receptor numbers estimated for other ligands (33), taking into consideration the imprecise nature of Scatchard analysis under certain conditions (34). The concentration of MuIFN-$\beta$ at which half of the plasma membrane receptors are occupied approaches 1 nM, indicating a very high affinity of the $L_{929}$ cells for IFN. The apparent positive cooperativity among receptors is suggested by the concavity of the binding curve toward the ordinate (Fig. 1). Although such apparent cooperativity has been shown for HuIFN-$\alpha$ receptors on human lymphoblastoid (Daudi) cells and Hs294T melanoma cells (5, 35), ligand–ligand interactions or heterogeneity of labeled ligand may also cause such an effect (33).

After binding, IFN molecules are internalized (3, 7, 8, 12). Whereas binding occurs at 0–4°C, internalization by endocytosis occurs at more physiological temperatures. Our studies show that internalization of MuIFN-$\beta$ occurs very

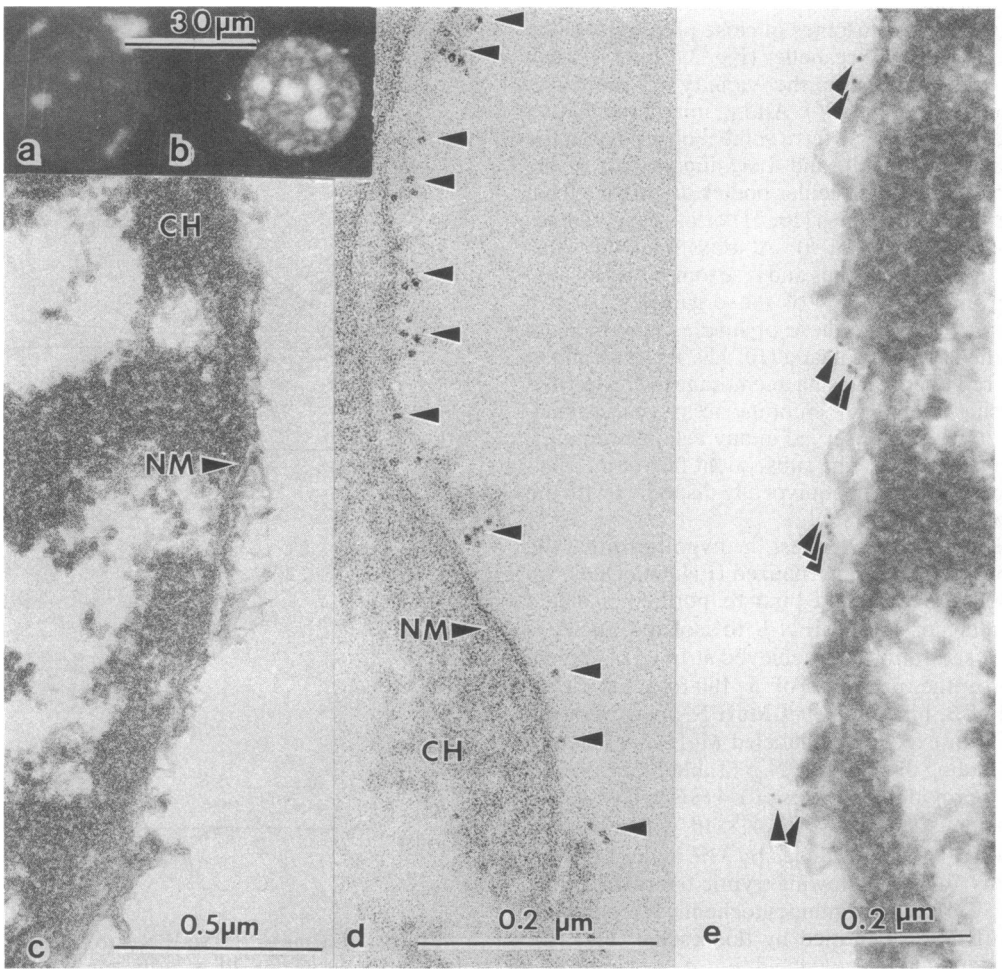


FIG. 5. Fluorescence (*a* and *b*) and electron (*c–e*) microscopy of binding of MuIFN-$\beta$ to isolated nuclei of $L_{929}$ cells. The nuclei were isolated, treated with MuIFN-$\beta$ like intact cells, and labeled either with fluorescein–IgG, ferroglobulin, or protein A–colloidal gold conjugates. (*a*) Control nucleus treated with fluorescein immunochemicals but with MuIFN-$\beta$ omitted. (*b*) MuIFN-$\beta$-treated nucleus showing fluorescence. (*c*) Control nucleus treated with ferroglobulin, but with MuIFN-$\beta$ omitted, showing no ferritin on the nuclear membrane (NM). (*d*) MuIFN-$\beta$-treated nucleus showing ferritin label (arrowheads). (*e*) MuIFN-$\beta$-treated nucleus showing colloidal gold conjugates (arrowheads). CH, chromatin.

rapidly, within a few minutes of incubation at 37°C. Subsequent events in processing of IFN have been less clear. Branca and Baglioni (7) have shown that HuIFN-$\alpha_2$($\alpha$A) was internalized by Daudi lymphoblastoid cells and then degraded after 1–2 hr. Yonehara *et al.* (12) confirmed these findings and made the correlation that IFN-sensitive cells internalized the IFN, whereas IFN-insensitive cells did not; further antiviral resistance failed to develop when internalization was blocked by incubation at 21°C or by treatment with sodium fluoride.

In our studies immunoferritin-labeled IFN was seen within receptosomes as early as 2 min at 37°C, around the Golgi apparatus or the nucleus by about 3 min, and within lysosomes after 5–15 min. Studies of other ligands such as asialoglycoprotein or transferrin (36) indicate that the degradation of the ligand permits recycling of the receptor to the plasma membrane. The apparent reappearance of a small amount of ferroglobulin-labeled MuIFN-$\beta$ on the surface at 15 min (data not shown) might be so interpreted or may represent persistence of the MuIFN-$\beta$–receptor complex at the surface. Our electron microscopic data, like those of Zoon *et al.* (8), indicate that the events involving the IFN–receptor complex follow the general pattern of receptor-mediated endocytosis (10, 11)—namely: (*i*) concentration of the ligand in coated pits, an event that can occur even at 0–4°C; (*ii*) gradual invagination of the coated pits into the cytoplasm after as little as 60 sec at 37°C, but with the mouth of the pit still open to the external milieu; (*iii*) the formation of "early" receptosomes containing the same amount (two to five cores) of ferritin label within them as in coated pits; and (*iv*) movement of the majority of the label by 5–15 min into lysosomes. The progressive, rapid disappearance of radiolabeled IFN from cell surfaces correlates well with the ultrastructural observations of individual cells.

Because of the possible dissociation of the ferritin label from IFN (or from the IFN–receptor complex) that may occur after internalization at the stage of multivesicular bodies (10, 11), the microscopy technique employed in this study did not allow direct observation of the ultimate fate of the ligand. Post-embedding immunocolloidal gold labeling of thin sections of whole cells treated with IFN shows IFN binding to the nuclear membrane (unpublished data). That the nucleus is a possible target of internalized MuIFN-$\beta$ is indicated by the binding of IFN to receptor sites on the nuclear membrane of isolated nuclei, demonstrable by biochemical and immunocytochemical methods. $L_{929}$ cells appear to have a relatively large number ($1.4 \times 10^4$) of specific receptors per nucleus. Their affinity is 7-fold higher ($K_d = 1.4 \times 10^{-10}$ M) than that for the plasma membrane receptors. The association of $^{125}$I-MuIFN-$\beta$ with its nuclear receptors seems to be strong, since treatment with 1 M NaCl, known to elute IFN from nucleic acids, reduced nuclear binding by less than a third. The relative specificity of these receptors is suggested by the fact that MuIFN-$\gamma$ did not compete for these receptors (Table 1). Since trypsin pretreatment of the isolated nuclei resulted in an 80% reduction in MuIFN-$\beta$ binding (Table 1), the nuclear membrane receptors appear to be protein.

The biological significance of IFN binding directly to the cell nucleus is still to be assessed. In addition to the known binding of IFN to plasma membrane receptors (an interaction leading directly to membrane alterations and possibly second messenger activities), we suggest that IFN may regulate some cellular functions by entering the cell and modulating regulatory functions of the nucleus. Various ligands, such as insulin, epidermal growth factor, and steroid hormones, bind to the nuclear membrane or the nuclear matrix (18–22, 37, 38). Goldfine *et al.* (20) have shown that treatment of isolated nuclei with insulin stimulates nuclear membrane nucleotide triphosphatase activity, mRNA efflux from the nucleus, and protein phosphorylation. Thus, it is conceivable that the interaction of IFN with the nucleus may afford additional mechanisms of IFN action, not heretofore suspected, to achieve its multiple cellular effects.

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