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Original Article

A Mouse Monoclonal Antibody Against Human IFN-γ and its Characters

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ABSTRACT

Background and Aims: A monoclonal antibody (mAb) can unambiguously identify, quantify, and purify an antigen or particular epitope at a large scale. The superiority of these antibodies lies in their specificity for the antigenic determinant. So, this study aims to prepare mouse mAb-secreting hybridoma against human gamma interferon (IFN-γ) and determine the produced antibody's characters.

Materials and Methods: Mouse splenic B lymphocytes immunized with recombinant human IFN-γ were fused with mouse SP2/0 cells. The hybridized cells were selected by hypoxanthine-aminopterin-thymidine and hypoxanthine-thymidine media to obtain monoclonal antibody-producing hybridoma cells. Finally, indirect enzyme-linked immunosorbent assay (ELISA), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and western blot were used to confirm the creation of antibody-secreting hybridoma cells.

Results: mAb against IFN-γ were produced by fusing SP2/0 mouse non-secretory myeloma cell line with the spleen cells of immunized mice. This antibody's indirect ELISA optical density was 2.055 on average, and the desired antibody bands were confirmed in SDS-PAGE compared to Septicol® (commercial antibody). Also, in the western blot, the desired antibody could bind to the antigen. IFN-γ transferred on nitrocellulose membrane. In ELISA and western blot tests, anti-mouse IgG conjugated antibodies were used; therefore, the mAb IgG isotype was taken into consideration.

Conclusion: In this study, a mouse mAb was obtained by immunization of Balb/C mice and fusion of spleen cells of these mice with the SP2/0 cells, which can specifically bind to recombinant human IFN-γ and can be used to detect IFN-γ secretion in all types of intracellular infections, including latent tuberculosis.

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Introduction

Interferons (INFs) were initially discovered as factors that interacted with the proliferation of viruses. Currently, they are divided into two types, I and II, based on the characters of the receptor and their sequence similarity. Type I includes INF-α, INF-β, INF-ω, and INF-τ, which bind to a general heterodimer receptor, and type II contains only INF-γ, which binds to different receptors and is expressed by a chromosomal locus. It is coded separately [1]. IFN-γ is an essential mediator of immune response and has antiviral, immunomodulatory, and antitumor properties. IFN-γ can generally exert antiviral function by directly attaching to its receptor and promoting pathogen killing by activating macrophages. IFN-γ can stimulate macrophages and T lymphocytes to enhance their antigen-presenting ability by expressing MHC class II molecules. Aberrant expression of IFN-γ is first identified when pathogens invade the host. Therefore, the level of IFN-γ can be used as a primary diagnostic index of diseases to evaluate the level of immunity and health status of the body [2-7]. In addition to its direct antiviral effects, IFN-γ also exerts antiviral efficacy indirectly via adjusting other immune responses. IFN-γ can also enhance Fc receptor-mediated phagocytosis. Therefore, IFN-γ can be used to assess the immune effect of vaccinations and the body's immune status [8-10]. Although enzyme-linked immunosorbent assays (ELISA) are well-known as ideal methods of measuring specific bimolecular interactions based on current principles and recent clinical applications, the fact remains that they are a rather older method than many others [11, 12]. One of the most famous techniques to investigate intracellular infections, especially latent mycobacterium tuberculosis, is the IFN-γ release assay (IGRA) [13]. IGRA demonstrated T cell release of IFN-γ following stimulation by specific antigens of the mycobacterium tuberculosis complex. Monoclonal antibody (mAb) against human IFN-γ is one of the essential components of the diagnostic technique of IGRA. By introducing the somatic cell hybridization method by Köhler and Milstein as a method to develop mAbs, they provided unparalleled access to a powerful tool for identifying various biomolecules, such as IFNs, through the isolation of their cells [14]. mAb are valuable tools for purifying, assaying, and monitoring many biologically active molecules [15]. In this study, we prepare mouse mAbs against human recombinant IFN-γ; moreover, the mAb can also be a valuable tool for IFN-γ diagnostic kits and colloidal gold test strips.

Materials and Methods

Animals

Female BALB/c mice were purchased from the Laboratory Animal Center of Isfahan University of Medicine, Iran. All treatments and animal care procedures were approved by the Animal Care and Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran (IR.SSU.MEDICINE.REC.1399.248). Mice reached their endpoints by spinal dislocation during collection experiments.

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Animal immunization

Briefly, young female BALB/c mice were immunized through subcutaneous injection with 25 µg of purified IFN-γ protein (Gifted by Dorsa Daru Company, Tehran, Iran) four times with an interval of 14 days. It should be noted that the first injection was assembled by a complete Freund's adjuvant (CFA) (Sigma-Aldrich), while the second, third, and fourth injections were accompanied by an incomplete Freund's adjuvant (IFA) (Sigma-Aldrich). After four injections, mice blood was collected from the venous sinus, and their sera were analyzed for the production of polyclonal antibodies against IFN-γ by the ELISA method. After checking the serum of mice, antigen without adjuvant was injected as a final booster intravenously (25 µg) three days before cell fusion. A mouse with the highest antibody titer in its serum was selected for cell hybridization.

Hybridoma lines production

Three days after the last boost injection, the selected mice were ethically sacrificed, and their spleen cells were removed and co-cultured with SP2/0 myeloma cells using polyethylene glycol 1450 (Sigma-Aldrich, USA) (5:1 ratio). In order to resuspension the fused cells, Roswell Park Memorial Institute Medium (RPMI)-1640 was used supplemented with 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 units of penicillin and streptomycin (Sigma-Aldrich, USA), and hypoxanthine-aminopterin-thymidine medium supplement (Sigma-Aldrich, USA). The cells were cultured in 96-well tissue culture plates at 37 °C and 5% CO_2. An indirect ELISA was conducted on the culture medium in the wells representing viable cells after an incubation period of seven to ten days to determine the presence of mAb of IFN-γ.

ELISA

The 96-well polystyrene microtiter plates were coated with IFN-γ protein (100 µL/well, 5 µg/mL) and cultured overnight at 4 °C. After three washes with phosphate buffered saline (PBS containing 0.05% tween-20), the plates were blocked with 0.1 M carbonate buffer containing 5% bovine serum albumin powder for two hours at 37 °C. As described above, samples (not immunized mouse serum or immunized mouse serum and cultured supernatant from the hybridomas) were transferred into each well and incubated at 37 °C for 1 hour. Following the previous step, 100 µL of horseradish peroxidase conjugated goat-anti-mouse IgG diluted in 1:1000 with PBS was added as a secondary antibody and incubated for one hour at 37 °C. After washing, 100 µL of 3,3′,5,5′-tetramethylbenzidine was used and kept for 18 min at room temperature. After adding 50 µL of chromogenic termination by 2 M H_2SO_4, absorbencies were measured with an automatic ELISA reader (Bio-Rad, California, USA) at 450 nm.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot assay

We used SDS-PAGE and western blot techniques to determine the specificity of the mAb. For the SDS-PAGE, 20 µL of commercial antibody solution as positive control and 20 µL of hybridoma cell culture medium were used., then 20 µL of 4x SDS sample loading buffer (8% SDS, 20% 2-

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mercaptoethanol, 40% glycerin, and 0.015% bromophenol) were added to each sample and tubes were heated at 95 ˚C for 1 min. The samples on a 12.5% SDS-PAGE gel were loaded, 5 µL of an unstained protein ladder in an additional lane was placed, and were separated by electrophoresis. According to the manufacturer's instructions, the gel was stained with coomassie brilliant blue (Thermo Fisher Scientific, Inc.).

For the western blot test, IFN-γ and plasma of a patient infected with toxoplasma were mixed with SDS sample loading buffer and denatured for 1 min at 95 ˚C. After separation of the mixtures by SDS-PAGE, they were transferred to nitrocellulose membranes under 90 V for 75 minutes in transfer buffer (58 mM Glycine, 71.8 mM Tris base, 20% Methanol). After the membranes were blocked with 5% bovine serum albumin in PBS for 2 hours and washed three times with Tris-buffered saline (TTBS plus 0.05% Tween-20), For the primary antibody, hybridoma culture supernatant was incubated at 25 °C for one hour. An horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich) secondary antibody was applied after washing three times with TTBS and incubated at 25 °C for one hour. The signal was then visualized using 3,3′,5,5′-tetramethylbenzidine.

Results

Generation and characterization of mAb against IFN-γ

Serum titration of the mice showed that the mice were well immunized and showed a high titer of anti- IFN-γ polyclonal antibody (Fig. 1A). At the time of cell fusion, $3x10^5$ SP2/0 cells and $15x10^5$ spleen cells were combined.

The first colonies appeared on day three after fusion (Fig. 2A), and hybridoma cells increased on day 10. As a result, a fresh medium was added to the culture medium to replace the old one. Therefore, Hybridomas were renewed every three days (Fig. 2B). Under this result, hybridomas were positive in the anti-IFN-γ indirect ELISA (Fig. 1B). SDS-PAGE and western blot assays were performed on the supernatant from hybridomas to confirm the ELISA test results. The coomassie brilliant blue staining determined that the mAb should be located on the gel (Fig. 3A), and its size was also determined. IFN-γ and toxoplasma gondii plasma samples were transferred to the nitrocellulose membrane, and the supernatant of the hybridoma culture medium was added to the paper as the primary antibody. It was found that anti-IFN-γ mAb specifically recognized IFN-γ (Fig. 3B).

Discussion

In the cellular immune response against intracellular infections, type I and II IFNs play critical roles. The immunomodulatory effects of IFN-γ are the most prominent among them, and establishing a long-term antiviral state is more beneficial. IFN-γ undergoes a signal transduction pathway linked to the production of an extensive range of enzymes, transcription factors, and cytokines that exert various antiviral effects on the body.

Therefore, the higher the level of IFN-γ secretion, the stronger the body's cellular immune function against viruses [4, 6, 16, 17].

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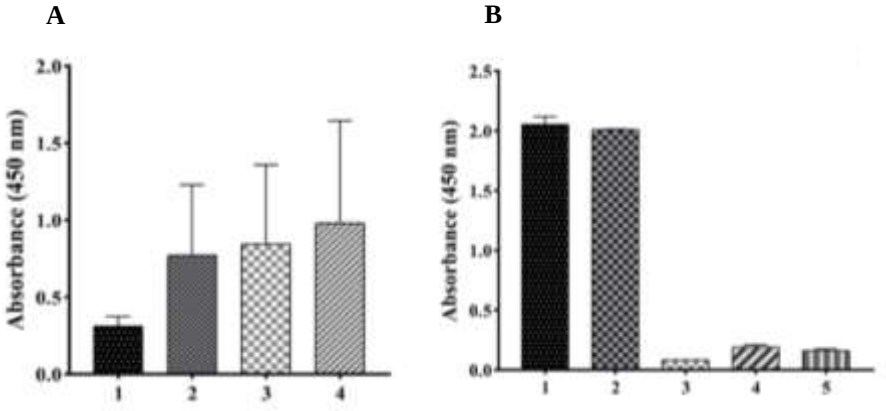


Fig1. 1. A. Comparing the absorption rate of polyclonal antibody in mouse serum with different concentrations of coated antigen on ELISA plate 1: Negative control, 2: 1/100 the concentration of antigen coated on the ELISA plate 3: 1/50 the concentration of antigen coated on the ELISA plate 4: 1/20 the concentration of antigen coated on the ELISA plate. B: Comparison of mouse mAb in culture medium supernatant with mouse polyclonal antibody in mouse serum. 1: Monoclonal antibody present in the supernatant of the cell culture medium, 2: Mouse polyclonal antibody, 3: mAb-free supernatant of cell culture, 4: Non-polyclonal mouse serum, 5: Negative control. The absorption rate is reported with Mean ± standard error



Fig. 2. Results obtained from fusion and hybridoma cells, A: Colony formed after one week of incubation in complete medium + HAT 2X (40X), B: Desired colony growth in complete medium + 2% HT for one week (10X), C: Desired colony growth in complete medium + HT 1% for one week after transfer to a 6-well plate

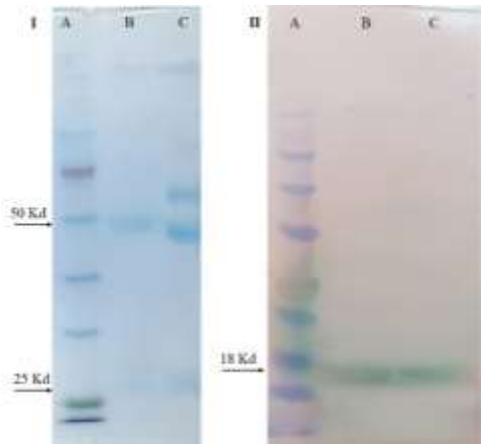


Fig. 3. The results of the presence of anti- IFN-γ mAb in hybridoma cell culture medium and its binding to IFN-γ. **I:** SDS-PAGE results, A: Protein ladder, B: Anti-IFN-γ mAb in cell culture medium supernatant, C: Purified commercial antibody (Septicol®), Kd: Kilodalton **II:** Western blot results, A: Protein Ladder, B: IFN-γ transferred to nitrocellulose paper, C: Toxoplasma-infected plasma

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In this study, after four injections with IFN-γ and CFA, and IFA, to ensure the mice's immunity, serum titration with IFN-γ was performed by indirect ELISA method. The results showed that the mice were immunized against IFN-γ. The hybridoma secreting for anti- IFN-γ was confirmed by indirect ELISA, SDS-PAGE, and western blot.

One of the first studies to produce mAbs against IFN-γ was the study of Novick et al., which used human IFN-γ purified by cation exchange high-performance liquid chromatography to develop mAbs. After immunization with purified human IFN-γ and CFA, and IFA, mouse spleen lymphocytes (18×10^7) were mixed with non-secreting murine myeloma (NSO) myeloma cells three days after confirmation of immunization 3×10^7. The produced mAb can bind to all types of human IFN-γ purified from different sources, and it has not shown cross-reaction with human interferon alpha and beta [18]. A similar study was conducted to produce mAbs against mouse IFN-γ in 1984 when female rats were immunized with 3 mg of protein emulsified in CFA inside the foot pad. Nine booster injections were performed at intervals of 3 weeks with the same amount of emulsified protein in IFA. It has been calculated that each mouse received a total of 2×10^5 units of mouse IFN-γ. Immunized rats' spleen cells (15×10^7) were fused with 7×10^7 P3. X63.Ag8 type 653 (murine myeloma) cells. In the mentioned study, the produced mAbs neutralized mouse IFN-γ from various sources, which included concanavalin A (ConA)-stimulated spleen cells, alloantigen-stimulated spleen cells, as well as monkey fibroblasts that had been transfected with cloned mouse IFN-γ gene [19]. In two previous studies, the mAb produced from different myeloma cells can bind to its antigen (human and mouse IFN-γ). Also, the number of immunization times was higher than in the present study, but a lower injection dose was used. It was assumed that human IFN-γ acted as a toxin and harmed the animal by increasing the number of booster injections. As a result, after four injections and confirmation of high antibody titer in mouse serum, the immunization process was stopped.

A study conducted by Ma et al. attempted to develop and use a mAb against goat IFN-γ in order to detect open reading frame Parapoxvirus ovis virus as an intracellular pathogen and stimulator of IFN-γ production. ELISA, western blot analyzed the mAb secreted by the hybridoma cell line. An ELISA test determined that this mAb could detect cultured goat peripheral blood mononuclear cells stimulated by ConA. Western blot analysis indicated that this mAb could specifically react with the purified IFN-γ protein of 34.9 kDa [20]. In the present study, the mAb produced from hybridoma cells had a strong binding to commercial IFN-γ by indirect ELISA technique. Also, this antibody could bind to commercial IFN-γ, and human IFN-γ present in the serum of patients with toxoplasma (considered an intracellular pathogen) in the western blot technique [21].

In a study by Novick et al. [22], after injection of IFN-γ for mice immunization, splenic lymphocytes were isolated from mice

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displaying the highest binding and neutralizing titers (18×10^7), fused with NSO mouse myeloma cells (3×10^7). ELISA was used to screen hybridomas for the presence of anti-IFN-γ mAb. In this study, a cell ratio of 6:1 was used, but in the present study, a ratio of 5:1 was used, and a smaller number of cells were used to produce hybridoma cells. The current study also concluded that the obtained mAb could bind to IFN-γ at a concentration of 5 µg/ml (optimal concentration) using ELISA. mAbs were suitable for a sensitive and rapid dual solid-phase radioimmunoassay that allowed IFN-γ detection at concentrations as low as 4 ng/mL (150 units/ml). Sp2/0-Ag14 fusion has been reported to have varying levels of efficiency. Nevertheless, in many experiments, fusions with Sp2/0 Ag14 cells have been more efficient than those with X63-Ag8, particularly when using lipopolysaccharide-stimulated spleen or large splenic cells. The Sp2/0-Ag14 cell line may be better for generating hybridomas that produce true mAbs [23].

ELISA and western blot analysis may use the mAb in this study to determine the secretion level of IFN-γ at the protein level. This study established an antibody that acts as a valuable tool for assessing the host's immunity to infection and studying the immune system's ability to fight infections.

Conclusion

In this study, a mouse mAb was obtained by immunization of Balb/C mice and fusion of spleen cells of these mice with SP2/0 cells, which can specifically bind to recombinant human IFN-γ. Further analyses indicated that the mAb could be used to detect IFN-γ secretion in all types of intracellular infections, including latent tuberculosis.

Conflict of Interest

The authors declare no conflicts of interest.

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