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# THE CELLULAR INTERNALIZATION OF RECOMBINANT GAMMA INTERFERON DIFFERS FROM THAT OF NATURAL INTERFERON GAMMA

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Purified natural and recombinant murine gamma interferons (MuIFN-γ) bind at 4°C to cultured L929 mouse fibroblasts with comparable receptor-binding affinity (Kd=9 x $10^{-10}$M). Both $^{125}$I-labeled MuIFNs are rapidly internalized by cells at 37°C, although recombinant IFN is internalized somewhat more slowly than natural IFN ($t_{1/2}$=90 sec and 45 sec, respectively). Immunoelectronmicroscopy showed that the majority of bound recombinant MuIFN-γ was located on the plasma membrane outside of coated areas, whereas natural interferon was found mainly in coated pits. At 37°C most of the recombinant molecules entered the cytoplasm in pinocytotic vesicles, while natural interferon was internalized by the specific mechanism of receptor-mediated endocytosis [1]. However, nearly equal amounts of immunocytochemically detectable molecules of both IFNs were found in the cell nucleus within 2-3 min incubation at 37°C. Thus, the process of translocation of the recombinant IFN-γ appears to differ from that of the natural product.


Much effort is currently directed toward producing recombinant proteins in bacteria for clinical use. Eukaryotic glycoproteins, including IFN-ß and -γ , that are made in bacteria are not glycosylated. The apparent molecular weights of native interferons can differ from their corresponding recombinant products greatly, as with MuIFN-ß (35 kDa compared to 19.5 kDa), or relatively little, as with MuIFN-γ (18 and 22 kDa compared to 15.5 kDa). Most studies indicate that natural murine interferon-γ and E. coli-produced recombinant murine interferon-γ compete for cellular receptor sites and that the lack of carbohydrate on the rMuIFN-γ does not change either the dissociation constant or the measure of the apparent number of plasma membrane receptors [2,3,4]. However, there are reports that natural and bacteria-made recombinant interferons differ in their

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Abbreviations: rMuIFN-γ, recombinant mouse interferon-γ; nMuIFN-γ, natural mouse interferon-γ; EMEM, Eagle's minimal essential medium; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CGPA, colloidal gold-protein A conjugate, BSA, bovine serum albumin; RME, receptor-mediated endocytosis.

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antiviral activities [5] as well as their in vitro antiproliferative effect on the tumor cell lines KB and G361 [6]. In this study nMuIFN-γ and rMuIFN-γ were compared with respect to the kinetics of their internalization and processing by L929 cells.

## MATERIALS AND METHODS

Mouse L929 fibroblasts were grown in suspension as previously described [1,7-10]. MuIFN-γ was induced in murine T lymphocytes and purified to a specific activity of $1.1 \times 10^6$ International Units (IU)/mg [11]. Recombinant MuIFN-γ with a specific activity of $4.7 \times 10^6$ IU/mg was obtained from Genentech (South San Francisco, CA). Antiviral activity of interferons was determined by a GDVII virus hemagglutinin yield-reduction assay [12], which measures about 5.4 times the unitage in the International Standard for MuIFN-γ Gg02-901-533.

Both IFNs were radiolabeled with $^{125}$I-Bolton-Hunter reagent (Amersham, Arlington Heights, IL) without loss of antiviral activity; specific activities were 1.04 Ci/μg and 1.32 Ci/μg for nMuIFN-γ and rMuIFN-γ, respectively. SDS-PAGE of $^{125}$I-rMuIFN-γ under reducing conditions indicated an apparent molecular weight of 15,500 daltons while $^{125}$I-nMuIFN-γ migrated as a doublet at 18,500 and 22,000 daltons, similar to measurements by others [13,14]. The $^{125}$I-MuIFN-γ was approximately 85% pure with several minor high-molecular-weight components.

Binding and internalization of IFNs by L929 cells were studied as described previously [1,7,9,10]. Briefly, cells pretreated with IFN at 4°C, were washed and transferred into 150 μl of EMEM prewarmed to 37°C in a waterbath. Aliquots were taken at one-min intervals and treated with 0.2M acetic acid to release surface-bound $^{125}$I-IFN, which by difference estimated the amount of internalized $^{125}$I-IFN [1,7,10]. Binding data were analyzed using the LIGAND program [15]. The $t_{1/2}$ was estimated from the internalization graph (Fig. 1) as the time the cells internalized 50% of the difference in cpm removable from the cell membrane before cells were shifted from 4°C to 37°C and the amount of cpm removable from plasma membrane after 15 min incubation of cells at 37°C.

To follow the intracellular transport of IFNs, L929 cells grown in suspension were washed three times with cold (4°C) EMEM without serum, incubated at 4°C for 30 min with 10,000 IU/ml of either recombinant or natural MuIFN-γ, washed again with cold EMEM and injected as a thick suspension ($10^8$ cells/0.5 ml EMEM) into 10 ml of EMEM prewarmed to 37°C. Aliquots of $10^7$ cells were taken for fixation in a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde for 30 min, and samples embedded in Lowicryl K4M as previously described [1,8,10]. Ultrathin sections were stained employing an indirect immunogold technique to locate the intracellular IFN [1,8,10]. The sections were first floated on a drop of 0.4% BSA for 1 hr, rinsed briefly, and incubated for 60 min on a drop of anti-MuIFN-γ rabbit IgG (1:900) preabsorbed with a suspension of L929 cells. These antibodies were specific for MuIFN-γ and did not neutralize MuIFN-ß. Sections washed with warm (60°C) PBS with 1% BSA were placed onto a drop of CGPA (E-Y Laboratories, San Mateo, CA) diluted 1:500-1:1,500. The level of nonspecific binding to Lowicryl K4M sections with these dilutions of CGPA was only 0.008 ± 0.002 ($p < 0.05$) particles/μm$^2$ [1,8, 10]. Sections of cells not treated with IFN and processed as described above served as a control for the immunostaining. CGPA was counted in a blind fashion on different parts of every section (i.e., extracellular plastic, cytoplasm, nucleus). The morphometric data were statistically analyzed by means of a VIAS Image Analysis System (Pelco, Tustin, CA) as described previously [1,10].

## RESULTS

The kinetics of internalization by L929 cells of radioiodinated nMuIFN-γ was shown to differ from that of rMuIFN-γ. The half-time ($t_{1/2}$) internalization

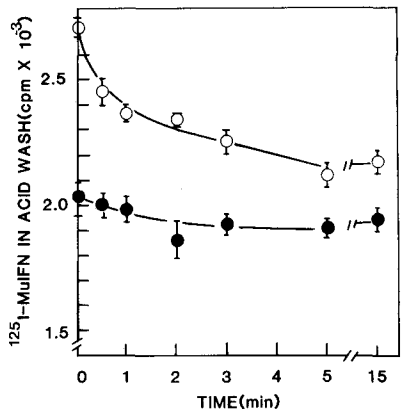


Figure 1. Internalization of $^{125}$I-nMuIFN-γ and $^{125}$I-rMuIFN-γ. L929 cells were pretreated with $^{125}$I-IFN at 4°C, then washed to remove any unbound IFN, and transferred to a water bath at 37°C. Cell samples were taken at intervals, and the surface-bound (not internalized) $^{125}$I-IFN was removed with 0.2 M acetic acid and counted by scintillation spectrometry. O-O, $^{125}$I-nMuIFN-γ; ●-●, $^{125}$I-rMuIFN-γ. Each point represents the mean ± standard deviation of 6 to 9 individual determinations. The $t_{1/2}$ was estimated from the internalization curve as the time at which the cells internalized 50% of the difference in cpm (i.e. IFN) bound to plasma membrane at 4°C at the zero time minus the cpm bound to plasma membrane at 15 min. of the cells' incubation at 37°C. For nMuIFN-γ $t_{1/2}$ is estimated at about 45 sec and $t_{1/2}$ for rMuIFN-γ is estimated at about 1.6 min.

rate of the natural MuIFN was about 45 sec, while rMuIFN-γ was internalized more slowly, with $t_{1/2}$ =1.5 min (Fig. 1).

Electron microscopy demonstrated that the entry and intracellular routes of recombinant and natural MuIFNs-γ also differed. We have shown previously [1] that at 4°C natural MuIFN-γ was located primarily in coated pits of the plasma membrane and upon warming of the cells at 37°C entered the cell via RME. In contrast, recombinant IFN-γ was only rarely found in coated pits (Fig. 2) and mainly located on the plasma membrane (Fig. 2a-e) as well as in pinocytotic vesicles not involving clathrin-coated areas. Recombinant MuIFN-γ was detected inside cytoplasm mainly in lysosomes (Fig. 2h) throughout the 15 min of observation while nMuIFN-γ was rarely seen in lysosomes [1]. Within 2-3 min incubation of 37°C some rMuIFN-γ was detected in vesicles of the Golgi apparatus (Fig. 2 f,g), in the perinuclear space (Fig. 2i), and in the nucleoplasm (Fig 2k). The dynamics of intracellular transport of both IFNs is depicted in Fig. 3. The natural MuIFN-γ, as detected immunocytochemically with CGPA, was rapidly transported at 37°C with maximal accumulation in the cell nucleus by 2 min incubation. The transport of the recombinant IFN-γ was slightly slower, with maximum accumulation in the nucleus observed at the third min. This difference in time is probably insignificant with regard to the 10-12 hours necessary to establish an antiviral state in L929 cells with MuIFN-γ (unpublished data). The amount of immunocytochemically detectable rMuIFN-γ increased in the nucleus and

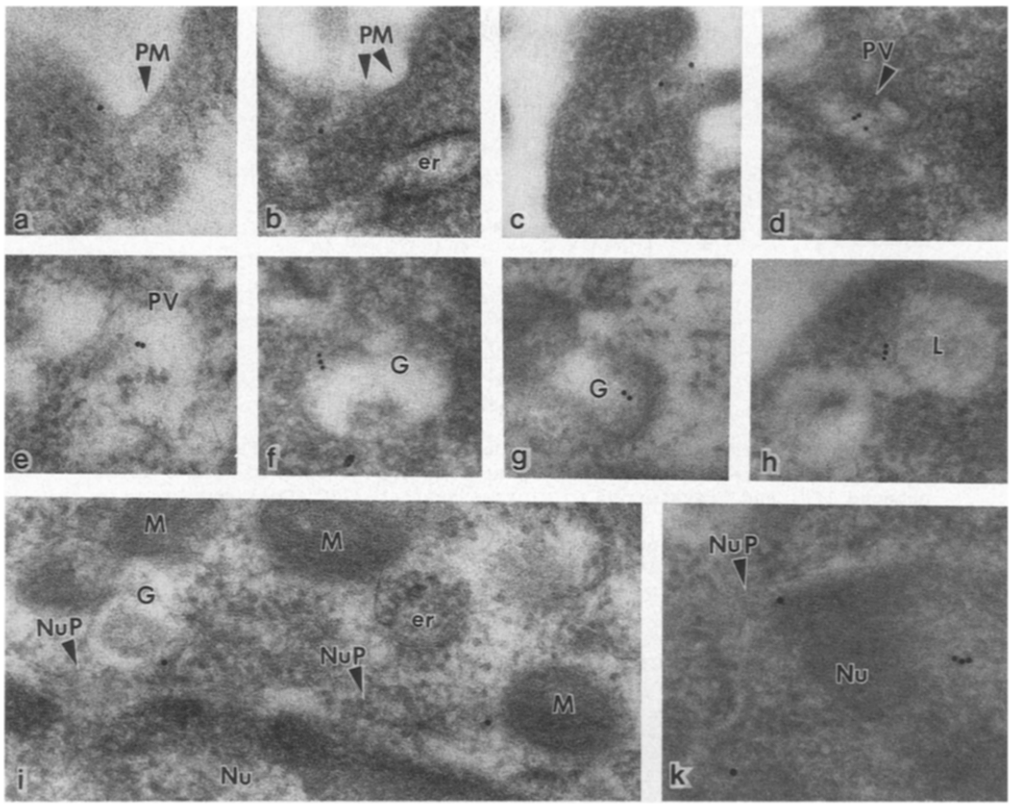


Figure 2. Electron micrographs of sections of Lowicryl K4M-embedded cells that had been treated with recombinant MuIFN-γ. Cells were first incubated with rMuIFN-γ at 4°C, then washed, and transferred to 37°C. The CGPA label appeared mainly in pinocytotic vesicles and much less in coated pits or receptosomes (a-e). However, some label was observed in Golgi vesicles (f, g, i) close to nuclear pores, as previously observed with nMuIFN-γ [1] and natural MuIFN-ß [8, 10]. Inside the nucleus, rMuIFN-γ was observed in dense chromatin (k). Substantial labeling was observed in lysosomes (h) and in the nucleus throughout incubation.

er-endoplasmic reticulum; G-Golgi apparatus; L-lysosome; M-mitochondria; Nu-nucleus; NuP-nuclear pore; PM-plasma membrane; PV-pinocytotic vesicles.

Magnification-a,b,e,k-x80,000; c,d,f-h,i-x60,000.

cytoplasm during 15 min of incubation at 37°C (Fig. 3) while the amount of natural MuIFN-γ was reduced to background level by 5 min at 37°C.

## DISCUSSION

Binding studies previously performed with mouse IFN-γ indicated that different cell types possess about 1-3 x $10^3$ receptor sites of high affinity (Kd=6.5 x $10^{-9}$ to 7.4 x $10^{-10}$M) [13] while others have shown that recombinant MuIFN-γ binds to L1210 cells with 10-fold lower affinity than does native MuIFN-γ [6]. Our results demonstrate that for L929 cells affinities of recombinant and native MuIFN-γ do

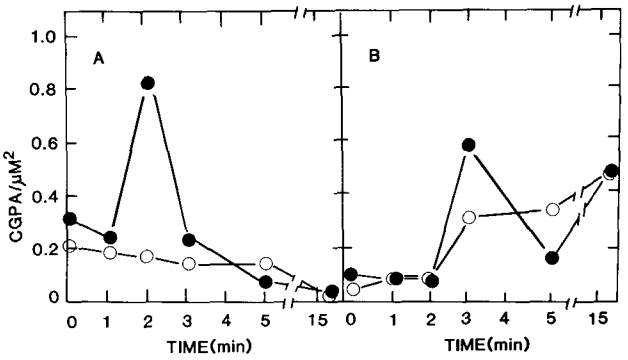


Figure 3. Dynamics of the migration of IFNs from the cytoplasmic compartment into the cell nucleus. The density of CGPA is represented per $\mu m^2$ of the surface of ultrathin sections of the cytoplasm or nucleus after subtraction of background counts. The scale on the ordinate indicates the difference in the mean CGPA counts on experimental and control sections, thus representing internalized IFN. Each point represents the mean of counts made on 20-40 random sections of cells, read in a blind fashion. Immunocytochemically recognizable rMuIFN-γ was detected in cytoplasm throughout incubation and in the nucleus with a peak at the third min. On the abscissa is the period of incubation at 37°C of cells pretreated with IFN at 4°C.
O--O - cytoplasm
●--● - nucleus

not differ (8.7 and 9.8 x $10^{-10}$M respectively, data not shown). Further, the number of high-affinity receptors on L929 cells demonstrable at 4°C was the same for both IFNs. Since two molecular-weight species were detected for nMuIFN-γ, the estimate of receptor affinities and numbers might be made less precise by the possible heterogeneity of radiolabeled proteins.

Here we report that $^{125}$I-rMuIFN-γ enters cells at a somewhat slower rate than natural $^{125}$I-MuIFN-γ; however, the total amount of IFN taken into the nucleus of the cells within 2-3 minutes is almost the same. Although the pathway from the plasma membrane to the nucleus is not established in the detail that is known for MuIFN-β [10], both IFNs are found in RME vesicles within the first 2 minutes of shift from 4°C to 37°C. Unlike natural IFN-γ, rIFN-γ is found in substantial amounts in pinocytotic vesicles, possibly related to its prevailing location on cell membrane outside of coated areas. We assume, as have others, that a ligand in pinocytotic vesicles is destined for hydrolysis [16] and may not be a functionally important pathway in regulating the early cell response to IFN although it may be of significance for IFN receptor recycling. Others have shown that IFN-induced RNA transcripts appear as soon as 5-15 minutes after IFN treatment [17]; the presence of IFN within the nucleus in 2-3 minutes suggests that this nucleus-associated IFN is there quickly enough to be involved in induction of these transcripts. The carbohydrate found on natural MuIFN-γ is probably not critical for the final effect of IFN on the cell nucleus for L929 cells but may influence molecular conformation and account for the differences from the recombinant IFN observed in cellular binding and processing.

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