Pulmonary Mononuclear Cell Responses to Antigens of *Mycobacterium tuberculosis* in Healthy Household Contacts of Patients with Active Tuberculosis and Healthy Controls from the Community


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Pulmonary Mononuclear Cell Responses to Antigens of Mycobacterium tuberculosis in Healthy Household Contacts of Patients with Active Tuberculosis and Healthy Controls from the Community


Protective immunity against Mycobacterium tuberculosis requires CD4+ lymphocyte-mediated immune responses and IFN-γ activity. As the primary portal of entry of M. tuberculosis is the lung, pulmonary immune responses against multiple M. tuberculosis Ags were compared between both M. tuberculosis-exposed tuberculin skin test-positive healthy household contacts (HHC) of patients with active sputum smear and culture-positive tuberculosis and tuberculin skin test-positive healthy control individuals from the community (CC). Frequencies of M. tuberculosis Ag-specific IFN-γ-producing cells, IFN-γ concentrations in culture supernatants, and DNA synthesis in bronchoalveolar cells (BAC) and PBMC were studied in HHC (n = 10) and CC (n = 15). Using enzyme-linked immunospot assay we found higher frequencies of IFN-γ-producing cells with specificity to M. tuberculosis-secreted Ag 85 (Ag 85) in BAC from HHC than in BAC from CC (p < 0.022) and relative to autologous PBMC, indicating compartmentalization of Ag 85-specific cells to the lungs. Further, IFN-γ-producing cells with specificity to components A and B of Ag 85 were specifically compartmentalized to the lungs in HHC (p < 0.05). IFN-γ concentrations in culture supernatants of BAC and Ag-specific DNA synthesis were low and comparable in the two subject groups. Increased immune responses to Ag 85 at the site of repeated exposure to M. tuberculosis (the lung) may represent an important component of protective immunity against M. tuberculosis. Correlates of protective immunity against M. tuberculosis are required for assessment of the efficiency of anti-tuberculosis vaccines. The Journal of Immunology, 2000, 165: 1479–1485.

There is abundant clinical and epidemiological evidence that supports the existence of protective immunity against Mycobacterium tuberculosis in humans. Approximately 90–95% of M. tuberculosis-infected humans successfully contain their primary M. tuberculosis infection and never develop tuberculosis. In fact, tuberculosis develops in only 5–10% of M. tuberculosis-infected individuals.

Active immune surveillance is required to maintain the latency of quiescent M. tuberculosis foci, and CD4+ T cells are critical to cell-mediated anti-tuberculosis immunity (1, 2), presumably because they are the primary source of the protective cytokine IFN-γ (3, 4). The importance of IFN-γ responses in mycobacterial infection is underscored by the observation that children with hereditary IFN-γ receptor 1 deficiency are prone to infection with ubiquitous mycobacteria or dissemination of Calmette-Guérin bacillus after vaccination (5–7). Further, IFN-γ was successfully used as adjuvant therapy in patients with refractory nontuberculosis mycobacterial infection (8) and has shown promising results in patients with multidrug-resistant pulmonary tuberculosis (9).

In patients with pulmonary tuberculosis, however, M. tuberculosis Ag-specific IFN-γ-producing cells (10) and spontaneous IFN-γ production (11) are compartmentalized to the site of infection (the lung) despite ongoing disease. Tuberculosis patients represent subjects who are at one end of the spectrum of M. tuberculosis infection, i.e., individuals who have lost immunological control, reactivated their infectious focus, and developed disease. Tuberculosis patients, thus, may not allow the study of protective immunity operative in the majority of M. tuberculosis-infected individuals.

Healthy household contacts of patients with active tuberculosis (HHC) are repeatedly exposed to M. tuberculosis aerogenically. Under situations of intense and prolonged exposure, the proportion of contacts found to be infected with M. tuberculosis at the time of...

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diagnosis of sputum smear-positive tuberculosis index cases is as high as 30–40% (12); however, infection rates can be even higher with extended exposure to untreated patients with tuberculosis (up to 80%) (13). The immunological study of M. tuberculosis-reactive HHC therefore may allow elucidation of protective immunity against M. tuberculosis. Because the lungs is the primary portal of entry for infection with M. tuberculosis in humans, in the present study pulmonary immune responses of tuberculin skin test-positive HHC to M. tuberculosis Ags were compared with autologous systemic (blood) immune responses and with pulmonary and systemic immune responses of tuberculin skin test-positive healthy control individuals from the community (CC). We found that IFN-γ-producing cells with specificity for Ag 85 complex (Ag 85co), which is considered to be a protective Ag of M. tuberculosis, were compartmentalized to the lungs of HHC, and that such cells were significantly more frequent in bronchoalveolar cells (BAC) from HHC than in those from CC.

Materials and Methods

Study subjects

Subjects identified as HHC and CC who were willing to volunteer to undergo bronchoalveolar lavage, venipuncture, and HIV-1 serology were screened for participation in this study between 1996 and 1999 at the National Institute of Respiratory Diseases (Mexico City, Mexico). Approval for the study was provided by Dr. John T. Bolton (Department of Microbiology, Colorado State University, Fort Collins, CO). Ag 85co is a major secreted protein of metabolically active M. tuberculosis, is fibronectin binding (18, 19), and functions as a mycolyltrans-

Preparation of PBMC

Between 40 and 60 ml of whole heparinized venous blood were obtained from each study subject. PBMC were prepared by centrifugation of the blood over Ficoll-Paque gradients (16) and were counted and resuspended in complete medium.

Preparation of BAC

Between 40 and 60 ml of whole heparinized venous blood were obtained from each study subject. PBMC were prepared by centrifugation of the blood over Ficoll-Paque gradients (16) and were counted and resuspended in complete medium.

Preparation of PBMC

PBMC and BAC were added to round-bottom 96-well plates (Nunc, Copenhagen, Denmark) at 5 × 10⁵ cells/well. Cells were stimulated with mycobacterial and nonmycobacterial Ags and the mitogen PHA (Sigma, St. Louis, MO) and SPSS for Windows (version 8.0, SPSS, Chicago, IL) statistical software. Statistical significance was set at P < 0.05.

ELISAs

Ag 85A, Ag 85B, and Ag 85C (17), were provided by Dr. John T. Belisle (Glostrup, Copenhagen, Denmark) at 5 × 10⁴ cells/well. Cells were stimulated with mycobacterial and nonmycobacterial Ags and the mitogen PHA (Sigma, St. Louis, MO). Complete medium was used as a negative control stimulus. Cells were pulsed with 1 μCi/well [³H]thymidine (Amersham, Aylesbury, U.K.; sp. act., 5 Ci/mmol) on day 4, and cells were harvested 24 h later. Incorporation of [³H]thymidine was determined with a scintillation counter and expressed as counts per minute.

ELISPOT assays

Because cytokine levels in cell culture supernatants reflect production, degradation, and consumption by cells, ELISPOT analysis was performed to determine the frequency of cytokine-producing cells and to assess cytokine production on a single-cell basis. ELISPOT analysis is 10- to 200-fold more sensitive than ELISA (24). Frequencies of cytokine-producing cells in PBMC and BAC were determined by ELISPOT assay as previously described (25). Briefly, T spot 96-well assay plates (Whatman, Clifton, NJ) were coated overnight at 4°C with primary Abs against either IFN-γ (Endogen) or IL-10 (ParrMingen, San Diego, CA) at 2–10 μg/ml. Plates were washed extensively and blocked with 1–2% BSA (Sigma). Then, PBMC and BAC were added to the Ab-coated wells at a concentration of 10⁶ cells/well and stimulated with mycobacterial (PPD, manLAM, Ag 85co, Ag 85A, Ag 85B, Ag 85C, ESAT-6, and ST-CF) and nonmycobacterial (Candida) Ags and PHA. Complete medium was used as a negative control stimulus. Cultures were incubated at 37°C, and cells were removed after 24 h (for IFN-γ and IL-10) by washing the wells three times with PBS. Biotinylated secondary Abs to IFN-γ (Endogen, Woburn, MA) and IL-10 (ParrMingen) were then added to the wells, and plates were incubated at 4°C overnight. Peroxidase-conjugated streptavidin (Dako, Glostrup, Denmark; 1/2000) was added, and spots were visualized with 1% 3-aminoo-9-ethylcarbazole (Pierce, Rockford, IL).

The frequencies of IFN-γ and IL-10-producing cells in each well were determined using a computerized series 1 ImmunoSpot Image Analyzer (Celldigm Technology, Guelph, ON). The average number of cytokine spots in duplicate or triplicate wells was then calculated.

Statistical analysis

Comparisons between the HHC and CC groups were made using nonparametric methods: Wilcoxon’s two-sample rank-sum test for comparisons of distributions or Fisher’s exact test when comparing proportions. In comparisons within groups examining paired samples of lung vs blood, a signed-rank test was used. All statistical tests were conducted using SigmaStat (version 2.0, Jandel, Chicago, IL) and SPSS for Windows (version 8.0, SPSS, Chicago, IL) statistical software. Statistical significance was set at P ≤ 0.05.
Characteristics of study groups and cells

Ten HIV-1-seronegative HHC (7 men and 3 women) and 15 HIV-1-seronegative CC (11 men and 4 women) underwent BAL and venipuncture to obtain BAC and PBMC. The median age of the HHC group (23.5 years) was not different from that of the CC group (24 years). An equal number (n=4) of participants in each group were smokers. HHC were family members (spouse (n=2), son (n=2), daughter (n=4), sister (n=1), life partner (n=1)) who lived in the same house (or the same room) with the index patient for a duration of at least 3 mo before the BAL.

The following median parameters were comparable between HHC and CC: body weight (62 and 67 kg), serum albumin (4.6 and 4.6 g/dl), peripheral white blood cell count (6.1 and 6.5 × 10^3/μl), and hemoglobin (16.3 and 15.9 g/dl), respectively. Median proportions of neutrophils were slightly higher in HHC than CC (63.8 and 56.1%, respectively; p = 0.045). In four of six HHC and seven of seven CC examined, scars indicating preceding vaccination with Calmette-Guérin bacillus were found. Tuberculin skin test indurations to 5 TU of PPD were comparable (20 and 18 mm in HHC and CC, respectively).

Retrieved proportions of BAL fluid, yield of BAC, and cellular profiles of BAC, including proportions of peroxidase-positive cells

FIGURE 1. DNA synthesis of PBMC and BAC from HHC and CC to nonmycobacterial and mycobacterial Ags and mitogen. PBMC (A and C) and BAC (B and D) from tuberculin skin test-positive healthy household contacts (HHC; n = 10; A and B) of patients with active tuberculosis and from healthy tuberculin skin test-positive community controls (CC; n = 13; C and D) were stimulated with Candida, PPD, Ag 85 complex from M. tuberculosis H37Rv culture filtrate (Ag 85co), ESAT-6, ST-CF, and manLAM. Control wells received culture medium (Medium) alone. PHA was used to test maximal induction of DNA synthesis. ESAT-6 and ST-CF were tested in PBMC and BAC from six individuals from each of the HHC and CC groups only. Incorporation of[^H]thymidine was determined after 5 days of culture. Standard box plots with median (25th and 75th percentiles) and whiskers at 10th and 90th percentiles are shown. ●, Minimum and maximum values.
(median, 2 and 1.5%) were comparable in HHC and CC, respectively. Proportions of alveolar neutrophils were ≤1% in BAC of both HHC and CC. Median proportions of alveolar lymphocytes were 14.1 and 14.0% and of alveolar macrophages were 85.8 and 83.9%, in HHC and CC, respectively, similar to those in CC in previous studies (10, 26).

**DNA synthesis of BAC and PBMC in response to M. tuberculosis Ags**

DNA synthesis (incorporation of \[^{3}H\]thymidine) was assessed in PBMC (Fig. 1, A and C) and BAC (Fig. 1, B and D) from HHC (A and B) and CC (C and D) to optimal concentrations of *Candida* (10 µg/ml), PPD (10 µg/ml), Ag 85co (5 µg/ml), ESAT-6 (2 µg/ml), ST-CF (1 µg/ml), manLAM (10 µg/ml), and PHA (1 µg/ml). As expected from the cell composition of BAC in HHC and CC, DNA synthesis in response to all stimuli was low in the two groups. DNA synthesis in response to PPD, Ag 85co, ST-CF, *Candida*, and PHA was significantly lower (\(p < 0.05\)) in BAC than in PBMC from both HHC and CC. Interestingly, DNA synthesis in PBMC from HHC in response to PPD, Ag 85co, and PHA was significantly lower than that in CC (\(p < 0.05\)).

**Frequencies of *M. tuberculosis* Ag-specific PBMC and BAC**

The concentrations of Ags used for optimal induction of IFN-\(\gamma\)-producing cells was significantly higher in BAC from HHC (\(n = 10\)) than in BAC from CC (\(n = 13\); \(p = 0.022\)). The frequencies of PPD-specific IFN-\(\gamma\)-producing cells were not statistically different in BAC of HHC compared with those of CC. Further, the frequencies of PPD- and Ag 85co-specific IFN-\(\gamma\)-producing cells were lower in BAC than in autologous PBMC from six and seven HHC, respectively. In contrast, in CC, the frequencies of PPD- and Ag 85co-specific IFN-\(\gamma\)-producing cells were higher in BAC than in autologous PBMC from HHC and CC, respectively. In only a limited number of CC (\(n = 2\)) were the frequencies of PPD- and Ag 85co-specific IFN-\(\gamma\)-producing BAC higher than those in PBMC, indicating possible exposure to *M. tuberculosis* within the community.

To assess whether increased frequencies of IFN-\(\gamma\)-producing Ag-specific cells were due to differences in absolute numbers of lymphocytes within BAC or PBMC of HHC and CC, frequencies of IFN-\(\gamma\)-producing cells were normalized to 10,000 lymphocytes in BAC and PBMC, respectively. Median numbers of Ag 85-specific IFN-\(\gamma\)-producing cells per 10,000 lymphocytes in BAC and PBMC were 29 (25th percentile, 17; 75th percentile, 90) and 7 (25th percentile, 2; 75th percentile, 11), and 13 (25th percentile, 5; 75th percentile, 34) and 6 (25th percentile, 2; 75th percentile, 14) in HHC and CC, respectively. Median numbers of PPD-specific IFN-\(\gamma\)-producing cells per 10,000 lymphocytes in BAC and PBMC were 56 (25th percentile, 39; 75th percentile, 148) and 17 (25th percentile, 9; 75th percentile, 23) and 34 (25th percentile, 23; 75th percentile, 92) and 8 (25th percentile, 7; 75th percentile, 27) in HHC and CC, respectively. Differences in frequencies of Ag 85-specific IFN-\(\gamma\)-producing cells per 10,000 lymphocytes in BAC and PBMC were significant in HHC only (\(p = 0.022\)).
To estimate the output of IFN-γ from IFN-γ-producing cells in PBMC and BAC from HHC and CC, sizes of spots were measured with the image analyzer. Proportions of IFN-γ spots from Ag 85co-stimulated PBMC and BAC of HHC (n = 8) and CC (n = 8) within each size category (10⁻¹⁻¹⁻³ mm²) were comparable between BAC and PBMC from HHC and CC, indicating comparable outputs of IFN-γ per cell (Fig. 3).

ST-CF-specific IFN-γ-producing PBMC and BAC were studied in subgroups of HHC (n = 6) and CC (n = 6). Median frequencies of ST-CF-specific IFN-γ-producing cells per 10⁵ BAC and PBMC in HHC and CC paralleled those of PPD responses and were 2-fold higher in BAC than in PBMC in four of six HHC and in zero of six CC (HHC BAC, 147 (25th percentile, 16; 75th percentile, 206); HHC PBMC, 51 (25th percentile, 34; 75th percentile, 101); CC BAC, 38 (25th percentile, 24; 75th percentile, 68); CC PBMC, 85 (25th percentile, 43; 75th percentile, 139; not significant). Median frequencies of ESAT-6-, manLAM-, and Candida-specific IFN-γ-producing cells were low (<30/10⁵ PBMC or BAC) in both HHC and CC. On the other hand, median frequencies of PHA-induced IFN-γ spots were high (580–810/10⁵ PBMC or BAC) and comparable in both HHC and CC (data not shown).

In a subset of study subjects (HHC, n = 6; CC, n = 6) frequencies of IFN-γ-producing cells were also assessed in response to the three components of Ag 85co, namely, Ag 85A (5 μg/ml), Ag 85B (5 μg/ml), and Ag 85C (5 μg/ml; Table I). The ratios of the frequencies of Ag 85A- and Ag 85B-specific, but not of Ag 85C-specific, IFN-γ-producing BAC and PBMC (BAC/PBMC) were significantly higher in HHC than in CC (p = 0.01).

The frequencies of PPD- and Ag 85co-specific IL-10-producing PBMC and BAC in HHC and CC, respectively, were comparable between cell populations and subject groups (data not shown).

**IFN-γ and TNF-α concentrations by ELISA**

In a subgroup of study subjects (HHC, n = 6; CC, n = 8), 24-h culture supernatants from in vitro Ag 85co- and PPD-stimulated PBMC and BAC were available for analysis of IFN-γ and TNF-α concentrations by ELISA. IFN-γ production in response to Ag 85co was significantly lower in BAC from HHC than in BAC from CC (p = 0.032). Thus, high frequencies of Ag 85co-specific IFN-γ-producing cells in BAC from HHC were not accompanied by a corresponding higher concentration of IFN-γ in cell culture supernatants.

Ag 85co-induced TNF-α production was significantly higher in BAC compared with PBMC in both HHC and CC. In HHC, the median TNF-α level in BAC was 6871 pg/ml (25th percentile, 4669; 75th percentile, 7513), and in PBMC it was 2457 pg/ml (25th percentile, 1537; 75th percentile, 3282; p = 0.043). In CC the TNF-α level in BAC was 3187 pg/ml (25th percentile, 2906; 75th percentile, 4113), and in PBMC it was 1688 pg/ml (25th percentile, 1377; 75th percentile, 1853; p < 0.043). TNF-α concentrations were higher (1.8-fold) in Ag 85co-stimulated BAC from HHC than in those from CC (not significant).

**Discussion**

This study is the first to compare the pulmonary immune responses of healthy individuals from households of patients with active smear- and culture-positive tuberculosis, and therefore with a high likelihood of exposure to *M. tuberculosis*, with those from healthy *M. tuberculosis*-sensitized individuals in a tuberculosis-endemic community. In HHC, Ag 85co-specific IFN-γ-producing BAC were compartmentalized to the alveolar spaces. Further, frequencies of Ag 85co-specific, IFN-γ-producing cells in BAC from HHC were higher than those in BAC from CC. Also, frequencies of Ag 85A- and Ag 85B-specific, but not of Ag 85C-specific, IFN-γ-producing cells were significantly higher in BAC from HHC than in those from CC. Considering that the proportions of lymphocytes in alveolar spaces of healthy subjects (i.e., both HHC and CC) are 4- to 5-fold lower than that in peripheral blood, the expansion of Ag 85co-specific IFN-γ-producing cells in BAC from HHC to proportions higher than those in PBMC is notable. These findings are consistent with aerogenic *M. tuberculosis* infection exposure in HHC of patients with tuberculosis. Thus, it appears that during the early phases of *M. tuberculosis* infection, Ag 85co-specific pulmonary mononuclear cells are expanded.

Ag 85co is a predominant product of live *M. tuberculosis* (19, 27) and may be critical in the development of protective immunity in *M. tuberculosis* infection (28). Recently, it has been shown that vaccination with Ag 85co conferred protection against *M. tuberculosis* challenge in mice (29–31) and guinea pigs (32, 33). In studies of human immune responses to Ag 85, PBMC from healthy tuberculosis skin test-positive subjects demonstrated strong DNA synthesis and IFN-γ secretion in response to both Ag 85co and Ag 85A (34). Further, IFN-γ production of Ag 85-stimulated PBMC from HHC was significantly higher than that of PBMC from patients with tuberculosis (35). In fact, DNA synthesis of PBMC in response to Ag 85co was low in 52% (33, 35) to 100% of patients with active tuberculosis (36) relative to that in healthy control individuals. Further, IFN-γ production in response to Ag 85A was

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**Table 1. Ratios of frequencies of IFN-γ-producing BAC and PBMC in response to the components A, B and C of Ag 85**

<table>
<thead>
<tr>
<th>IFN-γ Spots in BAC/Frequencies of IFN-γ Spots in PBMC</th>
<th>HHCC</th>
<th>CC</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag 85A</td>
<td>8 [4, 15]</td>
<td>1 [1, 1]</td>
<td>0.01</td>
</tr>
<tr>
<td>Ag 85B</td>
<td>9 [5, 47]</td>
<td>1 [1, 1]</td>
<td>0.01</td>
</tr>
<tr>
<td>Ag 85C</td>
<td>3 [1, 95]</td>
<td>1 [1, 2]</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Frequencies of Ag 85A-, Ag 85B-, and Ag 85C-specific IFN-γ spots were measured per 10⁵ of BAC and PBMC, respectively, in healthy household contacts (HHC, n = 6) and healthy tuberculin skin test positive community controls (CC, n = 6). Presented are medians and 25th and 75th percentiles (in brackets) of the ratios of frequencies of Ag 85A-, Ag 85B-, and Ag 85C-specific IFN-γ spots in BAC over that in PBMC. NS, not significant between the study groups.
low in patients with pulmonary tuberculosis (34, 37). Overall, it appears that T cell responses to Ag 85co may be indicative of host protective immunity against *M. tuberculosis* in humans. Our finding of increased frequencies of Ag 85co-specific IFN-γ-producing BAC in tuberculin skin test-positive HHC may indicate the expansion of local protective immunity in these subjects who have a high probability of having been infected with *M. tuberculosis*.

The observed differences between frequencies of Ag 85-specific IFN-γ-producing BAC in HHC and CC are unlikely to be due to differences in the composition of blood and lungs cells, as no differences in number or percentage of lymphocytes and mononuclear phagocytes were found by histochemical analysis of BAC and PBMC from HHC and CC. In particular, proportions of lymphocytes were comparable between the study groups. Additionally, frequencies of IFN-γ-producing cells that were specific for *M. tuberculosis* Ags other than Ag 85co (PPD, ST-CF, manLAM, ESAT-6), were comparable in BAC from both study groups. Thus, the increased proportions of Ag 85co-specific IFN-γ-producing BAC in HHC probably resulted from expansion of Ag 85co-specific BAC that occurred in response to recent aerogenic *M. tuberculosis* infection/exposure in the households of tuberculosis patients. This is consistent with the finding in HHC that when frequencies of Ag-specific IFN-γ-producing cells were normalized to 10,000 lymphocytes in both PBMC and BAC, numbers of PPDb- and Ag 85-specific IFN-γ-producing cells were significantly higher in lymphocytes in BAC than in lymphocytes in PBMC. However, because recent data indicate that *M. tuberculosis*-infected AM might be sources of IFN-γ (38), normalization of IFN-γ-producing cells to numbers of lymphocytes in BAC may not reflect the true range of IFN-γ-producing cell populations in BAC. It was beyond the scope of this study to unravel the cellular source of IFN-γ and to assess the influence of exposure to *M. tuberculosis* on the cell populations involved in production of IFN-γ. Elucidation of the cellular source of IFN-γ in HHC and CC is the subject of ongoing research.

Compartmentalization of Ag 85co-specific IFN-γ-producing BAC in lungs from HHC was detectable only by a highly sensitive assay (ELISPOT) and not by DNA synthesis or IFN-γ immunoreactivity in supernatants from cell cultures.

The implications of increased frequencies of *M. tuberculosis*-specific IFN-γ-producing cells in the lungs of HHC are not presently clear, as IFN-γ-producing *M. tuberculosis* Ag-specific BAC are also detectable in high frequencies in BAC from radiographically affected areas of lungs from tuberculosis patients (10).

Time points of conversion of tuberculin skin tests from negative to positive could not be assessed in HHC due to the cross-sectional nature of this study. Definitive diagnosis of new infection or re-infection with *M. tuberculosis* in HHC as a result of contact with the tuberculosis patients, therefore, was not possible. Several observations, however, might be indicative of recent exposure to or infection with *M. tuberculosis* in HHC in this study. First, slightly increased proportions of circulating neutrophils in the peripheral blood might indicate a systemic and recent inflammatory response. Further, TNF-α production by BAC from HHC in response to Ag 85co was increased (although the difference from CC did not reach statistical significance) and might indicate local inflammatory immune responses. Lastly, lower levels of DNA synthesis in PBMC of HHC compared with CC in response to PPD, Ag 85co, and PHA might suggest the activation of immunosuppressive circuits due to recent active *M. tuberculosis* infection, as also seen with other infections (39).

The frequencies of IFN-γ-producing ST-CF-specific cells were higher in BAC than in PBMC in the majority of HHC, but were not significantly different from those in BAC of CC. These differences may, however, reach significance if larger populations are studied. ST-CF is a crude mixture of secreted Ags and contains Ag 85co and a multitude of other Ags. Also, since the concentration of purified Ag 85co used in the current study (5 μg/ml) exceeded that in crude ST-CF (at most 1 μg/ml), differences in the detectable frequencies of IFN-γ-producing Ag 85co- and ST-CF-specific cells may have resulted from suboptimal stimulation of cells by the latter Ag mixture. Alternatively, ST-CF may contain components with suppressive activities for expression of IFN-γ. Demissie et al. have recently shown that ST-CF induces greater DNA synthesis and higher IFN-γ production in PBMC from HHC compared with those from patients with tuberculosis (40). Interestingly, levels of IFN-γ production were significantly higher in ST-CF-stimulated PBMC from HHC of patients with advanced tuberculosis compared with those from HHC of patients with minimal disease, suggesting an association with more intense exposure to *M. tuberculosis*.

We found that the frequencies of IFN-γ-producing BAC with specificity for the purified components of Ag 85co, namely, Ag 85A and Ag 85B, but not Ag 85C, were significantly higher in BAC of HHC than in BAC of CC, resembling the findings with the purified Ag 85co. The relative immunogenicity of the components of Ag 85co (A, B, and C) are not known to differ despite the predominance of Ag 85B in purified Ag 85co. Also, whether there are differences among Ag 85A, -B, and -C with regard to biological activity and/or induction of protective immunity is not known.

In conclusion, recent aerogenic exposure/infection with *M. tuberculosis* is associated with the accumulation of Ag 85co-specific IFN-γ-producing cells in the alveolar spaces of HHC. The finding of compartmentalization of Ag 85co-specific IFN-γ-producing cells to the alveolar spaces of HHC may reflect the activation of early protective immune responses in situ. The understanding of human protective immune responses to *M. tuberculosis* at sites of infection may facilitate the identification of correlates of protective immunity, which are urgently needed in the evaluation of the efficacy of new anti-tuberculosis vaccines.

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