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Preparation and characterization of labelled interferon-γ and the development of radioreceptor assay for interferon-γ

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Abstract: A ^{125}I-labelled recombinant interferon-γ (IFN-γ) was prepared by the lactoperoxidase–glucose oxidase method. The specific activity of the labelled IFN-γ was 31 Bq U^{-1} and its molecular weight, immunoreactivity and receptor binding ability remained the same after the labelling. Using the labelled IFN-γ and FL5-1 cells from human amniotic membrane, a radioreceptor assay was developed. Natural IFN-γ, recombinant IFN-γ and the labelled IFN-γ were observed to bind to the same binding sites on the cells with similar affinity (K_d = 1.3–2.2 × 10^{-10} M). The radioreceptor assay was more specific than a bioassay because the labelled IFN-γ did not compete with IFN-α or IFN-β. It was much more sensitive (90 pM) than conventional competitive radioimmunoassay (300 pM) using the same labelled IFN-γ, and as sensitive as immunoenzymometric assay (60 pM). The radioreceptor assay should be useful not only for research on IFN-γ but also for the determination of biological activity in process control and/or quality control of IFN-γ manufacturing.

Keywords: *Radioreceptor assay; radioiodine label; interferon-γ; competitive radioimmunoassay; immunoenzymometric assay.*

Introduction

Interferon-γ (IFN-γ), which has anti-virus and anti-tumour activities, and also can suppress cell growth, has recently been produced by recombinant DNA technology and used as a therapeutic agent for renal cancer [1].

The biological activity of IFN-γ is mainly measured by bioassay. One bioassay using FL5-1 cells of an epithelial cell strain from human amniotic membrane has been established [2] and is used for routine process control and/or quality control in large-scale production of recombinant IFN-γ. However, its procedure is complex and time consuming. Furthermore, it is affected by the presence of IFN-α and IFN-β. Immunoradiometric assay is popular for peptides like IFN-γ, offering high sensitivity and good precision [3] but the measured values do not always agree with the biological activities because of differences of antigenic epitopes from biological active sites.

One solution to these problems may be a radioreceptor assay because the biological activity of IFN-γ is triggered by binding to a receptor on the cell surface and the binding behaviour should reflect the biological activity [4]. Sarkar *et al.* and Nickloff detected receptors for IFN-γ using a monolayer of WISH cells and human keratinocytes, respectively [5, 6], and estimated the dissociation constants and the number of binding sites per cell.

The study of receptor binding requires radiolabelled IFN-γ with high affinity and specific radioactivity. In the past, ^{125}I-labelled IFN-γ has been prepared by the Bolton–Hunter method [5–7] or the chloramine-T method [8] but little information is available on the properties of labelled IFN-γ. In this study, the lactoperoxidase–glucose oxidase method was chosen for the labelling because less modification of the IFN-γ molecule occurs than with the Bolton–Hunter method and there is less damage to the protein/peptide molecule than that caused by the usual chloramine-T method. The receptor binding of the labelled IFN-γ as well as the molecular weight and immunoreactivity of the labelled IFN-γ were investigated.

Using the labelled IFN-γ, a radioreceptor assay with suspended FL5-1 cells was developed with the intention of finding a more precise and feasible assay, although a monolayer of cells is generally used for the detection of IFN-γ binding sites [4–6].

A radioimmunoassay and an immuno-

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enzymometric assay for IFN-γ were also developed in order to characterize the labelled IFN-γ and their sensitivities were compared with that of the radioreceptor assay.

Experimental

Reagents

Recombinant human IFN-γ (2×10^7 U mg^{-1}) was obtained from Shionogi & Co. (Osaka, Japan). FL5-1 cells (origin: human amniotic membrane) were kindly provided by Dr S. Yamazaki of the National Institute of Health, Japan. These cells were cultured and after confluency, were suspended by trypsin treatment [2]. The concentration was adjusted with Eagle's minimal essential medium (Nissui Pharmaceutical Co., Tokyo, Japan) which contained 10% foetal calf serum (Flow Laboratories, Inc., McLean, VA, USA) and 0.00025% amphotericin B (Sankyo Co., Tokyo, Japan) (FCS–MEM). Natural IFN-α_2, IFN-β, and IFN-γ were purchased from Schering Co. (Kenilworth, NJ, USA), Dr Rentschler Arzneimittel GmbH & Co. (Laupheim, FRG) and Japan Chemical Research Co. (Kobe, Japan), respectively. Rabbit antiserum against IFN-γ was prepared by the usual method [9]. Anti-IFN-γ monoclonal antibody was a gift from Dr M. Ide of our laboratories. Other reagents were of analytical grade unless otherwise specified.

Preparation of iodine-labelled IFN-γ

A 25-μl portion of 1% β-D-glucose in 0.5 M phosphate buffer (pH 7.4) was pipetted into a small test tube which contained 50 μl of IFN-γ [1 mg ml^{-1} in 0.1 M phospahate buffer (pH 7.4)], 20 μl of $Na^{125}I$ (3.7 GBq ml^{-1}, Amersham International Plc, Amersham, UK) and 50 μl of immobilized lactoperoxidase–glucose oxidase suspension (Enzymobead, Bio-Rad Laboratories, Richmond, CA, USA). The tube was incubated for 20 min at 20–25°C and then centrifuged. The supernatant and an equal volume of 500 mM $Na_2S_2O_5$ were mixed and left standing for 30 min at 20–25°C. The reactant was applied to a Sephadex G-25 (Pharmacia-LKB Biotechnology, Uppsala, Sweden) column [200 × 10 mm i.d.; eluent: 0.1 M phosphate buffer (pH 6.8) containing 0.5% human serum albumin (Sigma Chemical Co., St. Louis, MO, USA), 5% maltose and 0.03% L-cysteine] and the first radioactive peak was collected. Radioactivity was measured with a γ-counter (ARC-600, Aloka, Tokyo, Japan).

Gel-permeation high-performance liquid chromatography

Molecular weight of the labelled IFN-γ was estimated by gel-permeation high-performance liquid chromatography (HPLC), using an LC-6A system (Shimadzu, Kyoto, Japan), TSK-Gel 3000SW column (600 × 7.5 mm i.d., Tosoh, Tokyo, Japan), and 0.1 M phosphate buffer (pH 6.8) containing 0.1% sodium lauryl sulphate as a mobile phase. The flow rate and wavelength of the UV detector were 1 ml min^{-1} and 280 nm, respectively. In addition, the IFN-γ molecule should be monomeric under these conditions instead of a dimerized form in usual buffers without detergents.

Radioreceptor assay

The labelled IFN-γ (260,000 cpm ml^{-1}), 100 μl, and the FL5-1 cell suspension ($0.5–1 \times 10^7$ cell ml^{-1}), 100 μl, were pipetted into a polystyrene assay tube which contained an 100-μl solution of the IFN-γ. The FCS–MEM was used as the assay buffer. The tubes were incubated for 1 h at 37°C and the supernatants were aspirated off after centrifugation for 5 min at 2000 ***g***. The radioactivity of the iodine bound to the cells was measured with the γ-counter.

Competitive radioimmunoassay

The labelled IFN-γ (diluted to 200,000 cpm ml^{-1}), 100 μl, and 100 μl of the antiserum (diluted to 1:120,000) were added to the assay tube which contained 100 μl of IFN-γ standard solution. After incubation for 16 h at 4°C, 100 μl of immobilized anti-rabbit second antibody suspension (Immunobead, Bio-Rad Laboratories, Richmond, CA, USA) was added to each reaction tube. The tubes were incubated for a further 1 h at 4°C and centrifuged for 5 min at 2000 ***g***. The radioactivities of precipitates in each tube were measured.

Immunoenzymometric assay

The γ-globulin fraction of the antiserum [2 μg in 100 μl of 0.2 M Tris–HCl buffer (pH 7.4) for each well] was immobilized on a microtiter plate (Immulon 1, Dynatech Laboratories, Chantilly, VA, USA) for 16 h at 20–25°C. After two washings with a washing buffer [0.01 M phosphate buffer (pH 7.4)

containing 0.9% sodium chloride and 0.5% Tween 80], the binding sites remaining on the well were blocked with an assay buffer [0.01 M phosphate buffer (pH 7.4) containing 0.9% sodium chloride and 1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA)] for 1 h at 37°C. Sample solutions or standard solutions of IFN-γ, 100 μl, were added to the antibody immobilized plate and the plate was left standing for 2 h at 37°C. After two washings of the plates, 100 μl of an enzyme-labelled antibody (horseradish peroxidase conjugate with monoclonal anti-IFN-γ antibody prepared by Nakane's method [10]) was added to the plate, which was then left standing for 2 h at 37°C. The enzyme activity of each well of the plate was measured using a substrate solution of 0.03% hydrogen peroxide and 0.05% 2,2′-azinobis(3-ethylbenzthiazoline sulphonic acid) (Sigma, Chemical Co., St. Louis, MO, USA) in 0.1 M citrate buffer (pH 4.2).

Results

Preparation of iodine-labelled IFN-γ

Radioiodine-labelled IFN-γ which retained the property of intrinsic receptor binding as well as its chemical characteristics was prepared by the lactoperoxidase–glucose oxidase method. The labelled IFN-γ was bound to FL5-1 cells and this suggested that it could be used for a radioreceptor assay.

The molecular weights of the labelled compounds were estimated by gel-permeation HPLC and no significant difference from that of unlabelled IFN-γ was observed. The labelled compound also became bound to anti-IFN-γ antibody and the imunoreactivity was maintained after the labelling. The labelled IFN-γ gave a good standard curve of competitive radioimmunoassay, with a sensitivity of about 300 pM (30 fmol 100 μl^{-1}) (Fig. 1). The amount of IFN-γ in labelled IFN-γ was measured by immunoenzymometric assay, which could measure more than 60 pM (6 fmol 100 μl^{-1}) by colorimetry (Fig. 1), and the specific radioactivity was obtained. The mean specific radioactivity of three lots was 31.1 ± 0.3 Bq U^{-1} and the reproducibility of the preparation was very good. Its stability was also checked by gel-permeation HPLC; it was stable for more than 2 days in the buffer solution.

Radioreceptor assay conditions

Controlling the growth of cells in the monolayer is rather difficult and wells for the radioreceptor assay do not contain precisely the same number of cells. Thus, the suspension of FL5-1 cells after trypsin treatment, which was used for the inoculation, was adopted.

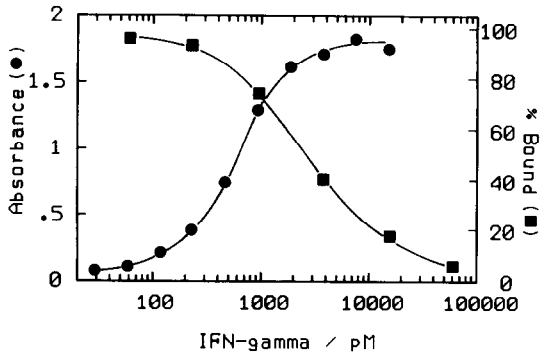

Figure 1
Standard curves of competitive radioimmunoassay (■) and immunoenzymometric assay (●) for IFN-γ.

The assay was carried out at 4°C because the per cent of bound labelled IFN-γ to the cell was smaller at 37°C than at 4°C and undesirable effects such as non-equilibrium binding or internalization should be negligible at this temperature. The reaction at 4°C reached equilibrium by 1 h. When labelled IFN-γ, *ca* 26,000 cpm (5×10^{-14} mol), was used for an assay tube, the optimum number of cells was around $0.5–1 \times 10^6$. FCS-MEM was chosen as an assay buffer with regard for cell stability. The incubation was carried out in air instead of a carbon dioxide atmosphere because of the short incubation time of 1 h.

Receptor binding of IFN-γ

Figure 2(A) shows that the labelled IFN-γ was bound to the FL5-1 cells and Fig. 2(B) is the Scatchard analysis. The competition curve of Fig. 3(A) shows that the binding of the labelled IFN-γ competed with unlabelled IFN-γ. These results of specific and reversible binding suggest that receptors for IFN-γ are present on the FL5-1 cell surface. The receptor should be homogeneous and have high affinity judging from the good linearity of the Scatchard plots [Fig. 2(B)]. The sensitivity of the competition curve in Fig. 3(A) was assessed to 30 pM of the final concentration in the assay tube, i.e. 90 pM (9 fmol 100 μl^{-1}) in standard/sample solution. This was enough to apply it for process and quality control analysis.

The concentration of the binding site, 4.9×10^{-11} and 9.0×10^{-11} M, from Scatchard

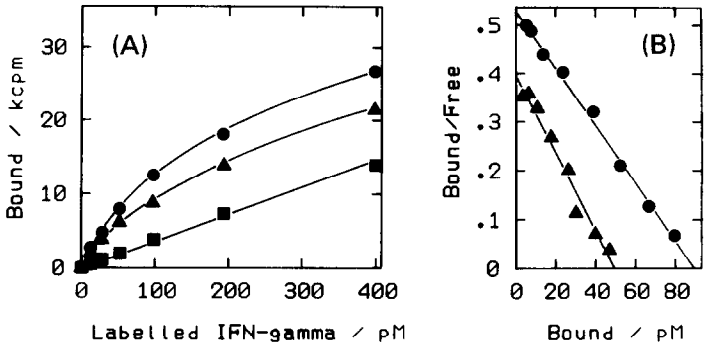

Figure 2
(A) Binding of the labelled IFN-γ to FL5-1 cells and (B) Scatchard plot of the binding. Cell concentration: 1 × 10^6 cells (●), 0.5 × 10^6 cells (▲), and no cells (■).

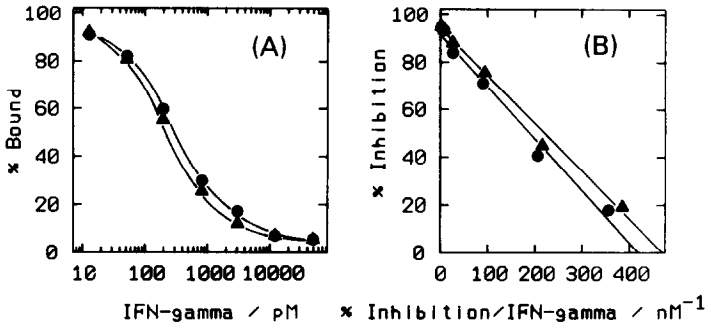

Figure 3
(A) Competition curves of the radioreceptor assay and (B) Hofstee plot from the inhibition. Cell concentration: 1 × 10^6 cells (●) and 0.5 × 10^6 cells (▲). The IFN-γ concentration on the horizontal axis is the final concentration in assay tube (300 μl).

analysis of labelled IFN-γ [Fig. 2(B)] was in proportion to a number of applied cells, 0.5 × 10^6 and 1 × 10^6 cells, respectively. Thus, the number of binding sites on the cell was estimated to be 16,000–18,000. Dissociation constants (K_d) of the labelled IFN-γ with binding at different cell concentrations [Fig. 2(B)] and inhibition constants (K_i) derived from 50% inhibition concentration [Fig. 3(A)] and from the Hofstee plot [Fig. 3(B)] are shown in Table 1. K_d of the labelled IFN-γ was similar to that of unlabelled IFN-γ.

Receptor binding of other interferons

As shown in Fig. 4, binding of the labelled IFN-γ to the receptor was equally inhibited with natural IFN-γ, which agreed with the results of the bioassay that IFN-γ has the same biological activity as natural IFN-γ [11]. Inhibition by IFN-α_2 and IFN-β was negligible, and receptors for IFN-α and IFN-β on the cell were different from those for IFN-γ as reported by Sarkar *et al.* and Nickloff [5, 6].

Discussion

Iodine-labelled ligands for radioreceptor assays can change their chemical properties even if they maintain receptor binding ability. Thus, the molecular size and immunoreactivity

Table 1
Dissociation constant (K_d) and inhibition constant (K_i) of IFN-γ receptor binding

Method	K_d (K_i) values (M)	
	0.5 × 10^6 cells	1.0 × 10^6 cells
^{125}I-labelled IFN-γ by Scatchard plot	1.3 × 10^{-10}	1.7 × 10^{-10}
IFN-γ by 50% inhibition	1.4 × 10^{-10}	1.5 × 10^{-10}
IFN-γ by Hofstee plot	2.0 × 10^{-10}	2.2 × 10^{-10}

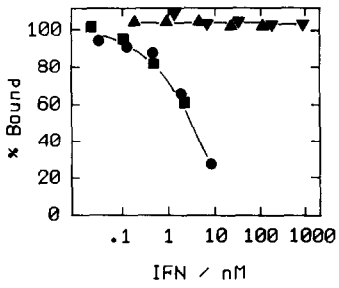

Figure 4
Competition curves with various interferons. Interferons: recombinant IFN-γ (●), natural IFN-γ (■), natural IFN-α_2 (▲), and natural IFN-β (▼).

as well as receptor binding need to be characterized for detailed investigation. The labelled IFN-γ prepared here, which fully maintained these activities, could be used not only for radioreceptor assay but also as a radioactive tracer such as that used *in vivo* distribution studies.

Using the novel radiolabelled IFN-γ and suspension of FL5-1 cells, the radioreceptor assay was developed. It was as sensitive (90 pM) as the immunoenzymometric assay (60 pM) and much more sensitive than the competitive radioimmunoassay (300 pM). Moreover, it was specific for IFN-γ, although the bioassay was greatly influenced by IFN-α and IFN-β. This assay should be useful not only for research on IFN-γ and its receptor but also for determination of biological activity of IFN-γ in process control and/or quality control of IFN-γ manufacturing because of its feasibility and specificity compared with the presently available bioassay. Further investigation for mass routine assay is now in progress.

As shown in Table 1, the labelled IFN-γ should have the same binding property for the receptors after the iodination. In addition, the dissociation constants and the number of binding sites on the cell obtained here were slightly different from those of previous reports [5, 6]. This discrepancy arose from the differences of cell strain, labelled compound, and incubation conditions.

Radioreceptor assays, which have the properties of bioassays, are expected to become more important for pharmaceutical analysis because drug concentrations determined by suitable radioreceptor assays should reflect the pharmacological or toxicological effects.

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