Development of a Human Gamma Interferon Enzyme Immunoassay and Comparison with Tuberculin Skin Testing for Detection of Mycobacterium tuberculosis Infection

NUKET DESEM AND STEPHEN L. JONES*
Biosciences Division, CSL Limited, Parkville 3052, Victoria, Australia

Received 12 November 1997/Returned for modification 5 February 1998/Accepted 29 April 1998

A sensitive two-step simultaneous enzyme immunoassay (EIA) for human gamma interferon (IFN-γ) has been developed and used as an in vitro test for human tuberculosis (TB) in comparison with tuberculin skin testing. The EIA was shown to be highly sensitive, detecting less than 0.5 IU of recombinant human IFN-γ per ml within a linear detection range of 0.5 to 150 IU/ml. The assay was highly reproducible and specific for native IFN-γ. Cross-reactions with other human cytokines or with IFN-γs derived from mice, cattle, or Old World monkeys were not evident. The assay was used to detect TB infection by incubating whole blood overnight with human, avian, and bovine tuberculin purified protein derivatives (PPDs), as well as positive (mitogen)- and negative-control preparations. The levels of IFN-γ in plasma supernatants were then determined. Blood from 10 tuberculin skin test-positive individuals responded predominantly to the human tuberculin PPD antigen and to a lesser extent to bovine and avian PPD antigens. By contrast, blood from 10 skin test-negative individuals showed minimal responses or no response to any of the tuberculin PPDs. Detectable levels of IFN-γ were present in all blood samples stimulated with mitogen. In vivo tuberculin reactivity was correlated with IFN-γ responsiveness in vitro. These results support the further study of the blood culture-IFN-γ EIA system as an alternative to skin testing for the detection of human TB infection.

Human tuberculosis (TB), which is caused by infection with Mycobacterium tuberculosis, was declared a global emergency by the World Health Organization in 1993 and each year leads to the deaths of 3 million people (24). One essential factor for controlling the spread of this disease is the ability to diagnose infection in its early stages.

TB infection has been detected for more than 100 years by the tuberculin skin test, which measures the cell-mediated immune (CMI) response generated by an intradermal injection of tuberculin purified protein derivative (PPD). However, despite widespread use of the skin test, frequent errors in the administration of PPD and in the reading of results, cross-reactivity with other non-TB mycobacterial infections, the booster phenomenon (19), and anergy in immunocompromised individuals (11, 25) make interpretation difficult and have an effect on determining the test’s true sensitivity and specificity (11). Therefore, alternate and improved diagnostic assays for TB have been sought. The ability to detect organisms directly in clinical specimens has been greatly improved by the availability of assays that detect circulating antibodies or antigen have been developed, and some of these assays appear to have diagnostic utility for patients with clinical TB (1, 7, 14, 27). However, in order to control the spread of TB, it is necessary to identify and treat infected individuals before they become infectious to others through progression to clinical disease. Active TB is associated with a heavy bacterial load and concomitant high levels of circulating antibody resulting from the inability of the immune system to contain bacterial growth (17). M. tuberculosis is an intracellular pathogen that replicates within host macrophages, and host defenses are believed to be largely dependent on T lymphocytes, with antibodies being of only minor importance (15). This suggests that an immunodiagnostic test for TB infection could be based on measuring specific T-cell reactivity.

The delayed-type hypersensitivity response, as measured by the skin test, has been shown to be dependent on the production of cytokines, including gamma interferon (IFN-γ), at the site of tuberculin injection (29). Based on these observations, Wood and colleagues in 1990 described an in vitro assay system for measuring CMI responses and applied this assay to the diagnosis of TB in cattle. The assay was based on stimulation of whole blood with PPDs and subsequent measurement of IFN-γ in a sandwich immunoassay specific for bovine IFN-γ (26). In large field trials, the assay was shown to be more sensitive (93.6%) than the traditional skin test (65.6%) for identification of Mycobacterium bovis-infected cattle (33). In the present study, we describe the development and characteristics of an enzyme immunoassay (EIA) for human IFN-γ and its application, in conjunction with a whole-blood culture system, for the detection of M. tuberculosis infection in humans.

**MATERIALS AND METHODS**

**MAbs.** Two noncompeting monoclonal antibodies (MAbs) specific for human IFN-γ (FA42.1F7 and FA26.7C2) were generated from mice immunized with recombinant IFN-γ (Bachem AG, Bubendorf, Switzerland) by methods described previously by Pietrzykowski et al. (23). MAb IgG was purified from ascites fluid with a ProSep-A (BioProcessing Ltd., Consett, England) column according to the manufacturer’s instructions. FA42.1F7 F(ab’)2 fragments were prepared by pepsin (Sigma-Aldrich Pty Ltd., Castle Hill, New South Wales, Australia) digestion, and FA26.7C2 was conjugated to horseradish peroxidase (HRP) (Sigma-Aldrich) as described by Jones et al. (13).

**Cytokines and antigens.** Recombinant human, murine, and bovine IFN-γ were purchased from Boehringer Mannheim (Castle Hill, New South Wales, Australia).
Australia), Sigma, and Ciba-Geigy (Basel, Switzerland), respectively. The international reference reagent for recombinant human IFN-γ (Gxg01-902-535) was kindly provided by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, Md.). Squirrel monkey IFN-γ, in the form of culture supernatants of concanavalin A (ConA)-stimulated peripheral blood lymphocytes (PBL), was supplied by R. Macfarlan (CSL Limited, Parkville, Victoria, Australia). Natural human IFN-γ and interleukin-2 (IL-2) were purchased from Boehringer Mannheim, recombinant human IL-5 was purchased from Endogen (Cambridge, Mass.), and recombinant human IL-6, IL-10, and IL-12 were gifts from P. Wood (CSIRO, Parkville, Australia). Human, avian, and bovine PPDs were manufactured by CSL, with their biological potencies standardized to international reference preparations. Phytohemagglutinin P (PHA) was purchased from Difco Laboratories (Detroit, Mich.).

**Human IFN-γ EIA.** MAb FA42.1F7 F(ab′)2 fragments were diluted in 50 mM carbonate buffer (pH 9.6) to a concentration of 5 μg/ml and were bound to 96-well EIA plates (Maxisorp Nunc, Roskilde, Denmark) (100 μl/well) overnight. The plates were postcoated with 150 μl of a 1-mg/ml solution of sodium casein per well in 0.01 M phosphate-buffered saline (PBS; pH 7.2) for 1 h. All traces of postcoating buffer were aspirated, and the plates were dried under vacuum. HRP-conjugated FA26.7C2 diluted in PBS−0.1% casein−20% normal mouse serum (NMS) was added (50 μl/well) to all wells. Then, 50 μl of sample per well was added and mixed, and the mixtures were incubated for 1 h. The plates were subsequently washed six times with PBS containing 0.05% Tween 20, and 100 μl of tetramethylbenzidine substrate per well (13) was added. After 30 min, the production of chromophore was stopped by the addition of 50 μl of 0.5 M H₂SO₄ per well, and optical densities at 450 nm were read with a 620-nm reference filter. All incubations and manipulations were performed at room temperature (22 ± 5°C). EIA optical density data were analyzed by generating a linear standard curve from four replicates of known dilutions of recombinant IFN-γ (in international units per milliliter) plotted against their mean optical density values in the EIA. The concentrations of IFN-γ in test samples were calculated from the standard curve with the software package KC-Jr (Bio-Tek Instruments, Inc.).

**Participants and skin testing.** The blood samples used in this study were obtained after informed consent from 14 male and 6 female (mean age, 35.1 years; range, 17 to 60 years) volunteers attending a Health and Community Services TB program in Melbourne, Australia. Participants were newly arrived immigrants previously identified by the Australian Immigration Service as being at risk of TB by virtue of country of origin and chest X rays. The study was approved by the Ethics Committee of the Victorian Government Department of Health and Community Services. Skin testing was performed according to the State Guidelines (3) with 10 IU of human tuberculin PPD (CSL). All skin tests were performed immediately after blood samples were taken and were read after 48 to 72 h.

**Whole-blood cultures.** Venous blood was collected into Exutainer tubes (Labco Ltd., London, England) containing lithium heparin (15 U/ml). Each blood sample was well mixed immediately prior to whole-blood culture such that 1-ml aliquots of each sample were dispensed into 5 wells of a 24-well tissue culture tray. Then, 120 μl of either sterile PBS (nil antigen control) or optimal concentrations of human PPD, avian PPD, bovine PPD, or PHA were added and thoroughly mixed with each 1-ml aliquot of blood. The 24-well culture plates were incubated for 16 to 24 h at 37°C in a humidified atmosphere. Plasma supernatants were then collected and stored at 2 to 8°C prior to assaying for IFN-γ.

**RESULTS**

**Range and detection limits.** The range and detection limits of the human IFN-γ EIA were determined by testing various recombinant human IFN-γ concentrations (0 to 300 IU/ml) diluted in pooled normal human plasma on two occasions by two operators and with two batches of reagents. The working concentration range of recombinant human IFN-γ detected by the assay was between 0.5 IU/ml (approximately 20 pg/ml) and 150 IU/ml (approximately 5 ng/ml) (Fig. 1). Concentrations of IFN-γ higher than 150 IU/ml, to as high as 100,000 IU/ml (highest tested), were also reactive, but their optical densities exceeded the upper limit of the microplate reader and were not quantifiable without dilution.

**Specificity for IFN-γ.** To assess the possibility that heterophilic antibodies (4, 5, 13, 16) caused interference in the IFN-γ assay, unstimulated human plasma samples from 201 healthy blood donations were tested in sample diluent without NMS. Of these plasma samples, 6% were highly reactive, generating an optical density in the EIA greater than twice that of the negative control (data not shown). Five of these samples were then used to determine the optimum concentration of NMS required in sample diluent to eliminate false reactivity. The addition of 20% (vol/vol) NMS reduced the optical densities of these five plasma samples to less than 0.1 (data not shown). The presence of NMS in the diluent had no effect on the ability of the EIA to detect IFN-γ added into human plasma derived from unstimulated blood (Table 1). The sample diluent for all further assays included 20% NMS.

To investigate the specificity of the assay, recombinant and natural human IFN-γ were denatured by heat (56°C, 1 h) or acidic pH (pH 2, 1 h) treatment and various other inducible cytokines were diluted in pooled human plasma and tested. As shown in Table 2, the EIA did not detect denatured human IFN-γ; natural human IL-2 (200 IU/ml); or recombinant human IL-4 (5 ng/ml), IL-5, IL-6, IL-10, or IL-12 (100 ng/ml). The species specificity of the IFN-γ EIA was also investigated. Supernatants of either PHA-stimulated whole blood or ConA-stimulated PBL from primate species chimpanzees, orangutans, gibbons (apes), and squirrel monkeys (New World monkeys), but not from colobuses, macaques, mandrills or baboons (Old World monkeys), were reactive in this assay (Table 2). Heat or acid treatment of the reactive samples abrogated this reactivity, supporting the conclusion that EIA reactivity was due to the presence of induced IFN-γ (12, 20). Recombinant bovine IFN-γ (100 ng/ml) was

---

**TABLE 1. Effect of adding NMS to sample diluent to remove false reactivity of plasma in the human IFN-γ EIA**

<table>
<thead>
<tr>
<th>Type of normal human plasma</th>
<th>Level of added human IFN-γ (IU/ml)</th>
<th>EIA optical density (450 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No NMS</td>
<td>0.069</td>
<td>0.062</td>
</tr>
<tr>
<td>20% NMS</td>
<td>1.419</td>
<td>1.419</td>
</tr>
</tbody>
</table>

*Plasma from unstimulated blood donation.*
only very weakly reactive in the EIA, and recombinant murine IFN-γ (5,000 IU/ml) was unreactive.

**IFN-γ EIA validation.** The linearity of standard curves expressed as a correlation coefficient (r) from 167 assays performed over a 14-month period was assessed to reproduce the reproducibility of the IFN-γ EIA. All r values were greater than 0.98, with a mean of 0.996 and a coefficient of variation of 0.3%. The precision of the human IFN-γ EIA was investigated by evaluating 20 plasma samples containing various concentrations of natural human IFN-γ (0 to 122 IU/ml) within the range of the human IFN-γ assay. These samples were tested undiluted as well as at a dilution of 2:1 in pooled human plasma. The mean difference between the IFN-γ concentrations determined for the undiluted and diluted samples was not significant (P = 0.713 [Student’s paired t test]). The accuracy of the human IFN-γ EIA was estimated by assaying four replicates of pooled human plasma spiked with various levels of recombinant human IFN-γ (150, 75, 37.5, 18.8, 9.4, and 4.7 IU/ml) on two occasions by two operators and with two batches of reagents. The average accuracy for the known concentrations was 105.8% ± 11.4%.

The correlation coefficient of the standard curve is a measure of linearity but does not take into account the slope of the standard curve. The linearity of the assay was examined by varying the sample incubation period. Standard concentrations of human IFN-γ in replicates of six were reacted for 60, 75, 90, and 120 min. All four standard curves produced were linear (r > 0.99), irrespective of assay incubation time (Fig. 2). While the slopes of the four standard curves were different (ranging from 0.012 for 60 min of incubation to 0.022 for 120 min), after log transformation the curves were parallel, indicating that the same IFN-γ concentration would be calculated for a sample regardless of the reaction time. This was confirmed by assaying 35 stimulated plasma samples for IFN-γ and various dilutions of recombinant human IFN-γ with either 60 or 120 min of incubation. The concentrations of IFN-γ in all samples were determined from their respective standard curves, and the mean difference between the two incubations was not significantly different to zero (P = 0.198 [Student’s paired t test]).

To determine the reactivity of the NIH recombinant human IFN-γ reactivity reagent in the EIA, six replicates of NIH and Boehringer IFN-γ at 150, 125, 100, 75, 50, 20, 10, and 5 IU/ml (in pooled human plasma) were assayed and compared by parallel regression analysis. The relative potency of the NIH IFN-γ reactivity reagent was 0.83 IU/ml (95% confidence interval, 0.78 to 0.88). The r values for both standard curves were 0.999 and 0.997, respectively.

**Detection of TB infection.** To investigate the utility of the whole-blood culture–IFN-γ assay system for detection of TB infection, blood samples from 10 tuberculin skin test-positive and 10 skin test-negative individuals were tested. The IFN-γ response of each individual to the five different stimulation antigens is listed in Table 3. The mean concentrations (± standard errors of the means) of IFN-γ of blood from tuberculin-positive individuals stimulated with human PPD and bovine PPD were 28.2 ± 12.9 and 20.3 ± 9.7 IU/ml, respectively (Fig. 3). These responses were significantly higher (P = 0.002 [Wilcoxon paired nonparametric signed rank test]) than those produced in response to avian PPD (11.6 ± 6.5 IU/ml). Blood from the skin test-negative individuals showed no or minimal production of IFN-γ in response to human (0.7 ± 0.3 IU/ml), bovine (0.4 ± 0.2 IU/ml), and avian (0.8 ± 0.4 IU/ml) PPDs. The differences in response to the human, avian, and bovine tuberculin PPDs between the skin test-positive group and the skin test-negative group were highly significant (P < 0.0001, P = 0.0011, and P < 0.0001, respectively [Mann-Whitney test]). All skin test-positive individuals produced higher levels

<table>
<thead>
<tr>
<th>Cytokine source and/or concn†</th>
<th>Conc. (IU/ml)††</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td></td>
</tr>
<tr>
<td>rIFN-γ (no treatment)</td>
<td>174</td>
</tr>
<tr>
<td>rIFN-γ (pH 2 treatment [HCl for 1 h])</td>
<td>4.7 (97.3)</td>
</tr>
<tr>
<td>rIFN-γ (heat treatment [56°C for 1 h])</td>
<td>0 (100)</td>
</tr>
<tr>
<td>nIFN-γ (no treatment)</td>
<td>232</td>
</tr>
<tr>
<td>nIFN-γ (pH 2 treatment [HCl for 1 h])</td>
<td>3.2 (98.6)</td>
</tr>
<tr>
<td>nIFN-γ (heat treatment [56°C for 1 h])</td>
<td>0 (100)</td>
</tr>
<tr>
<td>rIL-2 (200 IU/ml)</td>
<td>0</td>
</tr>
<tr>
<td>rIL-4 (5 ng/ml)</td>
<td>0</td>
</tr>
<tr>
<td>rIL-5 (100 ng/ml)</td>
<td>0</td>
</tr>
<tr>
<td>rIL-6 (100 ng/ml)</td>
<td>0</td>
</tr>
<tr>
<td>rIL-12 (100 ng/ml)</td>
<td>0</td>
</tr>
<tr>
<td>Nonhuman primate IFN-γ</td>
<td></td>
</tr>
<tr>
<td>Pan troglodytes (chimpanzee)†</td>
<td>73.1</td>
</tr>
<tr>
<td>Pongo pygmaeus (orangutan)†‡</td>
<td>35.9</td>
</tr>
<tr>
<td>Hylobates spp. (gibbon)‡</td>
<td>247</td>
</tr>
<tr>
<td>Saimiri (squirrel monkey)‡</td>
<td>98</td>
</tr>
<tr>
<td>Colobus satanas (guerezas)†</td>
<td>1.8</td>
</tr>
<tr>
<td>Papiro sphinx (mandrill)‡</td>
<td>0</td>
</tr>
<tr>
<td>Papio hamadryas (sacred baboon)†</td>
<td>0.1</td>
</tr>
<tr>
<td>Macaca fascicularis (cynomolgus macaque)‡</td>
<td>0</td>
</tr>
<tr>
<td>Murine rIFN-γ (5,000 IU/ml)</td>
<td>0</td>
</tr>
<tr>
<td>Bovine rIFN-γ (100 ng/ml)</td>
<td>2</td>
</tr>
</tbody>
</table>

*† rIFN-γ and nIFN-γ, recombinant and natural IFN-γ, respectively; rIL and nIL, recombinant and natural interleukin, respectively.
†† Values in parentheses are percent reduction values.
‡ PHA-stimulated whole blood.
§ ComA-stimulated PBL.

FIG. 2. Human IFN-γ EIA standard curves after 60, 75, 90, and 120 min of incubation of the assay.
of IFN-γ in response to human PPD (5.4 to 142 IU/ml) than did any of the skin test-negative individuals (0 to 2.6 IU/ml). Moreover, the correlation between skin test induration diameter and the magnitude of the IFN-γ response to human PPD was highly significant ($r = 0.82$ by the Spearman test [$P < 0.0001$]). All IFN-γ responses to human PPD in the skin test-positive group were greater than their respective responses to avian PPD. There was no IFN-γ detected in the nil antigen cultures from tuberculin skin test-negative individuals (0 to 2.6 IU/ml). The level of response to the mitogen of skin test-positive individuals (53.2 ± 8.4 IU/ml) was not significantly different ($P = 0.315$ [Mann-Whitney test]) from that of skin test-negative individuals (78.5 ± 15.9 IU/ml).

### DISCUSSION

This paper describes the development of a sensitive and specific EIA for human IFN-γ and its application to the detection of TB infection. The assay detected as little as 20 pg of recombinant human IFN-γ per ml, and was linear over a concentration range of 20 pg/ml to 5 ng/ml, and the relative potency of the NIH recombinant IFN-γ reference reagent was 0.83. The assay was shown to be specific for native IFN-γ, since neither denatured IFN-γ nor any of the other cytokines tested reacted in the EIA. The human IFN-γ EIA was found to be highly reproducible and exhibited very low between-assay variation in correlation coefficients for standard curves. The assay was also shown to be both accurate (by consistently generating the expected results for samples of known concentration under various conditions) and precise (by showing no significant difference between potency estimates for samples tested neat and dilute).

It is well-established that two-site sandwich EIAs which employ MAbs as both the solid-phase capture antibody and the HRP-conjugated antibody often suffer from false-positive problems due to the presence of heterophile antibodies in many serum-plasma samples (4, 5, 13, 16). To circumvent this problem, we followed the procedure described by Jones et al. (13) and coated microtiter plates with F(ab')2 fragments of the solid-phase capture antibody and added 20% NMS to the sample diluent. This effectively eliminated false-positive reactions with plasma samples in the EIA and did not have any effect on the sensitivity of the assay.

IFN-γs from some species of nonhuman primates (chimpanzees, orangutans, gibbons, and squirrel monkeys) were detected by the EIA, whereas bovine, murine, and other Old World monkey IFN-γs were not. The lack of cross-reactivity with Old World monkeys was surprising, given their close phylogenetic relationship and the high degree of amino acid sequence homology that exists between human and macaque (96%) and human and baboon (92%) IFN-γs (8, 31). For example, a similar assay described for the detection of bovine IFN-γ also detected IFN-γs from other members of the family Bovidae (26). Nonetheless, the amino acid sequences of human and Old World monkey IFN-γs are not identical, and, hence, it is possible that the substitutions and deletions in these sequences result in an alteration to at least one of the epitopes recognized by the MAbs employed in this assay. Since we did not test these preparations in a specific bioassay for IFN-γ, we cannot be certain that there was IFN-γ in the samples generated by either ConA or PHA stimulation.

A number of investigators have reported sensitive EIAs employing specific MAbs for human IFN-γ (2, 10, 22, 30). The EIA described in the present study, as well as that described by Galli et al. (10), was developed as a simultaneous assay in which only one incubation and washing step was required before the addition of substrate. Development of the EIA in this format did not result in a loss of sensitivity of the assay compared to that performed by using sequential steps (data not shown) (2, 30). Our assay showed sensitivity comparable to that of other previously described human IFN-γ EIAs, and it...
This study demonstrates the potential of the blood culture–IFN-γ EIA system for the detection of *M. tuberculosis* infection in humans. Further studies aimed at assessing its sensitivity and specificity and clinical and epidemiological utility in the wider population are warranted.

ACKNOWLEDGMENTS

We gratefully acknowledge Rod Macfarlan, John Cox, Andrew MacGregor, Anastasia Moisidis, Charles Quinn, and Elizabeth Pietrzykowski for their work in the selection of MABs. We are grateful to Deborah Bailey, Mary Randall, and the nursing staff at the Department of Health and Community Services TB program at Fairfield Hospital for collection of blood and to the Veterinary staff at Melbourne, Taronga Park (Sydney), and Adelaide Zoological Gardens for provision of the nonhuman primate blood samples. We also acknowledge Jim Rothel for his constructive comments in preparing the manuscript.

REFERENCES