Incubation of Whole Blood at 39°C Augments Gamma Interferon (IFN-γ)-Induced Protein 10 and IFN-γ Responses to Mycobacterium tuberculosis Antigens

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A rarely challenged dogma in cell-mediated immune (CMI) assays is the incubation temperature, 37°C. Fever augments proinflammatory immune responses in vivo, and the aim of this study was to explore whether incubation at fever-range temperature could increase antigen-specific biomarker responses. We compared CMI responses following incubation of whole blood at 37°C and 39°C. Whole blood was obtained from (i) 34 healthy subjects whose blood was incubated with TB10.4 antigen, present in the Mycobacterium bovis bacillus Calmette-Guérin vaccine and many environmental mycobacteria; (ii) 8 TB patients and 8 controls incubated with Mycobacterium tuberculosis-specific antigens in the QuantiFERON-TB Gold test (QFT-IT); and (iii) from both groups incubated with a T cell mitogen. T cell responses (gamma interferon [IFN-γ]) and responses from antigen-presenting cells (IFN-γ-induced protein 10 [IP-10]) were determined. We further evaluated the effect of adding interleukin-7 (IL-7) and blocking IL-10 during incubation. In TB patients, IFN-γ and IP-10 levels were increased 4.1- and 3.4-fold, respectively, at 39°C incubation (P < 0.001). Similar results were seen after mitogen stimulation. In subjects responding to TB10.4, the effects were less pronounced and significant only for IP-10. Incubation at 39°C increased IP-10 and IFN-γ responsiveness to both antigens and mitogen in persons with baseline or initial low responses. Adding IL-7 and blocking IL-10 augmented the effects in synergy with fever-range temperature. Incubation at fever-range temperature vividly increases CMI responsiveness to antigen stimulation in vitro in tuberculosis patients and may increase the sensitivity of CMI assays.

Cell-mediated immune (CMI) assays are important research tools used to monitor T cell-dependent immune responses toward antigens from, e.g., pathogens or vaccines. CMI assays rely on generation of an in vitro immune response and quantification of the proinflammatory immune response (26). In clinical medicine, CMI assays are used for the diagnosis of infections that cannot be detected with direct methods (28). An example is the diagnosis of latent infection with Mycobacterium tuberculosis, where either purified peripheral blood mononuclear cells (PBMCs) or whole blood is incubated with M. tuberculosis-specific antigens, followed by quantification of the gamma interferon (IFN-γ) response, hence the name IFN-γ release assays (IGRAs) (3).

A rarely challenged dogma in CMI assays—both in research and in diagnostic medicine—is that the incubation temperature is optimal at 37°C. Fever is a part of the innate immune response in vivo and can aid the immune defense by impairing the viability of pathogens with strict temperature preferences or by enhancing immunological reactions (reviewed in reference 38). Previous studies indicate that fever-range temperature in vivo and in vitro augments antigen presentation and coreceptor expression and skews antigen-presenting cells (APCs) toward activation, in concert with augmented T cell responsiveness and Th1 bias (5, 6, 17, 33).

One of the major challenges of CMI assays—and IGRAs, in particular—is achieving a consistent and strong response (i.e., high sensitivity) without generating nonspecific immune activation (i.e., compromising specificity).

We and others have demonstrated that, compared to T cell responses, the APCs interacting with the specific T cells generate stronger chemokine and cytokine signals that could enable simpler or more sensitive readouts of CMI assays (reviewed in reference 24). In several studies, the chemokine IFN-γ-induced protein 10 (IP-10) has shown promise as a novel marker for immunodiagnosis of infection with M. tuberculosis in both children and adults (1, 13, 15, 21–23, 30).

In an attempt to further improve CMI assays, we hypothesized that incubation at fever-range temperatures in vitro could lead to an increase in proinflammatory immune responses and potentially an increase in the sensitivity of IGRA and CMI assays. Interleukin-7 (IL-7) is a cytokine that promotes survival of T cells (8, 9). IL-10 is an anti-inflammatory cytokine released by T cells and monocytes/macrophages with immune activation (25). Both IL-7 and blocking of IL-10 have previously been shown to augment IFN-γ responses to M. tuberculosis-specific antigens (9, 10, 12).

The aim of this study was to explore if incubation of cells at fever-range temperature could potentiate biomarker responses in a simple whole-blood CMI assay and if IL-7 and anti-IL-10 had an additional effect. Antigen-specific T cell responses were evaluated using IFN-γ as marker for a T cell response and the

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chemokine IP-10 as a marker for the APC response. We assessed the effects in three models; (i) a TB10.4 antigen model detecting responses to antigens present in the Mycobacterium bovis bacillus Calmette-Guérin (BCG) vaccine and other mycobacteria in healthy controls, (ii) a tuberculosis (TB) diagnostic model measuring responses to M. tuberculosis-specific antigens (early secretory antigenic target 6 [ESAT-6], culture filtrate protein 10 [CFP-10], and TB7.7) in TB patients, and (iii) a model independent of antigen and infection using a T cell mitogen.

(Extracts of the data in this paper were presented as an oral presentation at the European Respiratory Society’s Annual Assembly in Barcelona, Spain, September 2010.)

MATERIALS AND METHODS

Setting and patient recruitment. The TB10.4 response study included healthy nurses and other employees with no history of TB exposure at the outpatient clinic at the Department of Infectious Diseases or at the Clinical Research Centre at Hvidovre Hospital, Copenhagen, Denmark. Information on BCG vaccination status was obtained from all individuals.

The TB antigen study included patients with active TB as well as healthy controls. Blood was prospectively collected from patients suspected of having active TB attending the outpatient clinic at the Department of Infectious Diseases, Hvidovre Hospital, less than 7 days after they started treatment. Healthy controls consisting of employees at the Clinical Research Centre, Hvidovre Hospital, with no history of prior TB or TB exposure were included.

Reagents and equipment. Peptide TB10.4 is a mix of 9 overlapping 18-mer peptides from TB10.4 that was diluted in phosphate-buffered saline (PBS) and used at a final concentration of 2.5 μg/ml (16). Phytohemagglutinin (PHA) was used as the mitogen at a final concentration of 1 μg/ml (16). Phytomitogen (PHA) was used as the mitogen at a final concentration of 5 μg/ml in the QFT-IT (16). Recombinant human IL-7 (2 ng/ml; R&D Systems) and monoclonal antibodies (MAbs) blocking human interleukin-10 (anti-IL-10; 1 μg/ml; MBL) were added before incubation. The contents of the QFT-IT tubes comprise dextrose as the nutrient (2% w/v; MBL) and were adjusted with PBS to reach a final pH of 7.2. The final concentration of dextrose was kept constant at 2% w/v.

Blood collection and incubation for biomarker measurements. Whole blood was collected in 10-ml heparinized tubes (BD, United Kingdom). One milliliter of blood was added to the QFT-IT tubes and, for the TB10.4 study, to Nunc cryotubes (1.5 ml; Thermo Fisher Scientific, Denmark), optionally with IL-7 and anti-IL-10. Blood was incubated in incubators, water baths, and heating blocks. An incubator was used for incubation at 37°C, while a water bath placed within an incubator was used for incubation at 39°C. All tubes were incubated with closed lids for precisely 18 h without addition of CO₂. Temperatures were measured using a mercury thermometer and were checked at least 4 times during each incubation at least 2-h intervals and did not vary by more than 0.2°C.

Measurements. Following incubation, IP-10 was measured in a 1:9 dilution using an in-house sandwich enzyme-linked immunosorbent assay (ELISA) with a linear range from 8 to 2,500 pg/ml. IFN-γ levels were measured using the QuantiFERON ELISA. To improve analytical precision, the standard curve was extended (with 8 IU/ml [400 pg/ml] and 16 IU/ml [800 pg/ml]); other standards were retained as per the manufacturer’s protocol. In all other aspects, the manufacturer’s instructions were followed. IP-10 levels are presented after multiplying by the dilution factor. In the data presented, background levels of biomarkers (nil) are subtracted. White blood cell counts were done at the Department of Clinical Biochemistry, Hvidovre Hospital (Cobas, Roche, Switzerland).

Statistical analysis and result interpretation. Data were analyzed using SAS, version 9.2, software (SAS Institute) and two-sided nonparametric tests. A P value of ≤0.05 was considered significant. In TB patients and controls, IFN-γ values were converted to test results using the manufacturer’s recommendations and software.

Ethical considerations. Permission to conduct the study was obtained from the Ethical Committee of the Municipality of Copenhagen (KF-01-278477). All study participants gave written informed consent to participate.

RESULTS

Identification of optimal incubation temperature. Antigen-dependent IFN-γ and IP-10 responses after stimulation with TB10.4 increased with increasing temperature from 20°C, reaching a maximum at 39.5°C (Fig. 1). At 40°C, biomarker responses were lower in some (30%) of our samples, and most often the responses purged at 41°C. At the risk of underestimating the potential of fever-range incubation, we prioritized robustness and chose incubation at 39°C for the following studies.

TB10.4 antigen study. A total of 34 donors, of whom half (17/34) were BCG vaccinated, were recruited. The donors were divided into 23 responders and 11 nonresponders on the basis of IP-10 responses above and below 500 pg/ml, respectively, after incubation of their blood with TB10.4 peptides at 37°C without additional stimulus (Fig. 2). Among responders and nonresponders, 65% (15/23) and 18% (2/11), respectively, were BCG vaccinated and 70% (7/23) and 82% (9/11), respectively, were female (P = 0.68), and the median ages were 39 years (range, 26 to 54 years) and 30 years (range, 26 to 48 years) (P = 0.09), respectively.

In nonresponders, we found no significant differences in IFN-γ or IP-10 levels under any incubation conditions, and all...
nonresponders had low IFN-γ and IP-10 responses at 37°C (Fig. 3A and 4A). Two nonresponders responded after incubation at 39°C by both IP-10 and IFN-γ production both with and without anti-IL-10 and IL-7. These two donors were, as the only ones in the nonresponder group, BCG vaccinated. None of the other nonresponders had an increase in IFN-γ or IP-10 levels, regardless of incubation conditions.

In responders, antigen-dependent IP-10 levels, but not IFN-γ levels, were significantly higher at 39°C than at 37°C (P = 0.02 and P = 0.41, respectively); the median fold changes were 1.3 (range, 0.3 to 4.1) for IP-10 and 1.0 (range, 0.6 to 2.4) for IFN-γ (Fig. 3B and 4B). Adding anti-IL-10 and IL-7 gave higher levels of IP-10 and IFN-γ at both 37 and 39°C (P < 0.001 for all); median fold IFN-γ increases were 1.3 (range, 0.8 to 7.8) and 2.5 (range, 1.3 to 21.8), respectively, after adding anti-IL-10 or IL-7; and for IP-10, the fold increases were 1.4 (range, 1.0 to 3.1) and 1.8 (range, 0.9 to 4.6), respectively. IL-7 alone increased IFN-γ responsiveness at both 37°C and 39°C, but IP-10 was increased only at 39°C. There was no significant effect of blocking IL-10 alone. The effects of adding IL-7 or anti-IL-10 MAbs separately at 37°C and 39°C are shown in Fig. S1 and S2 in the supplemental material. The combined effect of increased incubation temperature and anti-IL-10 and IL-7 was synergistically augmenting for both IP-10 (P < 0.0001) and IFN-γ (P < 0.01); i.e., the effect of the two interventions used together was greater than addition of the effect of each intervention used separately (data not shown).

Two responders with consistently high IP-10 responses to TB10.4 antigens under all incubation conditions (>500 pg/ml) had low IFN-γ responses when their blood was incubated at 37°C. Incubation at 39°C both with and without anti-IL-10 and IL-7 brought IFN-γ responses in these two donors as well (Fig. 3B).

**TB antigen study.** For the TB diagnosis study, 8 TB patients and 8 healthy controls were recruited. Among the TB patients, the median age was 43 years (range, 25 to 60 years) and 38% (3/8) were female. Six were from regions where TB is endemic and two were from Scandinavia. Six patients had culture-confirmed pulmonary TB; one had nucleic acid amplification test (NAAT)-confirmed TB spondylitis; and one was diagnosed with TB meningitis on the basis of a positive tuberculin skin test (35 mm), cerebrospinal fluid pleocytosis, and characteristic magnetic resonance imaging findings, while NAAT and culture results were negative. Among controls, 3 were female (38%) and the median age was 36 years (range, 27 to 45 years).

There were no significant differences between TB patients and controls in gender distribution (38% [3/8] and 38% [3/8] female, respectively; P = 1.00) or age (median, 43 years [range, 25 to 60 years] and 36 years [range, 27 to 45 years], respectively; P = 0.09).

Among controls, there was no significant difference in IP-10 or IFN-γ responses to the antigens when incubation was at 39°C with or without anti-IL-10 and IL-7 compared to those at 37°C (Fig. 5A and 6A). In TB patients, antigen-dependent IP-10 and IFN-γ levels were increased at 39°C (P < 0.01 for all), especially when anti-IL-10 and IL-7 were added (P < 0.001 for all) (Fig. 4B and 5B). The effects of fever-range incubation temperature were comparable for both biomarkers: for 37°C versus 39°C, median fold changes were 4.1 (range, 1.6 to 8.1) for IFN-γ versus 3.5 (range, 1.4 to 8.1) for IP-10 (P = 0.95); for 37°C versus 39°C with IL-7 and anti-IL-10, median fold changes were 6.4 (range, 0 to 21.6) for IFN-γ and 3.5 (range, 1.5 to 25) for IP-10 (P = 0.95).

The increase in IP-10 and IFN-γ responses at the 39°C incubation temperature was significantly more pronounced in
TB patients responding to the TB antigens than in healthy individuals responding to TB10.4 antigens ($P < 0.001$ for both).

There were no significant differences in background (nil) release of IFN-γ levels after incubation at 37°C and 39°C without anti-IL-10 and IL-7 (median, 0.14 IU/ml versus 0.08 IU/ml [$P = 0.14$]) and with anti-IL-10 and IL-7 (median, 0.14 IU/ml versus 0.26 IU/ml [$P = 0.08$]) (data not shown). Nil IP-10 levels were similar when incubation was at 37°C and 39°C (median, 620.0 pg/ml versus 620.6 pg/ml [$P = 0.58$]), but they were higher when anti-IL-10 and IL-7 were added (620.0 pg/ml versus 2,740 pg/ml [$P = 0.01$]) (graphs not shown).

For both biomarkers, responsiveness to mitogen stimulation was increased at 39°C compared to that at 37°C ($P < 0.01$), with median 3.8-fold (range, 1.0- to 100.3-fold) changes for IFN-γ, compared to median 1.4-fold (range, 1.0- to 1,979.0-fold) changes for IP-10 ($P = 0.127$) (Fig. 7). The effect of fever-range temperature was further increased when anti-IL-10 and IL-7 ($P < 0.001$) were added. Importantly, the effects were especially pronounced in the patients with the lowest responses to mitogen stimulation.

Effects of fever-range incubation temperature and IL-7 plus anti-IL-10 on sensitivity of QFT-IT. At the standard 37°C incubation temperature, all controls were QFT-IT negative. Six of 8 TB patients were QFT-IT positive and 2 patients with culture-confirmed TB had indeterminate results, on the basis of low IFN-γ levels in the mitogen sample: one patient converted to QFT-IT positive at the 39°C incubation temperature, with increases in antigen-dependent IFN-γ levels from 0.25 to 2.00 IU/ml and mitogen-induced IFN-γ levels from 0.17 to 11.00 IU/ml; the other converted from indeterminate to a (false) negative result, with an increase in mitogen-induced IFN-γ levels from 0.25 to 7.49 IU/ml, but maintained a low antigen-dependent IFN-γ response (0.00 to 0.12 IU/ml). We have no previous set cutoff for IP-10 as a diagnostic marker for TB using the ELISA, so no firm conclusion can be drawn, but we observed a pattern for IP-10 similar to that for IFN-γ. At 39°C, there was a complete segregation between TB patients and controls using an IP-10 cutoff in the range of 2,050 to 3,100 pg/ml. When incubation was at 39°C with anti-IL-10 and IL-7, two controls had an increase in antigen-dependent IFN-γ and IP-10 levels, and one of these controls converted to QFT-IT positive.

**DISCUSSION**

Summary of major findings. This study shows that incubation at fever-range temperature augments cell-mediated immune responses. In three different models, we demonstrate that T cell IFN-γ and monocyte IP-10 responses are increased at a 39°C incubation temperature. In addition, we found that the CMI responses were further augmented by the addition of the T cell survival cytokine IL-7 and neutralizing antibodies against the anti-inflammatory cytokine IL-10. Finally, we ob-

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**FIG. 5.** IFN-γ responses to M. tuberculosis-specific antigen stimulation at 37°C, 39°C, and 39°C with IL-7 and blockage of IL-10. Whole blood was drawn and incubated 18 h at 37°C or 39°C and at 39°C with IL-7 and blocking antibodies to IL-10 (denoted “plus”). One milliliter of whole blood from 8 controls (A) and 8 TB patients (B) was stimulated with overlapping peptides specific for M. tuberculosis using QFT-IT tubes. For all values, the levels in the unstimulated sample were subtracted. *, $P < 0.01$ compared to 37°C; **, $P < 0.001$ compared to 37°C.
served that incubation at fever-range temperatures could bring low responders and nonresponders to respond with high levels of both IP-10 and IFN-γ.

Results in light of previous studies. Fever is a physiological reaction when the immune system combats invading microorganisms; therefore, it is intuitively appealing that the proinflammatory reaction in vitro would be more potent if it is done under conditions simulating the in vivo situation. While incubation under both hypothermic temperature (below 37°C) and heat shock temperature (41 to 43°C) conditions have been shown to impair immune cell function (18, 27, 35, 37), a number of studies suggest that incubation at fever-range temperatures (38 to 41°C) can augment the responsiveness of murine and human immune cells compared to that at the conventional 37°C incubation: at fever-range temperatures, stimulated PBMCs have been shown to increase antigen presentation and activity of APCs and to augment T cell responses to mitogen stimulation (5, 17, 33, 37). The production of mainly Th1-specific proinflammatory cytokines, e.g., IFN-γ, tumor necrosis factor alpha, IL-1, and IL-2, has been found to increase with incubation at fever-range temperatures, while the production of others, mainly Th2-specific cytokines such as IL-10, is downregulated (4, 5, 14, 38). Previous studies have primarily been carried out in PBMC cultures stimulated with mitogens or whole bacteria and never in a simple whole-blood assay. To our knowledge, this is the first study to show that fever-range incubation temperature can augment the antigen-specific responses to peptide stimulation in both T cells and monocytes in vitro.

Effects of adding and blocking cytokines. Previous studies have demonstrated that adding IL-7 or blocking IL-10 can augment M. tuberculosis-specific antigen responsiveness in vitro (9, 10, 12). In the TB10.4 study, we compared anti-IL-10 and IL-7 head-to-head and found that the effects of added IL-7 appeared to be superior to those of anti-IL-10 alone. IL-7 functions as a survival cytokine for T cells, and the effects of IL-7 were more pronounced when incubation was at higher temperature, where cells are likely to be under more stress (8). In contrast to the bovine data from Denis et al (9), we were not able to demonstrate an effect in IFN-γ responses of anti-IL-10 added alone.

Can fever-range incubation temperature improve IGRAs? Incubation at 39°C did not seem to compromise specificity in either the TB10.4 or the TB diagnostic model. On the contrary, the 39°C incubation temperature led to better separation between responders/nonresponders and patients/controls, suggesting that it might reduce the phenomenon of IGRA converters and reverters (7, 32, 34). Incubation at 39°C converted the only two BCG-vaccinated TB10.4 nonresponders to strong responders by both IFN-γ and IP-10 as well as one TB patient from an indeterminate to a positive QFT-IT result. This suggests that incubation at 39°C might increase assay sensitivity. While adding anti-IL-10 and IL-7 further added to this differentiation, it caused one control to convert to a false-positive
QFT-IT result. Furthermore, while background IFN-γ levels were not affected, incubation at 39°C increased background IP-10 levels, although only in individuals with very strong antigen responses as well. Larger studies are needed in order to confirm whether incubation at fever-range temperatures can improve IGRA performance.

Differences between vaccine responses and infection. An interesting observation was that incubation at fever-range temperatures seemed to have a greater effect in patients with an ongoing infection. This might be due to the fact that the TB10.4 response in healthy individuals is likely to derive mainly from central memory T cells (2, 11), whereas the TB antigen responses in patients with ongoing infection is derived from effector memory T cells (20, 31, 36). However, this study does not shed light on the exact mechanisms underlying the findings.

Limitations. In our preliminary studies, we identified 39.5°C to be superior to 39°C. However, as we observed a negative effect in some of the patients and donors at incubation temperatures of ≥40°C, we choose 39°C to get a stable system. Further studies in the 38 to 40°C range are needed to dissect the truly optimal temperature point and hereby the full potential of the findings described in this report. Although the observed effects of incubation at fever-range temperatures were consistent, a larger sample size, especially the inclusion of more TB patients, is needed in order to substantiate these findings and assess the effect on diagnostic assays.

Perspectives and conclusions. With this study, we present proof of principle of a simple no-cost method by which immune-based diagnosis of tuberculosis in infected cattle. Furthermore, we observed that incubation at fever-range temperatures might be improved. Incubation at fever-range temperatures appears to be superior to the standard 37°C incubation temperature for both peptide antigen-dependent and mitogen-induced biomarker production. Adding IL-7 and anti-IL-10 to the full protocol of the IGRA and CMI assay with increasing temperatures and might be improved. Incubation at fever-range temperatures of Mycobacterium bovis infections in cattle. Clin. Vaccine Immunol. 14:418–426.

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