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## Demonstration of the presence of a specific interferon- $\gamma$ receptor on murine astrocyte cell surface

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### Summary

Interferon gamma (IFN- $\gamma$ ) is a pleiotropic lymphokine produced by T-lymphocytes which acts as a soluble mediator in immunological reactions. In addition to several immune target cells, such as monocytes and macrophages, it acts on the principal glial population, the astrocytes, inducing Ia antigen expression.

We have developed a binding assay for  $^{125}\text{I}$ -labeled recombinant murine IFN- $\gamma$ , and show that, using this assay, IFN- $\gamma$  interacts with a single specific receptor on the murine astrocyte cell membrane. The binding is specific and saturable and it takes place with a  $K_d = 1.64 \times 10^{-9}$  M, with 11,100 receptor molecules per astrocytic cell. The binding shows, as for macrophages, species specificity.

Using an immune assay including rabbit antibodies to IFN- $\gamma$  and  $^{125}\text{I}$ -labeled protein A, we have demonstrated an internalization of the ligand. This is an energy-dependent process, as around 50% of the bound IFN- $\gamma$  is endocytosed after 4 h at 37 °C when cultures are maintained in complete culture medium.

### Introduction

A paradox exists in neuroimmunology as the central nervous system (CNS) does not express class II major histocompatibility complex (MHC) antigens (Williams et al., 1980) and pathological immune reactions take place within the CNS. This paradox can be resolved by the finding that

interferon gamma (IFN- $\gamma$ ) and some viral infections, such as those induced by murine coronavirus (Suzumura et al., 1986), Theiler's murine encephalomyelitis virus (Rodriguez et al., 1987) or neurotropic murine hepatitis virus (Massa et al., 1986) can induce the expression of new antigens in astrocytes. Several studies have shown that IFN- $\gamma$ , also known as immune interferon, induces the expression of class II MHC antigens in macrophages (Stegg et al., 1982) and astrocytes (Hirsch et al., 1983; Wong et al., 1985).

The fact that a T-lymphocyte lymphokine produces such a striking effect on glial cells may

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have high functional significance. It has been demonstrated that IFN- $\gamma$  induces Ia antigen expression on astrocytes (Fierz et al., 1985) and that astrocytes activated in this manner can present antigens such as myelin basic protein to T-lymphocytes (Fontana et al., 1984). These autoaggressive T-cells can lyse presenting astrocytes (Sun and Wekerle, 1986). Conversely, astrocytes do not produce IFN- $\gamma$ , even when subjected to a superinduction regimen (Tedeschi et al., 1986).

We have undertaken the study of the interaction between recombinant IFN- $\gamma$  and its receptor on the murine astrocyte cellular membrane from a physicochemical point of view. Here we describe in detail the binding characteristics of this important neuroimmunological reaction.

## Material and methods

### *rIFN- $\gamma$ radioiodination*

Recombinant mouse gamma interferon, free of protein stabilizers was purchased from Holland Biotechnology, Leiden, The Netherlands and was labeled with  $^{125}\text{I}$  (Amersham International, U.K.) utilizing the enzymobead iodination lactoperoxidase reagent (Bio-Rad, Richmond, CA, U.S.A.) (Marchalonis, 1969). The  $^{125}\text{I}$ -rIFN- $\gamma$  and free iodine were separated on a disposable PD-10 Sephadex G-25M column (Pharmacia, Uppsala, Sweden). The specific activity obtained was 203 Ci/mmol and was 96% trichloroacetic acid (TCA) precipitable. The iodinated rIFN- $\gamma$  migrated mostly as a single peak with an apparent molecular weight of 20,000 when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis slabs. Protein A from *Staphylococcus aureus* (Sigma Chemical Co., St. Louis, MO, U.S.A.) was labeled by the chloramine T method (Greenwood et al., 1963).

### *Astrocyte cultures*

Astrocyte cultures were prepared by mechanical dissociation of the cerebral cortex from newborn Wistar rats or SJL/J mice. The cortex was isolated under a dissecting microscope and carefully cleaned of choroid plexus and meninges. Cell suspensions were filtered through 80  $\mu\text{m}$  pore size mesh into Dulbecco's modified Eagle

medium (DMEM) containing 10% fetal calf serum (FCS) and gentamicin (Flow Laboratories, U.K.). After centrifugation, cells were filtered through a 20  $\mu\text{m}$  mesh sieve, plated in 75  $\text{cm}^2$  tissue culture flasks (Costar, Cambridge, MA, U.S.A.) and cultured at 37 °C. The medium was changed after 4 days of culture and, subsequently, 3 times a week for the entire culture period. Cultures were enriched in astrocytes by removal of less adherent oligodendrocytes by shaking for 18 h at 37 °C and 250 rpm in an orbital shaker. Cellular confluence was observed 10 days after plating with a polygonal flat cell morphology. The content of a mean of 98.4% astrocytes was confirmed by indirect immunofluorescence staining of methanol-fixed cultures using rabbit anti-glial fibrillary acidic protein (GFAP) antiserum (Dakopatts, Denmark) and fluorescein-labeled goat anti-rabbit IgG (Miles Laboratories, Elkhart, IN, U.S.A.). The lack of noticeable oligodendrocytes and microglial/macrophage cells was determined by using a guinea pig anti-myelin basic protein (MBP) antiserum prepared by ourselves (Rubio and Cuesta, 1989) and a monoclonal anti-Mac-1 antibody (Serotec, Oxford, U.K.). Secondary fluorescein-labeled antibody against guinea pig and rat IgG were purchased from Sigma.

The rat astrocytoma C-6 was obtained from the American Type Culture Collection, U.S.A., and cultured in DMEM supplemented with 10% FCS and gentamicin.

### *Binding assay*

Confluent astrocyte monolayers in 16 mm diameter 24-well plates (Costar) were used in the binding experiments. Cells were incubated at different temperatures, times and with or without unlabeled IFN- $\gamma$ , as stated in the text. The buffer used was phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) from Sigma. After washing 3 times with the above buffer, cells were detached from the plastic surface with 1 N NaOH at 60 °C and counted in an LKB-Wallac 1282 Compugamma counter.

### *Endocytosis experiments*

Monolayers of astrocytes in 16 mm diameter 24-well plates were incubated with a saturating amount of unlabeled IFN- $\gamma$ . Binding and endocytosis

tosis were allowed to take place at 4 °C or 37 °C for different periods of time. After two washes with PBS-BSA to remove free IFN- $\gamma$ , cells were fixed for 30 min at room temperature in freshly prepared 3% formaldehyde in PBS. After cells were washed, they were incubated with a rabbit anti-murine IFN- $\gamma$  antiserum (kindly provided by Dr. Robert C. Curry, Bristol-Myers, Wallingford, CT, U.S.A.) diluted 1:100, at 37 °C for 30 min. After two further washes, the antibody bound to IFN- $\gamma$  available on the astrocytes surface was detected by incubation with radioiodinated staphylococcal protein A (100,000 cpm, 30 min, 37 °C). After washing, cells were detached with 1 N NaOH and counted. To assess total internalized ligand, we removed surface-bound  $^{125}\text{I}$ -IFN- $\gamma$  from twice-washed cells with 2 ml of 0.15 M NaCl-50 mM glycine, pH 3.0 at 4 °C for 2 min (Costlow and Hample, 1982). After two more washes with PBS, cells were detached and counted in the gamma counter.

#### Statistical analysis

Scatchard and Hill plot analysis of the data from binding assays was performed employing the LIGAND program (Munson and Rodbard, 1980). The data shown represent mean results of three reproducible experiments  $\pm$  standard deviations.

## Results

#### IFN- $\gamma$ binding to cultured astrocytes

Fig. 1 shows the kinetics of  $^{125}\text{I}$ -labeled IFN- $\gamma$  binding to pure astrocyte cultures. Care has been exercised to use only cultures containing around 99% astrocytes, free of oligodendrocytes or microglial/macrophage cells according to criteria explained under Materials and methods. Saturation of the binding capacity of  $1 \times 10^5$  astrocytic cells takes place in 60 min at 37 °C. At 4 °C, a 120 min incubation period is required to reach maximal binding. Maximal binding is found to be around 5% of the input, which is kept constant in 100,000 cpm per well in this work.

#### Specificity of IFN- $\gamma$ binding to astrocytes

To demonstrate that the binding shown in Fig. 1 is specific, labeled IFN- $\gamma$  was allowed to bind

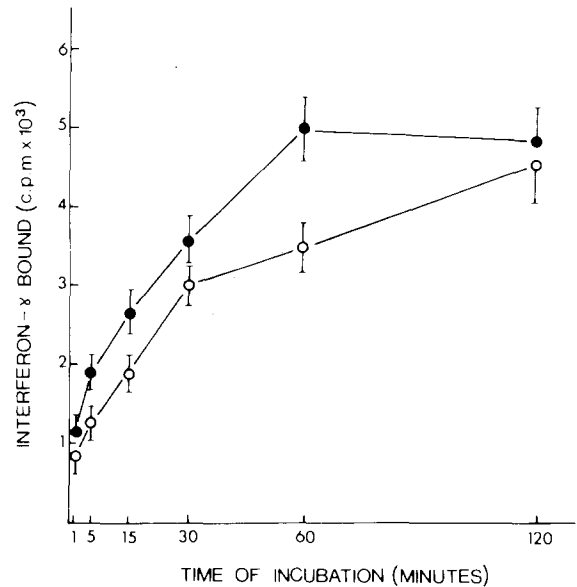


Fig. 1. Kinetics of  $^{125}\text{I}$ -IFN- $\gamma$  binding to cultured mouse astrocytes. The binding assay was performed at 37 °C (●—●) or 4 °C (○—○). Mean results of three experiments  $\pm$  standard deviations are shown. 500 pmol of labeled IFN- $\gamma$  were used per well.

cells in the presence of a 100-fold excess of the unlabeled recombinant molecule. The saturation of specific receptor sites is depicted in Fig. 2, and shows that the binding of IFN- $\gamma$  to cells is inhibited by the presence of a constant amount of unlabeled ligand. If specific binding is defined as that binding which is inhibited in the presence of an excess of 'cold' IFN- $\gamma$ , it is approximately 65% in this system.

When these data are analyzed by the LIGAND program, a Scatchard plot (Scatchard, 1949) is obtained showing a  $K_d$  of  $1.64 \times 10^{-9}$  M and a  $B_{\text{max}}$  of  $3.78 \times 10^{-11}$  M (Fig. 2, inset). The number of receptor molecules per cell is calculated to be 11,100. The Hill plot shows that the astrocyte surface receptor binds IFN- $\gamma$  in a homogenous, noncooperative manner. Half-saturation is achieved at a concentration of free ligand of about 200 pmol ( $0.2 \times 10^{-9}$  M) that is in the range of the  $K_d$  reported.

#### Cellular specificity of the receptor

Different numbers of mouse astrocytes were allowed to attach to 16 mm diameter wells and

incubated with 100,000 cpm of IFN- $\gamma$ . As shown in Fig. 3, the binding reaches a plateau when  $5-10 \times 10^4$  cells are plated per well. This indicates that the binding is receptor-specific and cell number-dependent.

As described by other authors in studies about IFN- $\gamma$  receptor in macrophages, rat cells do not recognize murine IFN- $\gamma$  (Celada et al., 1984). Wistar rat astrocytes do not bind any ligand during the time of incubation used by us (Fig. 3). C-6 cells, an astrocytoma cell line of rat origin, do not either show any binding (not shown). These data confirm the species specificity of the receptor molecule, present also in astrocytes.

The above results provide direct evidence for high affinity binding of 'immune' interferon to a specific cell surface receptor on murine astrocytes.

### Endocytosis of the receptor-IFN- $\gamma$ complex

The use of a rabbit antibody against murine IFN- $\gamma$  and  $^{125}\text{I}$ -labeled protein A allows us to study the availability of the leukin on the astrocyte surface. When cell receptor-bound unlabeled IFN- $\gamma$  remains at  $37^\circ\text{C}$  in complete culture medium (DMEM + 10% FCS) it begins to be internalized after 30 min. After 4 h only 50% of the maximal amount of bound IFN- $\gamma$  remains available to the specific antibody plus protein A complex. If the binding and further incubation are performed at  $4^\circ\text{C}$ , internalization does not occur (Fig. 4). No endocytosis takes place when the complete culture medium is substituted by PBS.

We assume that the detection of lower antibody binding is due to an energy-dependent in-

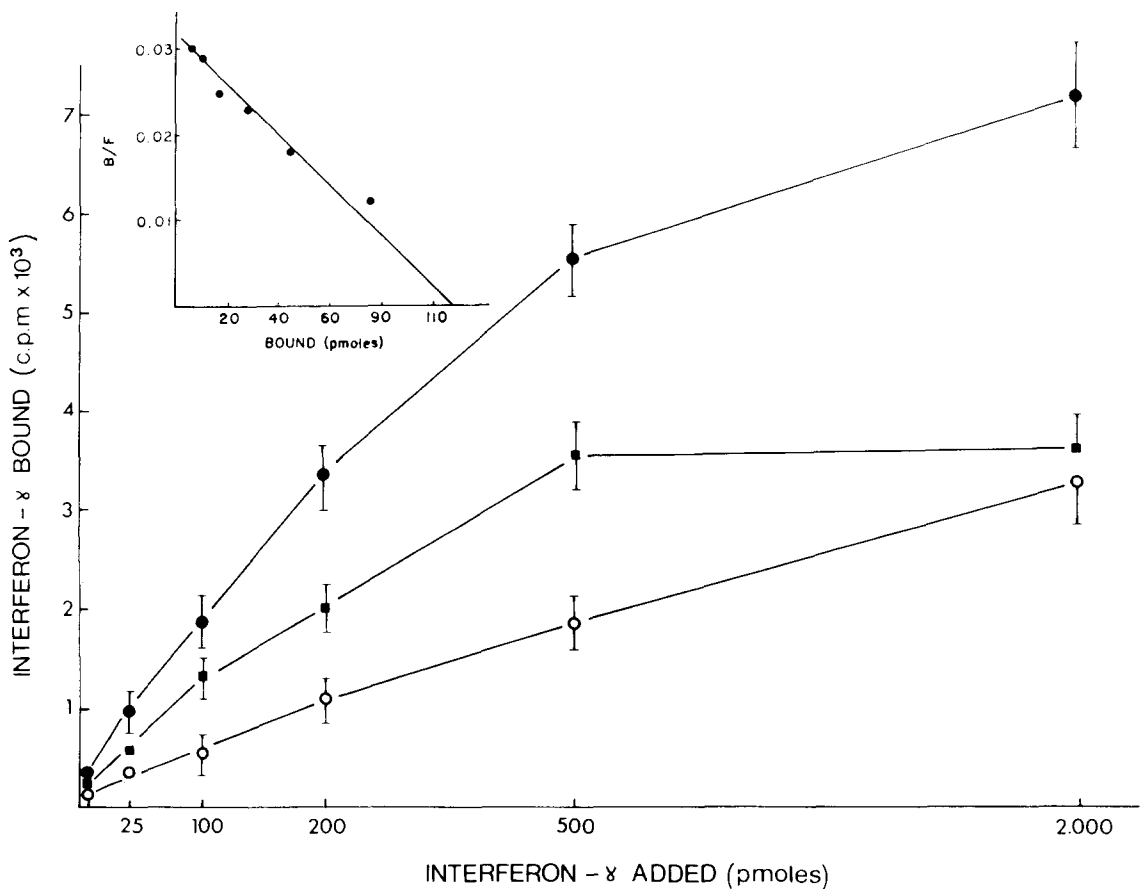


Fig. 2. Saturation of  $^{125}\text{I}$ -interferon binding to mouse astrocytes. Specific binding (■—■) takes place in the presence of an excess of unlabeled IFN- $\gamma$ . Total binding (●—●) and non-specific binding (○—○) are also shown. The Scatchard analysis obtained using the LIGAND program is shown in the inset.

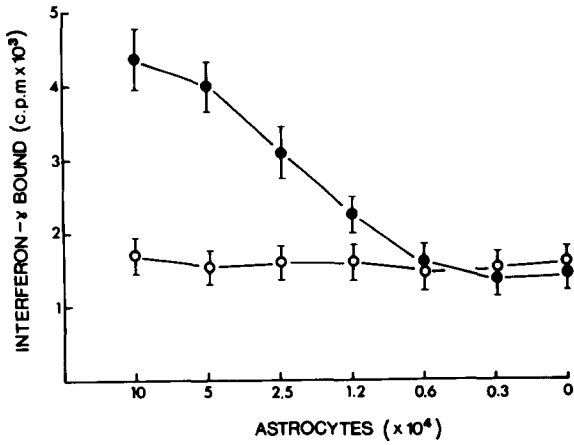


Fig. 3. Dose-dependent absorption of <sup>125</sup>I-IFN-γ by astrocytes. Different numbers of mouse (●—●) or Wistar rat (○—○) astrocytes were incubated for 1 h at 37 °C, as described in Materials and methods, with 500 pmol of labeled INF-γ per well.

TABLE 1  
INTERNALIZATION OF <sup>125</sup>I-IFN-γ BY CULTURED MURINE ASTROCYTES

Temperature	Time of incubation	% pH 3.0 resistant (internalized) <sup>a</sup>
4 °C	60 min	6 ± 2.6 <sup>b</sup>
	120 min	9 ± 3.7
	240 min	8.5 ± 3.1
37 °C	60 min	11 ± 3.3
	120 min	38 ± 4
	240 min	56 ± 5.2

<sup>a</sup> Mean ± SE, n = 3.

<sup>b</sup> 100% binding was defined as bound cpm after 30 min at 37 °C.

ternalization process rather than to a release from the cell receptor, because of the high affinity of the binding ( $K_d = 1.64 \times 10^{-9}$  M) and be-

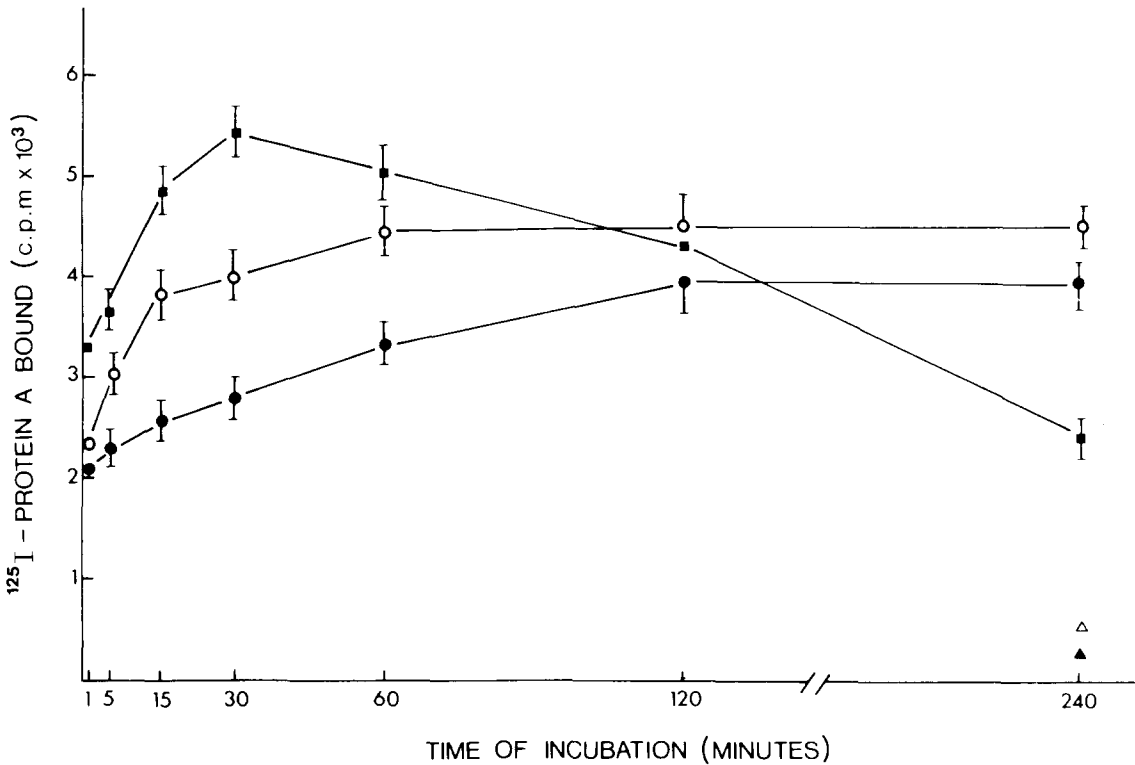


Fig. 4. Internalization of receptor-bound IFN-γ by murine astrocytes. The availability of the lymphokine molecules to antibody was measured at 37 °C (■—■) or 4 °C (●—●) for different periods of time. Complete culture medium was substituted in some experiments by PBS at 37 °C (○—○). Negative controls include normal rabbit serum instead of antiserum (Δ—Δ) and no IFN-γ added (▲—▲).

cause the hypothetical release does not take place either at 4°C or in PBS at 37°C. These data demonstrated an energy-dependent internalization of IFN- $\gamma$  by astrocytes, as described by others for macrophages (Anderson et al., 1983).

To further demonstrate internalization of the ligand, we assessed the amount of intracellular radioactivity after removing surface-bound INF- $\gamma$  at low pH (glycine buffer, pH 3.0). Table 1 shows that at 4°C no internalization takes place in a significant manner when the time of incubation is increased. Conversely, at 37°C the pH 3.0-resistant label (internalized) increases, reaching  $56 \pm 5.2\%$  of maximal binding after 240 min of incubation. The amount of cpm bound after 30 min at 37°C is defined as 100% of binding.

## Discussion

Interferon gamma is a lymphokine with a wide array of biological effects on immune and non-immune cells (Trinchieri and Perussia, 1985). These effects are thought to be mediated by a cell surface receptor (Aguet, 1980) which does not bind IFN- $\alpha$  or IFN- $\beta$  (Branca and Baglioni, 1981). The human IFN- $\gamma$  receptor has been purified and characterized from a biochemical point of view showing a molecular weight of around 90,000 (Novick et al., 1987, 1989). Monoclonal antibodies with the ability to block the binding of  $^{125}\text{I}$ -labeled IFN- $\gamma$  to its receptor have been raised against it (Novick et al., 1989; Depla and De Ley, 1990). Finally, the gene encoding the human IFN- $\gamma$  receptor has recently been cloned (Aguet et al., 1988).

In addition to the macrophages, its main target cell (Schreiber et al., 1985), glial astrocytes have been reported to be committed to an important immune role when in contact with IFN- $\gamma$  (Hirsch et al., 1983; Wong et al., 1985).

Our results clearly demonstrate the presence of a specific receptor for IFN- $\gamma$  on murine astrocytes. The binding takes place in a bimolecular reversible manner with high affinity,  $K_d = 1.64 \times 10^{-9}$  M. This affinity constant is higher than that found for rIFN- $\gamma$  on human fibroblasts ( $1-5 \times 10^{-8}$  M) (Anderson et al., 1982) and mouse macrophages ( $1.18 \times 10^{-8}$  M) (Celada et al., 1984)

and lower than that reported for human monocytes ( $5-10 \times 10^{-9}$  M) (Finbloom et al., 1985).

The number of binding sites per cell reported here (11,100 sites/cell) is similar to the number found for murine macrophages ( $\approx 12,000$ ) (Celada et al., 1984) and human fibroblasts (Anderson et al., 1982) and somewhat higher than that observed on human monocytes (4000 sites/cell) (Finbloom et al., 1985). The binding is specific and saturable (Fig. 2).

It is also species-specific, as Wistar rat astrocytes and the rat astrocytoma cell line C-6 do not bind murine IFN- $\gamma$ , maintaining the species specificity found in macrophages. There is a somewhat paradoxical situation concerning the apparent lack of specific binding of mouse IFN- $\gamma$  to rat cells and the biological effects detected in rat astrocyte cultures (Fiertz et al., 1985). This could be explained by the presence of a low affinity receptor only noticeable after very long periods of incubation (Celada et al., 1984).

The human fibroblast receptor for IFN- $\gamma$  has been reported to be internalized at 37°C (Anderson et al., 1983). We have observed the same phenomenon in an immune assay set up with specific antibodies and protein A labeled with  $^{125}\text{I}$ . The endocytosis of IFN- $\gamma$  bound to the cell surface receptor takes place at 37°C but not at 4°C and a rich culture medium (DMEM + 10% FCS) is required, probably to maintain an active metabolic state in the cultured astrocytes.

Further demonstration of internalization by an energy-dependent process is provided for the direct measure of the intracellular labeled ligand which takes place at 37°C but not at 4°C.

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