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Immunology

Use of monoclonal antibodies as sensitive and specific probes for biologically active human γ-interferon

(lymphokines/macrophage activation factor/immunoradiometric assay)

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ABSTRACT **Mouse monoclonal antibodies B1 and B3 are specific for natural and *Escherichia coli*-derived recombinant human γ-interferon (IFN-γ). The two antibodies recognize different epitopes of the IFN-γ molecule and do not compete with each other's binding. We have used these two antibodies to construct a solid-phase, sandwich immunoradiometric assay for human IFN-γ. Purified antibody B1 was coated on polystyrene beads (0.64 cm in diameter) and used as the solid-phase immunoadsorbent and antibody B3 was labeled with ^{125}I and used as tracer. This assay can be completed in about 4 hr and is capable of detecting IFN-γ levels in human serum or tissue culture fluids as low as 0.1 NIH reference unit/ml. Recombinant human IFN-γ derived from *E. coli* was detectable at a concentration of 0.02 ng/ml. The assay appears to be specific for the biologically active forms of IFN-γ, since after exposure to pH 2, 37°C, or 56°C, biological activity and reactivity in the immunoradiometric assay decreased in parallel. The immunoradiometric assay can be employed for the analysis of the structural characteristics of the human IFN-γ molecule.**

γ-Interferon (IFN-γ), also called immune IFN, is a lymphokine secreted by T lymphocytes upon stimulation with antigens or nonspecific mitogens (1–6). Like IFN-α and -β (7–9), IFN-γ induces the resistance of cells to viral infection and also exerts a range of immunoregulatory activities *in vivo* and *in vitro*, such as the augmentation of natural killer cell tumoricidal activity (4). In addition, IFN-γ activates macrophages by triggering their morphological transformation, secretion of soluble factors, and expression of surface Ia antigens and Fc receptors (10, 11). Recent evidence also strongly suggests that IFN-γ is indistinguishable from macrophage activation factor (12–16).

Routine laboratory assays for IFNs are generally based on their ability to inhibit the lysis of cultured cells by viruses (17). Recently, two mouse monoclonal antibodies, B1 and B3, specific for natural and *Escherichia coli*-derived recombinant human IFN-γ have been developed (18, 19). The two antibodies recognize different epitopes, since B1 and B3 differ in their reactivity with the IFN-γ molecule (18) and they do not compete with each other's binding to IFN-γ (unpublished data). Using these two antibodies, we have developed a sensitive and rapid solid-phase immunoradiometric assay (IRMA) for IFN-γ. Our report shows that this assay can be used as a specific probe for biologically active human IFN-γ.

MATERIAL AND METHODS

Preparation of IFN-γ and Sources of Other IFNs. Human IFN-γ was produced in the cultures of lymphocyte-rich plateletpheresis residue induced with phytohemagglutinin and phorbol 12-myristate 13-acetate. It was partially purified by a four-step protocol (20) and used as IFN-γ standard for the IRMA. NIH IFN-γ reference standard was obtained from Research Resources Branch, National Institute of Allergy and Infectious Diseases, Bethesda, MD. Purified recombinant human IFN-γ derived from *E. coli* (21) was provided by Genentech (San Francisco, CA). Human IFN-α was produced by Meloy Laboratories (Springfield, VA) and human IFN-β by Rentschler Arzneimittel (Laupheim, FRG).

Purification of B1 and B3 Monoclonal Antibodies. Approximately 15–20 ml of ascitic fluid from mice bearing the hybridomas secreting B1 and B3 antibodies was diluted by adding 0.1 vol of Tris buffer (1.0 M, pH 8.0). This material was filtered through glass wool, passed through a column packed with 15 ml of protein A-Sepharose 4B, and eluted with Tris buffer (0.1 M, pH 8.0). The two antibodies, both of which are IgG1 (18), were retarded by the column and hence separated from albumin, IgM, and other immunoglobulin subclasses. The fractions that were contaminated with albumin and IgM were further purified by concentration and second passage through the protein A column. HPLC analysis indicated that the B1 and B3 antibody preparations thus obtained contained at least 95% IgG1.

Preparation of B1 Antibody-Coated Polystyrene Beads. Polystyrene beads, 0.64 cm in diameter (Precision Plastic Balls, Chicago), were washed with ethanol and phosphate-buffered saline (P_i/NaCl, pH 7.0). Coating of the beads with B1 antibody was performed by a procedure similar to that described earlier (22). Briefly, 1000 beads were incubated in a slow-moving shaker with 150 ml of P_i/NaCl (pH 7.0) containing 5.0 mg of B1 antibody at room temperature for 16 hr. The beads were then washed with P_i/NaCl three times and then incubated with 150 ml of P_i/NaCl containing 1% bovine serum albumin at 37°C for 6 hr to seal nonspecific reactive areas. After two washings with P_i/NaCl the beads were incubated with 150 ml of stabilizer solution (aqueous solution of 4% polyvinylpyrrolidone and 10% sucrose) at room temperature for 30 min. The stabilizer solution was removed by centrifugation and the dried beads were stored in capped tubes at 4°C.

Preparation of ^{125}I-Labeled B3 (^{125}I-B3) Antibody Tracer. B3 antibody was labeled with ^{125}I by using the chloramine-T method (23). ^{125}I-B3 had a specific radioactivity of 12–14 μCi/μmol (1 Ci = 37 GBq) of antibody and was diluted with P_i/NaCl containing 0.5% bovine serum albumin and 1% normal CAF1 mouse serum (Hazelton-Dutchland Laboratories, Denver, PA) to 100,000 cpm/200 μl for the assay.

IRMA for IFN-γ. Partially purified natural IFN-γ (20) diluted with heat-inactivated (56°C, 1 hr) pooled normal human serum, unless otherwise specified, was used as assay standard. The B1 antibody-coated polystyrene beads, used as solid-phase immunoadsorbent for human IFN-γ, were incubated with 200 μl of test samples in an assay tray at room temperature for 2 hr without shaking. The beads were then washed with water and incubated with 200 μl of tracer solu-

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Abbreviations: IFN, interferon; IRMA, immunoradiometric assay.

tion containing 100,000 cpm of ^{125}I-B3 antibody at room temperature for 2 hr. After washing, the beads were assayed for ^{125}I in a γ counter.

Antiviral Assay of IFN. IFN-γ titers were determined by a semi-micro method in human FS-7 cells challenged with encephalomyocarditis virus (24). An internal laboratory standard was included with each assay; the potency assigned to the internal laboratory standard was based on the value of the NIH reference standard.

RESULTS

Fig. 1*A* shows the typical results of an experiment in which a wide range of natural IFN-γ concentrations was examined in the IRMA. The background of this IRMA is 50–100 cpm (using 100,000 cpm tracer input). The sensitivity at the lower limit of the assay is about 0.1 unit/ml of IFN-γ—i.e., 0.1 unit/ml of IFN-γ gave a signal twice that of background. The IRMA is reproducible, because standard deviations were generally <10% of the mean. The sensitivity of the IRMA was also examined with purified recombinant *E. coli*-derived human IFN-γ and found to be about 0.02 ng/ml (Fig. 1*B*).

Unlike IFN-γ, human IFN-α (leukocyte) or IFN-β (fibroblast), added to B1 antibody-coated beads at concentrations ranging from 10 to 10,000 units/ml, did not produce binding of ^{125}I-B3 antibody tracer above background (results not shown). Thus, unlike most of the biological assays, the present IRMA detects only IFN-γ and not IFN-α or IFN-β.

To further examine the specificity and utility of the assay, we diluted human IFN-γ in three types of animal serum before adding the samples to the B1 antibody-coated beads. Neither unheated pooled normal human serum nor fetal bovine or mouse serum caused an increase in the ^{125}I radioactivity bound to the beads. These results indicate that no other substances in normal human serum cross-react with the two antibodies. They also show that the IFN-γ levels in sera of normal individuals are <0.1 unit/ml, the sensitivity limit of the assay.

The B1 antibody-coated immunoadsorbent beads, the ^{125}I-B3 tracer, and the IFN-γ standard could be used for at least 8 wk when stored at 4°C, although the signal intensity decreased somewhat over time due to the decay of ^{125}I in the B3 tracer. However, when the individual components were examined for stability at 37°C, the IFN-γ standard was found to be the only component showing decay after 1 wk of storage. This finding prompted us to determine whether other treatments known to inactivate IFN-γ (17) decrease or abolish its reactivity in the IRMA. A short exposure to pH 2 completely abolished reactivity in the IRMA (Fig. 2). This finding is in agreement with the known lack of stability of IFN-γ at pH 2 (1, 17). Exposure to 56°C resulted in a more gradual loss of reactivity in the IRMA, with an 80% loss observed after 2 hr.

We also compared the reactivity of IFN-γ in the IRMA and its activity in the standard bioassay after denaturing

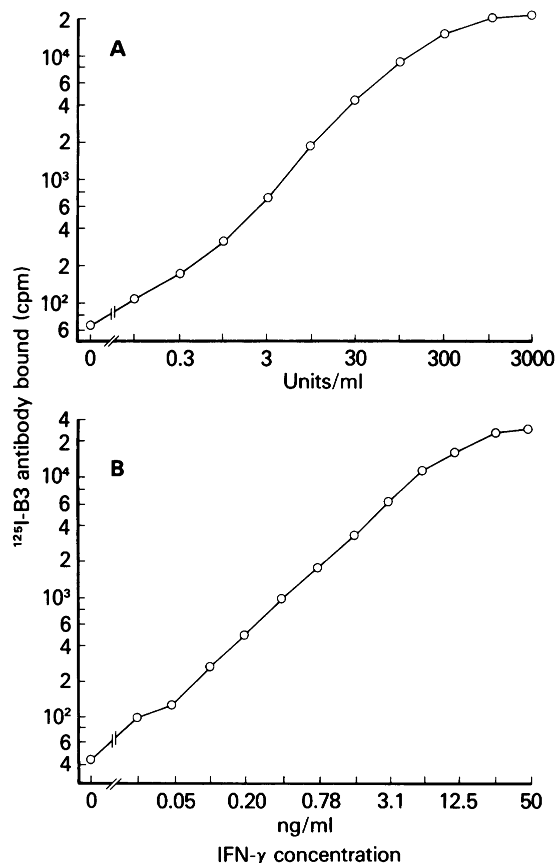

FIG. 1. IRMA of human IFN-γ using B1 antibody-coated polystyrene beads and ^{125}I-B3 antibody tracer. (*A*) Natural IFN-γ, partially purified from the culture supernatant of phytohemagglutinin- and phorbol 12-myristate 13-acetate-activated human lymphocytes (20), was diluted with heat-treated (56°C, 1 hr) pooled human serum. Each point represents the mean ± SD of two determinations. (*B*) Purified *E. coli*-derived recombinant human IFN-γ diluted with human serum before IRMA. Each point represents the mean ± SD of three determinations.

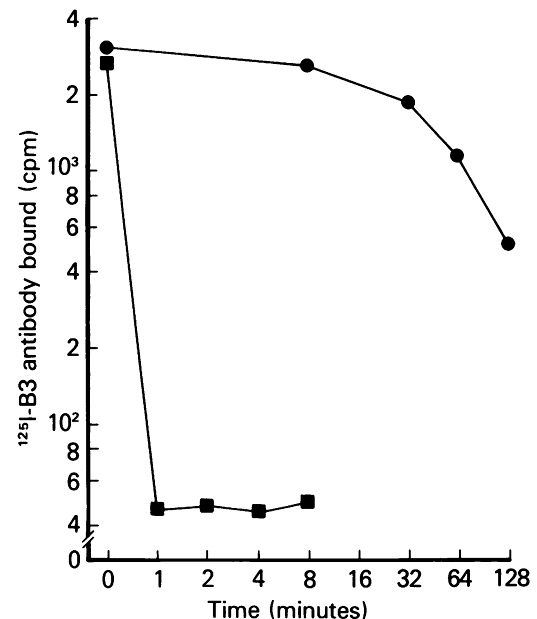

FIG. 2. Reactivity of IFN-γ in the IRMA after exposure of IFN-γ to pH 2 or 56°C for different periods. Treatment at pH 2 (■): P_i/NaCl (pH 7.2) containing 0.5% bovine serum albumin was adjusted to pH 2.0 with 1 M HCl, and 14.9 ml of this buffer was pipetted into a 15-ml tube at room temperature. One hundred microliters of IFN-γ preparation containing 830 units of IFN-γ was added into the tube and mixed well immediately. At 1-, 2-, 4-, and 8-min time points from the moment of mixing, 900 μl of the acidified IFN-γ was removed and mixed with 100 μl of Tris·HCl (1 M, pH 8.0) in a tube on ice. The pH of the resulting solution was found to be pH 7.5. A zero time point control was prepared with the acidic and Tris buffers mixed before adding the IFN-γ stock sample. Treatment at 56°C (●): A tube containing 14.9 ml of P_i/NaCl (pH 7.2) with 0.5% bovine serum albumin was incubated in a 56°C water bath. One hundred microliters of IFN-γ preparation containing 750 units of IFN-γ was pipetted into the tube and mixed well immediately. At the time points indicated, 1 ml of the heated IFN-γ was pipetted into tubes on ice. Each point on the graph represents the average of two determinations.

Table 1. Simultaneous loss of antiviral activity and reactivity in IRMA upon incubation of IFN-γ at 37°C

Time of incubation at 37°C, day	Antiviral activity		IRMA activity	
	Units/ml	% original	cpm*	% original
0	200	100	8674	100
1	50	25	1258	14.5
2	<4	<2	162	1.9
3	<4	<2	23	0.3
4	<4	<2	30	0.3

Partially purified natural IFN-γ preparation was diluted about 1:500 in P_i/NaCl to 200 units/ml and incubated at 37°C. Samples removed at daily intervals were quantitated in the antiviral assay and IRMA.

*Mean of duplicate samples after subtraction of background.

treatment, which is less drastic than the pH 2 or 56°C exposure—i.e., after natural IFN-γ, diluted in P_i/NaCl, was incubated for various periods at 37°C (Table 1). Incubation at 37°C for 24 hr resulted in a 75% drop in biological activity and an 85% decrease in the amount of radioactivity bound in the IRMA. After 2 days at 37°C, >98% of the original activity was lost in both the bioassay and the IRMA. These data suggested that the IRMA is specific for IFN-γ in its biologically active conformation and that there is a close correspondence between the quantitation of IFN-γ by bioassay and by the IRMA described in this paper. These conclusions have been corroborated by many other experiments.

DISCUSSION

A great deal of new information about the structure and functions of IFN-γ has accumulated since its successful purification (20) and, in particular, since the cloning and microbial expression of the IFN-γ cDNA (21). It is now recognized that IFN-γ is an important lymphokine exerting numerous actions on the function of monocyte/macrophages and various lymphoid cells (reviewed in ref. 4). Newly recognized actions of IFN-γ are still being reported (e.g., ref. 25) and, obviously, more needs to be learned about the role of IFN-γ in immune regulation. The possible role of IFN-γ in human diseases (26–30) also needs to be further explored.

The fact that until now the demonstration and quantitation of IFN-γ has depended on the use of a suitable biological (antiviral) assay has hampered an even faster progress in the research of IFN-γ and its various activities. The IRMA described in this paper should facilitate work with both natural and recombinant *E. coli*-derived human IFN-γ. Unlike monoclonal antibody GIF-1 (31), which recognizes only natural and not *E. coli*-derived recombinant IFN-γ (14, 19), both antibodies B1 and B3 react equally well with glycosylated natural and unglycosylated *E. coli*-derived human IFN-γ (18, 19). Since the IRMA can detect as little as 0.1 NIH reference unit/ml of IFN-γ, the IRMA is more sensitive than the common biological assay in which the limit of detectability usually is around 4 units/ml. Recently, Novick *et al.* (32) reported the preparation of several mouse monoclonal antibodies and their use in a radioimmunoassay with a sensitivity limit of 4 ng/ml. The greater sensitivity of our IRMA might be due to differences in the inherent properties of the monoclonal antibodies, such as affinity and specificity, or to differences in the preparation of immunoadsorbents and tracers.

The fact that, after inactivating treatments of IFN-γ, the reactivity in the IRMA and biological activity decrease in parallel suggests that the antibodies recognize the tertiary structure characteristic for the active conformation of the molecule. A similar discrimination between active and inactive forms of *E. coli*-derived human recombinant IFN-α_2 (IFLrA) by monoclonal antibodies was reported recently by Pestka *et al.* (33).

The sandwich IRMA with antibody B1 as the immunoadsorbent and B3 as the tracer was found to be slightly better in sensitivity and signal intensity than the opposite configuration, probably because B3 has a higher affinity for human IFN-γ than B1 (results not shown). Somewhat surprisingly, assays in which only one antibody, either B3 or B1, was used both as the immunoadsorbent and the tracer showed a similar performance as the B1/B3 configuration with either natural or recombinant *E. coli*-derived human IFN-γ. Since the amino acid sequence of IFN-γ rules out the presence of two identical antigenic sites on a single monomeric molecule (21), these results suggest that human IFN-γ molecules exist mostly as dimeric or higher polymeric forms. This conclusion is in agreement with the notion that in native form, both natural (34) and *E. coli*-derived recombinant human IFN-γs (35) form oligomers, presumably dimers. Additional experiments in which this IRMA is used together with appropriate physicochemical measurements could provide a formal proof of this notion.

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