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CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO HUMAN INTERFERON-GAMMA AND THEIR APPLICATION TO ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Makoto HIRAI*, Yoshitake TERANO and Yoshiaki MIURA

Suntory Institute for Biomedical Research, 1-1 Wakayamadai, Shimamoto-cho, Mishima-gun, Osaka 618

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SUMMARY: The authors obtained two mouse monoclonal antibodies, G-208 and G-166, to recombinant human interferon- γ (rH-IFN- γ). Immunologically, they were classified as IgG1- κ subclass. G-208 neutralized the antiviral activity of natural and recombinant human IFN- γ , but did not bind to heat-denatured rH-IFN- γ . G-166 was able to bind to rH-IFN- γ as well as to heat-denatured rH-IFN- γ , but it did not bind to natural human IFN- γ (nH-IFN- γ). A sandwich enzyme immunoassay specific to H-IFN- γ molecule was developed using polyclonal rabbit anti-nH-IFN- γ antibody and G-208. This assay monitors only biologically active H-IFN- γ molecule. Thus, this method may be used for the direct determination of H-IFN- γ instead of determination of antiviral activity of H-IFN- γ .

INTRODUCTION

IFN- γ is a lymphokine secreted by T-lymphocytes upon stimulation with antigens, non-specific mitogens, or drugs like OK-432 (1-3). IFN- γ exerts a wide range of immunoregulatory activities in vitro and in vivo, such as the

平井 誠・寺野由剛・三浦義彰(サントリー株式会社生物医学研究所 大阪府三島郡島本町若山台1-1-1)

*Present address: Suntory Co. Ltd., Mori Bldg., 5-7-2, Kojimachi, Chiyoda-ku, Tokyo 102.

augmentation of tumoricidal activity by natural killer cell (4), as well as induction of the resistance of cells to viral infection (5,6). Furthermore, IFN- γ activates macrophages (7,8) and enhances the expression of Ia antigens (9) and Fc receptors (10,11).

In the early stage of interferon studies, those of IFN- γ were particularly difficult, owing to a very limited quantity obtained by the stimulation of cells with mitogens and specific antigens, as well as by its lability to heat and changes in pH.

Through recombinant technology, human IFN- γ cDNA was cloned and expressed in *E. coli* (12). Due to the production of a large amount of recombinant H-IFN- γ (rH-IFN- γ), much progress has been made, but many problems still remain to be solved, because of its pleiotropic effects on cells.

Routine laboratory assays for IFNs are generally based on their ability to inhibit the lysis of cultured cells by viruses. The advantage of the solid phase immunoassay for IFN- γ is not only shortened assay time to 3 to 4 hr from 36 to 48 hr for biological assays but also specific recognition of H-IFN- γ . With the advancement of technologies to produce monoclonal antibodies and to purify molecules to homogeneity, it is now possible to develop immunochemical assays for the detection of biologically active cytokines (13,14). Mouse monoclonal antibodies to H-IFN- γ are effectively utilized for the purification of natural H-IFN- γ , the sensitive IFN assay, and the identification of H-IFN- γ (15-18). Chang et al. (19) and Novick et al. (20) reported the preparation of several mouse monoclonal antibodies and their use in radioimmunoassay.

In the study reported here, we obtained monoclonal antibodies to rH-IFN- γ , designated as G-208 and G-166, recognizing different epitopes. Utilizing the monoclonal antibody (G-208) and polyclonal antibody to nH-IFN- γ , we developed a sandwich enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

IFN preparations: Recombinant human IFN- γ was produced in *E. coli* and purified to 4.5×10^6 Japan Reference Units (JRU)/mg (21). The product appeared homogeneous when analyzed by zone electrophoresis or immunoelectrophoresis with rabbit anti-nH-IFN- γ (data not shown). Natural human interferon- α , β , and γ were purchased from Meloy Laboratories (Springfield, VA).

Polyclonal antibody to nH-IFN- γ : The purified nH-IFN- γ (1 mg) was injected at weekly intervals with Freund's complete or incomplete adjuvant into the footpads and dorsal skin of a New Zealand white rabbit. After 4 weeks with four injections, the serum was collected and the immunoglobulins were purified with Affi-gel Protein A (Bio-Rad Lab., Richmond, VA).

Monoclonal antibodies to nH-IFN- γ : [1] Immunization, fusion, and cloning. An emulsion of equal volumes of purified rH-IFN- γ and Freund's complete adjuvant was injected intraperitoneally into BALB/c mice (5 μ g/mouse). Two and 4 weeks later, the mice received the same amount of antigen mixed with Freund's incomplete adjuvant. Three weeks after the third immunization, the mice were boosted with 10 μ g of rH-IFN- γ in phosphate-buffered saline (PBS). Splenocytes from the immunized mice were mixed with 8-azaguanine-resistant murine myeloma P3-X63-Ag8.U1 (P3U1) cells at a ratio of 4:1 and fused by a modification of the procedure described by Kohler and Milstein (1975). Positive hybridomas were cloned by limiting dilution in the presence of splenocytes as feeder cells.

[2] Selection of antibodies to human IFN- γ . rH-IFN- γ was suspended in 0.1 M sodium bicarbonate buffer (pH 9.6) at a concentration of 10 μ g/ml, and a 100- μ l aliquot of the suspension was added to each well of a 96-well microtiter plate (Immulon 2, Dynatech Lab., VA). The plate was incubated overnight at 4 C and the fluid was removed. Residual protein-binding sites were blocked with phosphate-buffered saline (PBS) containing 1% ovalbumin for 1 hr at 37 C. After washing with PBS containing 0.1% Tween 20 (PBS-T), 100 μ l of hybridoma culture supernatant was added and the plate was incubated for 1 hr at 37 C. Then the plate was washed five times with PBS-T and 100 μ l of 1:1,000 goat anti-mouse IgG (H) antibody labeled with alkaline phosphatase (P-L Biochemicals, Uppsala, Sweden) was added to each well. After incubation for 1 hr at room temperature and washing with PBS-T, 1.7 μ g of 4-methyl umbelliferyl phosphate (50 μ l) was added. After incubation for 10 min at room temperature, 150 μ l of 1 M K₂HPO₄-KOH (pH 10.3) was added to stop the reaction. The fluorescence of the reaction product was measured with a Corona multispectrofluorometer (excitation 365 nm, emission 450 nm).

Binding of monoclonal antibodies: Natural and recombinant H-IFN- γ was suspended in 0.1 M sodium bicarbonate buffer (pH 9.6) at a concentration of 10 μ g/ml and immobilized on the microtiter plate. Residual protein-binding sites were blocked with PBS containing 1% ovalbumin (37 C, 1 hr). Antibodies (1 mg/ml) were serially diluted and each dilution was added to each well. The plate was incubated for 2 hr at room temperature and the fluid was removed. Then, the plate was washed five times with PBS-T and 100 μ l of 1:1,000 goat anti-mouse IgG (H) antibody labeled with alkaline phosphatase (P-L Biochemicals) was added to each well. After incubation for 1 hr at room temperature and washing with PBS-T, 100 μ l of *p*-nitrophenyl phosphate (1 mg/ml) was added. After incubation for 10 min at room temperature, 10 μ l of 5 M NaOH was added to stop

the reaction. The absorbance at 405 nm of the reaction product was measured. The binding specificities of monoclonal antibodies to heat-denatured (70 C, 30 min) rH-IFN- γ were also examined by the same procedure as described above.

Biotin-labeling of G-208: Biotinyl-N-hydroxysuccinimide (BNHS) was used for introducing biotin moieties into the mouse monoclonal antibody (G-208). It was prepared according to the procedure described by Jasiewicz et al. (22) with N,N-carbonyl diimidazole (Aldrich Chemical Co., Milwaukee, WI) to form an intermediate biotin derivative which was subsequently converted to the N-hydroxysuccinimide ester by addition of N-hydroxysuccinimide. 0.1 M NaHCO₃ containing 10 mg/ml of G-208 was mixed with equal volume of 0.1 M solution of BNHS in distilled dimethylformamide (34.1 mg/ml). The reaction mixture was incubated at room temperature for 1 hr and then dialyzed for 24 hr at 4 C against several changes of PBS.

Enzyme-linked immunosorbent assay (ELISA) for human IFN- γ : A 96-well microtiter plate was coated with rabbit anti-nH-IFN- γ IgG (polyclonal antibody: R6, 30 μ g/ml). Various concentrations of IFN- γ were allowed to react for 2 hr at room temperature. After washing, 100 μ l of biotinylated G-208 (100 μ g/ml) was added and the plate was incubated for 2 hr at room temperature. After washing, 100 μ l of 1:1,000 streptavidin-biotinylated peroxidase (Cappel Lab. Inc., Malvern, England) was added and the plate was incubated for 1 hr at room temperature. Then, the mixtures were incubated with o-phenylenediamine (0.43 mg/ml in 0.1 M citrate buffer, pH 4.8) and H₂O₂ (0.002%) (in a final volume of 200 μ l) for 30 min at room temperature in the dark. The absorbance of the product was measured at 490 nm with a Dynatech Microelisa autoreader.

Biological assay for IFN antiviral activity: The antiviral activity of IFN was assayed by inhibition of the cytopathic effect of Sindbis virus in human FL cells (23). In most cases, the titers of human IFN- γ were expressed in JRU.

RESULTS

Monoclonal Antibodies to Recombinant Human IFN- γ . [1] Selection of hybridomas. The spleen cells from the mice showing high titers of antibody in their sera were fused with P3U1 cells. Two weeks after fusion, culture supernatant was harvested from each well and assayed by ELISA for the secretion of anti-IFN- γ antibody. Eleven hybridomas were cloned by limiting dilution. Among them, two monoclonal antibodies, G-208 and G-166, were determined for their serological activities.

[2] Serological activities of anti-human IFN- γ monoclonal antibodies, G-208 and G-166. The immunoglobulin subclasses of both G-208 and G-166 were determined by the Ouchterlony method. Both monoclonal antibodies belonged to IgG₁- κ subclass.

The abilities of these monoclonal antibodies to neutralize the antiviral activity of IFN- γ was examined. As shown in Table I, G-208 neutralized the antiviral activities of both recombinant and natural IFN- γ , but G-166 did not neutralize the antiviral activity of recombinant nor of natural human IFN- γ . Both G-208 and G-166 neutralized neither natural IFN- α nor IFN- β .

Binding specificity of these monoclonal antibodies to immobilized IFNs on microtiter plates were examined with ELISA (Fig. 1). Neither G-208 nor G-166 bound to natural IFN- α nor IFN- β . The reactivities to recombinant and natural IFN- γ were different between the two monoclonal antibodies. While G-208 bound to both recombinant and natural human IFN- γ immobilized on the microtiter plate, G-166 bound to recombinant IFN- γ but not to natural IFN- γ .

To find whether the two monoclonal antibodies are able to react with heat-denatured IFN- γ , recombinant human IFN- γ was heat-treated at 70 C for 3 hr (Fig. 2). G-166 bound to inactivated IFN- γ , albeit with a lower affinity, but G-208

Table I. Neutralization of antiviral activity of human IFNs

Human IFNs		The remaining activity after treatment with monoclonal antibodies		
		untreated	(+) G-208	(+) G-166
Natural	IFN- γ	64	< 2	64
Recombinant	IFN- γ	64	< 2	64
Natural	IFN- α	64	64	64
Natural	IFN- β	64	64	64

IFNs (128 units/ml) were each mixed with an equal volume of culture supernatant of hybridomas and the mixtures were incubated overnight at 4 C. The remaining antiviral activity was measured as described in MATERIALS AND METHODS.

Fig. 1. Binding of monoclonal antibodies to natural and recombinant H-IFN- γ determined by ELISA. Binding ability of serially diluted monoclonal antibodies to immobilized natural (—○—) or recombinant (—●—) H-IFN- γ on the microtiter plate was examined by ELISA as described in MATERIALS AND METHODS. Each point represents the relative absorbance at 405 nm of each well of the microtiter plate.

did not bind to inactivated IFN- γ .

[3] Quantitative determination of IFN- γ by ELISA methods. To establish the optimal conditions to detect human IFN- γ , we developed the ELISA system using rabbit anti-natural human IFN- γ IgG (R6) and biotinylated G-208. The amount of IFN- γ bound to R6-coated wells was assayed by the method described in MATERIALS AND METHODS. As shown in Fig. 3, binding of IFN- γ to immobilized R6 was detected in a dose-dependent manner at concentrations between 5-100 ng/ml. These results indicate that the method is quite useful for rapid and reliable determination of IFN- γ .

Fig. 2. Binding of monoclonal antibodies to heat-denatured rH-IFN- γ determined by ELISA. Binding ability of serially diluted monoclonal antibodies to immobilized heated (—●—) or unheated (—○—) rH-IFN- γ on the microtiter plate was examined by ELISA as described in MATERIALS AND METHODS. Each point represents the relative absorbance at 405 nm of each well of the microtiter plate.

Fig. 3. Quantitation of H-IFN- γ by ELISA using rabbit anti-nH-IFN- γ (R6) IgG and G-208. ELISA was carried out as described in MATERIALS AND METHODS.

DISCUSSION

To develop rapid and quantitative assay methods for preclinical and clinical studies of rH-IFN- γ , we obtained the monoclonal antibodies against rH-IFN- γ . Immunochemical assay can be completed within 8 hr compared to 72 hr for the viral cytopathic effect assay. Apart from the time factor, the antiviral assay requires sterile propagation and manipulation of target cells as well as virus, whereas the immunochemical assay requires neither cells nor virus and can be performed on an open bench top. Additionally, the biotinylated antibody in our system is very stable.

These monoclonal antibodies were useful also for the study of the mechanism of the action of IFN- γ and purification of rH-IFN- γ . Two monoclonal antibodies, G-208 and G-166, were different in their reactivities with natural and recombinant IFN- γ . Our results showed G-208 bound to and neutralized both recombinant and natural IFN- γ , whereas G-166 did not neutralize recombinant nor natural IFN- γ and bound to recombinant but not to natural IFN- γ .

As G-208 was a neutralizing antibody, the epitope recognized by the antibody is likely to be located at or near the region of the active sites of the IFN- γ molecules. Since G-166 failed to neutralize the antiviral activity of H-IFN- γ , the portion recognized by G-166 is not implicated in the biological activity. These data might suggest that the epitope recognized by G-166 on the rH-IFN- γ molecule is absent in the nH-IFN- γ molecule. It seemed likely that the discrimination of natural and recombinant IFN- γ by G-166 involved a difference between the tertiary structure between these two IFN- γ molecules. The reason for the difference in the tertiary structure of natural and recombinant IFN- γ is not clear. In eukaryotic cells the process of glycosylation is performed during translation (24). The presence of branched carbohydrate chains on the nascent polypeptide could affect folding and lead to a conformation of natural IFN- γ different from that of recombinant IFN- γ . It was suggested that discrimination between natural IFN- γ and recombinant IFN- γ by G-166 is due to the difference in the polypeptide folding structure.

Le et al. (25) reported that the monoclonal antibody raised against nIFN- γ could not react with rIFN- γ , but reacted with the deglycosylated nIFN- γ . They suggested that discriminative recognition of nIFN- γ and rIFN- γ by the monoclonal antibody might be due to a conformational difference between them.

Nocvick et al. (20) reported the preparation of several mouse monoclonal antibodies and their use in radioimmunoassay with a sensitivity limit of 4 ng/ml, similar to that of our system.

The fact that G-208 did not bind to heat-inactivated IFN- γ , suggests that it recognized a conformational epitope present only on the native IFN- γ molecule. These data indicate that the ELISA employing rabbit anti-natural human IFN- γ polyclonal antibody (R6) and mouse anti-recombinant human IFN- γ (G-208) can be used for detection of both recombinant and natural IFN- γ , for monitoring of recombinant IFN- γ levels in the sera from patients receiving IFN- γ therapy, or in certain pathological cases which might result in increased IFN- γ levels. It also suggests that G-208 recognizes only biologically active H-IFN- γ in this ELISA system.

In a similar study, Pestka et al. (26) reported that several IgG₁-class monoclonal antibodies specific for IFN- α failed to bind to heat-inactivated IFN- α , and a sandwich radioimmunoassay using two of these monoclonal antibodies showed a close correlation with an assay based on the antiviral activity. However, it seemed that the specificity of such monoclonal antibodies for the native form was not absolute one. G-166 reacted with denatured IFN- γ , albeit with a lower affinity.

The application of both monoclonal antibodies with different characteristics will be useful in the analysis of various effects of human IFN- γ on cells and of the structure-function relationships.

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