Differential T and B Cell Responses against *Mycobacterium tuberculosis*
Heparin-Binding Hemagglutinin Adhesin in Infected Healthy Individuals and Patients with Tuberculosis

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Because only 10% of individuals infected with *Mycobacterium tuberculosis* will eventually develop disease, antigens that are recognized differently by the immune systems of infected healthy and diseased subjects may constitute potential vaccine candidates. Here, the heparin-binding hemagglutinin adhesin (HBHA) is identified as such an antigen. Lymphocytes from 60% of healthy infected individuals (*n* = 25) produced interferon (IFN)–γ after stimulation with HBHA, compared with only 4% of patients with active tuberculosis (*n* = 24). In the responders, both CD4+ and CD8+ cells secreted HBHA-specific IFN-γ, and the antigen was presented by both major histocompatibility complex class I and II molecules. In contrast to the reduced ability of patients with tuberculosis to produce HBHA-specific IFN-γ, most of them (82%) produced anti-HBHA antibodies, compared with 36% of the infected healthy subjects. These observations indicate that HBHA is recognized differently by the immune systems of patients with tuberculosis and infected healthy individuals and might provide a marker for protection against tuberculosis.

Received 28 June 2001; revised 3 October 2001; electronically published 31 January 2002.


Informed consent was obtained from all subjects enrolled in this study. The study was approved by the ethical committee of the Université Libre de Bruxelles.

Financial support: Centre de Recherche Interuniversitaire en Vaccinologie; INSERM and Institut Pasteur de Lille; Fond de Recherche dans l’Industrie et l’Agriculture (fellowship to S.T.); French Ministère de la Recherche (fellowship to K. P.).

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The Journal of Infectious Diseases 2002;185:513–20 © 2002 by the Infectious Diseases Society of America. All rights reserved. 0022-1899/2002/18504-0014$02.00

Tuberculosis, which causes 3 million deaths annually, remains the world’s leading cause of mortality mediated by a single infectious agent, and there are ~8–10 million new cases each year worldwide [1]. About one-third of the world’s population is infected with *Mycobacterium tuberculosis* and, thus, is at risk for developing the disease. The clinical expression of the disease may vary substantially between individuals and depends on both host- and microbe-related factors. Only ~10% of infected persons develop active disease during their lifetime. This suggests that, in 90% of infected subjects, the immune system successfully controls the infection; however, the details of the underlying immunologic mechanisms involved remain largely unknown. A deeper understanding of these mechanisms may evolve from studies comparing the immune responses of patients with tuberculosis with those of infected healthy control subjects and would undoubtedly be of benefit for the development of new, effective vaccines against tuberculosis.

The essential role of T cell responses [2], especially interferon (IFN)–γ production and signaling, has been documented substantially in animal models and in human studies [3–6]. Disruption of the gene encoding IFN-γ renders mice hypersusceptible to *M. tuberculosis* infection [3], and mutations in the IFN-γ receptor gene increase the susceptibility to mycobacterial infections in humans [6]. Furthermore, IFN-γ has been used successfully as adjunctive therapy, especially in patients with multidrug-resistant pulmonary tuberculosis [7]. The induction of T cell responses against mycobacterial antigens, especially antigen-specific IFN-γ production, is therefore considered to be of pivotal importance for the development of protective immunity against *M. tuberculosis*. One of the major challenges in tuberculosis vaccine development thus resides in the identification of IFN-γ–inducing mycobacterial antigens and formulations that promote such induction.

Because, in contrast to infected healthy subjects, patients with tuberculosis have lost their ability to control *M. tuberculosis* infection, a comparison of the immune responses between these 2 groups may help to elucidate the mechanisms of protective immunity against tuberculosis. Antigens that can induce the production of IFN-γ only in healthy infected individuals may constitute attractive candidates for inclusion in tuberculosis vaccines.

Surface and secreted proteins have been reported to be particularly immunogenic. The mycobacterial heparin-binding hemagglutinin adhesin (HBHA) is a surface-associated protein in-
volved in adherence to epithelial cells [8] and recently has been shown to be required for extrapulmonary dissemination of *M. tuberculosis* [9]. This study was designed to determine whether *M. tuberculosis*–infected subjects mount an immune response against HBHA and whether this immune response is different between healthy infected individuals and patients with active tuberculosis.

**Subjects and Methods**

**Subjects.** Blood samples were obtained from 31 patients with active tuberculosis, 25 healthy *M. tuberculosis*–infected subjects, 16 bacillus Calmette-Guérin (BCG)–vaccinated individuals, and 12 control subjects. All subjects were followed in a Belgian hospital and were living in Europe at the time of enrolment. The patients with tuberculosis had documented infection with *M. tuberculosis*, as evidenced by sputum examination and culture. Twenty-four of them were included in the study before treatment, whereas 7 were tested after ≥4 weeks of treatment. Among the patients with tuberculosis, 3 had lymph node tuberculosis, 1 had bone tuberculosis, 1 had pleural tuberculosis, 1 had peritoneal tuberculosis, and the other 25 had pulmonary tuberculosis. Infected healthy individuals were selected on the basis of positive skin test results, as determined by standard criteria (i.e., >10 mm of induration). Active tuberculosis was excluded by the absence of clinical signs and by normal chest radiographs.

The recipients of BCG vaccine had a known record of BCG vaccination with or without a positive skin reaction (<10 mm of induration). They were included in the study regardless of the length of time between vaccination and this study. The negative control subjects were defined as having a negative skin reaction after tuberculin injection and as having no known history of tuberculosis, contact with patients with tuberculosis, or BCG vaccination.

**Antigen preparation.** HBHA was purified from *Mycobacterium bovis* BCG by heparin-Sepharose chromatography, as described elsewhere [8, 10], followed by reverse-phase high-pressure liquid chromatography (HPLC; Beckman Gold System), using a Nucleosil C18 column (TSK gel Super ODS; Interchim) equilibrated in 0.05% trifluoroacetic acid. The protein was eluted by a linear 0–80% acetonitrile gradient prepared in 0.05% trifluoroacetic acid. HBHA was fluoroacetic acid. The protein was eluted by a linear 0–80% acetonitrile gradient prepared in 0.05% trifluoroacetic acid. The HPLC chromatogram revealed a single peak corresponding to the HBHA molecule. Analysis by SDS-PAGE showed a single band after Coomassie-blue or silver staining. The band corresponded to the apparent molecular weight of HBHA, indicating that the final preparation was not contaminated with additional proteins, as far as can be detected by HPLC and gel electrophoresis. Analysis by gas chromatography indicated that the final fraction did not contain detectable levels of lipooarabinomannan (LAM) or other glycolipids. The limulus test, which is used for possible lipopolysaccharide (LPS), contamination revealed that the LPS concentration was <10 pg/mL.

**Antigen-specific cytokine determinations.** Peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation of venous blood samples on Lymphoprep (Nycoderm Pharma). They were resuspended at 2.10^6 PBMC/mL in X-vivo medium (BioWhittaker) supplemented with 40 μg/mL gentamicin and 50 μM 2-mercaptoethanol. The cells were stimulated with 800 ng/mL HBHA. This concentration was chosen on the basis of a dose-response curve ranging from 0 to 4 μg/mL: 0.8 μg/mL was found to provide the best discrimination between the positive sample and the negative control. In parallel, the cells also were stimulated with 4 μg/mL purified protein derivative (PPD; Statens Serum Institut, batch RT 49) or with 2 μg/mL phytohemagglutinin (PHA; Abbott Laboratories). The cells were incubated for 4 days at 37°C under 8% CO₂. The culture supernatants then were collected, and IFN-γ and interleukin (IL)–4 concentrations were determined by use of a sensitive flow cytometry–based immunofluorescent assay (IFA; Immunoflow, BioErgonomics) [11]. The samples and different standards were incubated successively with paramagnetic beads coated with anti–IFN-γ/IL-4 monoclonal antibody (MAb) and with fluorescence-labeled secondary antibodies. The mean channel of fluorescence then was determined for each sample and standard on a flow cytometer (FACScan; Becton Dickinson) by drawing a gating bitmap around the main population of beads. The cytokine concentrations in the samples were determined by comparing the mean channel of fluorescence of the samples with those of the standard curve. The standard curves were designed to measure cytokine concentrations in the range of 0.5–5000 pg/mL.

To afford a maximum of sensitivity for IL-4 detection, an ELISPOT technique also was used, as described elsewhere [12]. In brief, PBMC were preincubated in tubes for 4 h at 37°C, at a concentration of 2.10^6 PBMC/mL, in the presence of HBHA (800 ng/mL), PPD (4 μg/mL), or PHA (2 μg/mL). Serial 2-fold dilutions of the cells suspensions then were dispensed into the ELISPOT plates coated with anti–IL-4 MAb (clone 82-2; Mabtech) and were incubated for 48 h (37°C) in the presence of the antigens or mitogen. The cells then were removed by extensive washing, and biotinylated anti–IL-4 MAb (clone 12.1; Mabtech) was added for 3 h at room temperature. The presence of spots was revealed by the addition of extravidin horseradish peroxidase (Sigma) followed by 3-aminophenylcarbazole (Sigma). The spots in wells containing <150–200 spots were counted under low magnification (×40).

To control for the absence of nonspecific stimulation of the monocytes by HBHA, the monocytes from an HBHA responder were enriched by the clumping method, which was successively applied twice [13]. The resulting monocyte population was incubated with HBHA (800 ng/mL), PPD (4 μg/mL), or LPS (1 μg/mL), and culture supernatants were collected after 48 h of culture. IL-12 (p70) concentrations were determined by ELISA (Endogen).

**Anti-HBHA antibody detection.** Serum anti-HBHA antibodies were detected by standard immunoblotting procedures [14]. In brief, after SDS-PAGE, using 12% polyacrylamide gels loaded with 0.5 μg/mL purified HBHA, the proteins were transferred onto nitrocellulose membranes (BA85; Schleicher & Schuell). After being blocked, the membranes were cut into 5-mm–wide strips, which then were probed for 1 h at room temperature with the 250-fold diluted human serum samples. Antibodies were detected with an alkaline phosphatase–conjugated goat anti–human IgG (ProtoBlot System; Promega).

**Inhibition of HBHA-specific IFN-γ secretion with blocking antibodies.** For 7 patients, the effect of blocking anti–major histocompatibility complex (MHC) I (IgG2a, clone W6/32; Dako) and anti–MHC II (IgG1, clone CR3/43; Dako) antibodies on the HBHA-specific IFN-γ secretion was tested by adding 2 μg/mL of either antibody 1 h before addition of the antigen. The effect of these anti-
bodies was compared with the effect obtained by the addition of isotype-matched control antibodies (IgG2a, clone DAK-G05; IgG1, clone DAK-G01, both from Dako).

**Phenotypic analysis of the IFN-γ-producing cells.** The percentages of IFN-γ–secreting cells and the phenotypes of the cytokine-secreting cells were determined simultaneously by use of the IFN-γ secretion assay (Miltenyi Biotech) [15]. In brief, isolated PBMC were stimulated in vitro for 24 h with HBHA (5 μg/mL) or PPD (1 μg/mL) to induce the secretion of IFN-γ. The secreted IFN-γ was captured immediately by an anti–IFN-γ antibody attached to the cell surface via an anti-CD45 antibody. This reagent binds IFN-γ as it is secreted by the activated antigen-specific T cells. The bound IFN-γ was detected subsequently by fluorescence, using an anti–IFN-γ antibody–conjugated to phycoerythrin. The cells then were stained for surface antigens and analyzed by flow cytometry (FACScan; Becton Dickinson) by first gating lymphocytes (forward vs. side scatter) and then the IFN-γ–secreting cells.

**Statistical analysis.** The nonparametric Mann-Whitney U test was used to determine statistical significance, except for the comparison of the percentages of anti-HBHA antibody producers, for which the χ² test was used.

**Results**

**Induction of HBHA-specific IFN-γ secretion by human lymphocytes.** The ability of purified HBHA to induce the production of IFN-γ was tested in 4 different populations: patients with tuberculosis, infected healthy individuals, BCG vaccinees, and negative control subjects. The results shown in figure 1A indicate that a considerable number (15 [60%] of 25) of infected healthy individuals produced >100 pg/mL HBHA-specific IFN-γ, compared with only 1 (4%) of 24 patients with active tuberculosis, and the IFN-γ concentrations were significantly lower in the latter group (P = .0025). In contrast, none of the BCG-vaccinated subjects and none of the control subjects produced HBHA-specific IFN-γ levels >100 pg/mL. The low percentage of patients with tuberculosis who responded to HBHA was not a consequence of general anergy in these patients, since most of them produced PPD-specific IFN-γ (figure 1B) and all of them produced IFN-γ in response to an in vitro polyclonal stimulation with PHA (figure 2). The levels of IFN-γ produced after stimulation with PPD were not significantly different between patients with tuberculosis and infected healthy individuals.

The same subjects were tested for their ability to produce IL-4, using both the flow cytometry–based IFA and ELISPOT assay to afford maximal sensitivity. None of the subjects produced HBHA- or PPD-specific IL-4, regardless of their infection or vaccination status (data not shown). However, all produced IL-4 in response to PHA, indicating that the absence of HBHA- and PPD-specific IL-4 was not due to a general inability to produce IL-4.

**Effect of treatment of the patients with tuberculosis on their immune response to HBHA.** The apparent lack of HBHA-specific IFN-γ production by the patients with tuberculosis prompted us to investigate their ability to produce IFN-γ after HBHA stimulation after treatment. Ten patients with active tuberculosis who had been treated for ≥4 weeks with antituberculosis drugs were analyzed for HBHA-specific IFN-γ production after treatment. All patients who were still expectorating at that time had culture-negative results. Three of these patients also had been analyzed before treatment. As shown in figure 2, 7 of the 10 patients produced IFN-γ levels >100 pg/mL after stimulation with HBHA. This level was significantly different than that for patients analyzed before treatment (P = .001). Of interest, all 3 patients who were analyzed both before and after treatment showed increased HBHA-specific IFN-γ production after treatment. In contrast to the effect on HBHA-specific IFN-γ produc-

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**Figure 1.** Heparin-binding hemagglutinin adhesin (HBHA)–specific and purified protein derivative (PPD)–specific interferon (IFN)–γ secretion by human peripheral blood mononuclear cells (PBMC). PBMC were isolated from patients with tuberculosis (TB), healthy infected individuals (Inf.), BCG vaccinees (BCG), or noninfected control subjects (controls) and stimulated with HBHA (A) or PPD (B). IFN-γ then was measured in the culture supernatants. Horizontal lines represent the medians, and the dotted line shows the cutoff corresponding to 100 pg/mL IFN-γ. NS, not significant.
tion, the treatment had no significant effect on IFN-γ production after stimulation with either PPD or PHA (figure 2).

**Phenotypes of the HBHA-specific IFN-γ-producing cells.**

To determine whether the IFN-γ production observed after stimulation with HBHA was the result of T cell stimulation rather than of nonspecific stimulation of monocytes, an enriched monocyte suspension from an HBHA-specific IFN-γ producer was stimulated in vitro with LPS (10 μg/mL) or HBHA (0.8 μg/mL). The concentrations of released IL-12 p70 were 3501 pg/mL for the LPS stimulation and only 92 pg/mL for the HBHA stimulation, indicating that the secreted IFN-γ was the result of a specific stimulation of lymphocytes by HBHA.

The phenotype of the IFN-γ-secreting lymphocytes was determined in the cell cultures incubated with HBHA by simultaneous immunostaining for secreted IFN-γ and surface markers followed by flow cytometry. The HBHA-specific IFN-γ-secreting cells comprised 0.15%–3.71% (median, 0.6%) of the lymphocytes, as evaluated after subtraction of the percentage of IFN-γ-secreting cells in the absence of HBHA (figure 3). CD3+ T cells, rather than CD56+CD3- NK cells, were the main source of IFN-γ (49%–98% CD3+ T cells [median, 75%]; 2%–42% CD56+CD3- NK cells [median, 11%]). Among CD3+ T lymphocytes, both CD4+ and CD8+, as well as a low percentage of CD4+CD8+ lymphocytes, participated in IFN-γ secretion (table 1). The relative proportions of CD4+ and CD8+ lymphocytes secreting IFN-γ after stimulation with HBHA varied among patients.

**MHC restriction of HBHA-induced IFN-γ secretion.**

Since both CD4+ and CD8+ T cells contributed to the secretion of IFN-γ after stimulation with HBHA, we analyzed the MHC restriction of the HBHA-specific IFN-γ secretion by testing the effect of blocking antibodies against class I and class II MHC molecules on IFN-γ secretion. The effect of these antibodies was expressed as the percentage of inhibition of the HBHA-induced IFN-γ secretion obtained in the presence of the blocking antibodies, compared with an isotype-matched control antibody. As shown in table 2, the addition of anti–MHC II antibody inhibited the HBHA-induced IFN-γ secretion in all cases by >80%. Anti–MHC I antibodies had a more variable effect on IFN-γ secretion (23%–89% inhibition). These results suggest that both MHC class I and MHC class II molecules are involved in the recognition of HBHA and that they both are required for the optimal secretion of IFN-γ by PBMC in response to stimulation by HBHA. They also confirm the involvement of both CD4+ and CD8+ lymphocytes in HBHA-induced IFN-γ secretion.

**Antibody production against HBHA.**

Although most patients with tuberculosis did not produce IFN-γ after HBHA stimulation before treatment, it has been reported that at least some patients can mount an HBHA-specific antibody response [8]. We therefore analyzed serum samples from 14 randomly chosen infected healthy subjects and 14 patients with tuberculosis for the presence of anti-HBHA IgG by immunoblotting. As shown in figure 4, anti-HBHA antibodies were detected in serum samples from 82% of the patients with active tuberculosis and 36% of the infected healthy individuals. This difference was significant (P < .01) and is in strong contrast to the proportions of HBHA-specific IFN-γ producers in both groups (figure 4). The negative control subjects and the BCG vaccinees did not produce anti-HBHA antibodies at a detectable level.
Discussion

Infection with *M. tuberculosis* results in active disease in only a minority of individuals. About 90% of those infected will contain their infection [1], suggesting that they mount an appropriate immune response. The immune systems of patients with tuberculosis probably do not recognize protective antigens or recognize them in an inappropriate way. Therefore, antigens that are recognized differently by the immune systems of diseased and healthy infected individuals might perhaps provide surrogate markers of protection in human tuberculosis.

It has been well demonstrated that the generation of antigen-specific IFN-γ is crucial for protection against tuberculosis [3], and individuals who produce low levels of IFN-γ or who are deficient in IFN-γ signaling are at a high risk for developing severe mycobacterial disease. In this study, we found that HBHA was recognized by human lymphocytes from most *M. tuberculosis*–infected healthy subjects. They secreted high levels of IFN-γ in response to in vitro stimulation with HBHA. In contrast, the lymphocytes of only 1 (4%) of 24 of the tested patients with active tuberculosis could secrete significant levels of IFN-γ in response to HBHA. Of interest, this 1 patient was the only tested
Phenotypic characterization of these T lymphocytes indicated that both CD4+ and CD8+ cells were involved in HBHA-specific IFN-γ secretion. However, the proportion of both cell types varied between individuals. Experiments using blocking anti-MHC antibodies were consistent with IFN-γ secretion by both CD4+ and CD8+ cells, since both anti-MHC I and anti-MHC II antibodies inhibited IFN-γ secretion. MHC I and MHC II molecules are known to present peptide antigens predominantly to CD8+ and CD4+ T cells, respectively. Of interest, although the percentages of inhibition of IFN-γ secretion obtained with the anti-MHC I antibodies varied between 23% and 89%, the anti-MHC II antibodies blocked IFN-γ secretion by ≥80% in all subjects tested. It is therefore likely that the secretion of IFN-γ by CD8+ T cells, dependent on MHC I presentation of HBHA peptides, requires the help of CD4+ T cells, depending on the presentation of HBHA peptides by MHC II molecules. The requirement of CD4+ T cell help for the induction of antigen-specific IFN-γ by CD8+ T cells has been illustrated recently in healthy tuberculin reactors with respect to PPD [19].

The involvement of both CD4+ and CD8+ T cells in the production of HBHA-specific IFN-γ may be of relevance to protective immunity against *M. tuberculosis*. The importance of IFN-γ–secreting CD4+ T cells in protective immunity has long been recognized both in animal models [20] and in humans [21]. However, more recently, increasing evidence also suggests an important role of MHC class I–restricted CD8+ T cells in controlling *M. tuberculosis* infection [5, 22–24]. Mice lacking functional CD8+ T cells are more susceptible than CD8+ T cell–producing mice to mycobacterial infection [25]. Human studies have shown that CD8+ T cells can be expanded after in vitro stimulation with antigen-presenting cells infected with mycobacteria, such as live *M. bovis* BCG, and can act as cytokine producers and cytotoxic T lymphocytes [22, 24, 26]. Although IFN-γ was produced by T lymphocytes in response to HBHA stimulation, as evidenced by flow cytometry. Its production was the result of an antigen-specific stimulation of T lymphocytes by HBHA processed by antigen-presenting cells rather than of a nonspecific indirect stimulation via HBHA-activated monocytes. This contention is supported by the fact that the release of IL-12 by monocyte-enriched cell suspensions stimulated with HBHA was very low, compared with the release induced by LPS. Although during human infection with *M. tuberculosis*, IFN-γ can be produced by NK cells [17], we show here that NK cells represent only a minor source of IFN-γ secretion, as reported elsewhere for the immune response to PPD [18].
we have shown that both CD4+ and CD8+ T cells isolated from infected healthy individuals produce IFN-γ after HBHA stimulation and that these responses are MHC restricted, the potential role of these T cell subsets in controlling M. tuberculosis infection, perhaps via their cytotoxic activity, remains to be investigated. It is certainly reasonable to assume that the HBHA-specific IFN-γ produced by both types of T cells can activate infected macrophages to protect against disease.

Consistent with this notion, most patients with active tuberculosis did not produce HBHA-specific IFN-γ. The defect in the production of HBHA-specific IFN-γ was not the result of general anergy in this group of patients, since they responded to PPD and to PHA as well as did the infected healthy group. There was also no evidence for a direct association with a specific HLA haplotype (data not shown). In addition, patients with tuberculosis who had undergone antimycobacterial drug treatment produced HBHA-specific IFN-γ, which strongly suggests that the failure to do so was associated with active disease.

Of interest, patients with tuberculosis produced anti-HBHA antibodies much more frequently than did the infected healthy subjects. A negative correlation between antibody production and T cell responses has been reported elsewhere for other antigens, such as for the 16-kDa α-crystallin protein [27] and the 30-kDa [28] and 32-kDa [29] antigens. However, this negative correlation has not been found for all mycobacterial antigens [28]. Although we have not studied the isotype profile of the anti-HBHA antibodies, previous studies using other mycobacterial antigens, such as the 30-kDa antigen [30], total protein extracts, or LAM [31], have not been able to link specific isotypes to cytokine profiles.

Despite the use of the sensitive ELISPOT assay and flow cytometry–based IFA, we were unable to correlate the enhanced humoral response in patients with tuberculosis with increased levels of IL-4 secretion, since IL-4 production was below detectable levels in both groups. Although antigen-specific IL-4 production has been detected previously by the ELISPOT assay in patients with tuberculosis [32], the numbers of IL-4–secreting cells were extremely low even when tested against total soluble M. tuberculosis extracts.

In conclusion, this study shows that untreated patients with active tuberculosis, unlike most M. tuberculosis–infected healthy individuals, generally produce insignificant amounts of IFN-γ in response to HBHA. In contrast, the vast majority of patients with tuberculosis produce anti-HBHA serum antibodies, compared with a much lower proportion of infected healthy subjects. Analysis of the immune response to HBHA appears, therefore, to allow for a good discrimination between infected healthy persons and patients with active tuberculosis. The identification of immunologic differences between these 2 groups of subjects, as identified here, may help to establish surrogate markers for protective immunity against tuberculosis and thus could be of benefit for the development of new vaccines against this dreadful disease.

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