Long-Lived Immune Response to Early Secretory Antigenic Target 6 in Individuals Who Had Recovered from Tuberculosis

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We sought to understand the persistence and relevance of the long-lived immune response to early secretory antigenic target (ESAT-6) of Mycobacterium tuberculosis in humans. ESAT-6 is recognized by memory cells involved in protection of animals against tuberculosis (TB). Recent reports also showed that ESAT-6 response can be recovered in patients with TB and in those soon after anti-TB therapy. We chose 18 individuals who had recovered from pulmonary TB (some in remission for >5 years), and 14 bacille Calmette-Guérin–vaccinated healthy individuals for this study. The results showed that peripheral blood mononuclear cells of 10 (55.6%) of 18 patients with TB remission responded to ESAT-6 with stimulation indices ≥3.0, whereas none of the healthy controls responded. Functional analysis showed that 13 (72.2%) of 18 patients with TB remission produced significant amounts of IFN-γ in response to ESAT-6, whereas only 1 (7.1%) of the 14 healthy control subjects did so. It appears that responses to ESAT-6 can persist in individuals who had recovered from pulmonary TB.

Proteins secreted by Mycobacterium tuberculosis induce strong immune responses in patients with tuberculosis (TB). The 6-kDa early secreted antigenic target (ESAT-6) antigen from M. tuberculosis is a dominant target for cell-mediated immunity in mice [1], guinea pigs [2], and cattle [3], as well as humans [4–6]. Experimentally infected cattle and field cases in the early stages of infection are characterized by strong IFN-γ responses directed toward ESAT-6 [3]. ESAT-6 is also one of the antigens most frequently recognized by peripheral blood mononuclear cells (PBMC) from patients with TB or from T cell lines induced against complex or single antigens of M. tuberculosis [6]. T cells from a high percentage of patients with TB proliferate and release IFN-γ into culture when challenged with ESAT-6 [4–6], and T cell responses to ESAT-6 increase after anti-TB chemotherapy [6, 7].

In studies elsewhere reporting human responses to ESAT-6, most of the individuals recruited were those with active disease [4, 5]. Therefore, responses to ESAT-6 in these patients mostly represent antigen recognition in individuals with recognized active disease. Studies showed that recent converters, as well as patients with TB, have positive T cell responses to ESAT-6 [7]. Moreover, ESAT-6–specific T cells could be maintained in an asymptomatic contact for >2 years. Thus, ESAT-6–specific CD8 T cells were implicated in long-term control of TB [8]. Protective immunity in the mouse model was found to be mediated by highly reactive
memory of CD4+ and CD8+ T cells triggered after reinfec- tion to produce large amounts of T helper type 1 (Th1) cytokines [1, 9], and ESAT-6 was one of the major antigenic targets for this response [1]. These results indicate that ESAT-6 epitopes have the potential to be candidates for inclusion in subunit vaccines. Thus, evaluation of the longevity of the long-lived immune response to ESAT-6 in humans becomes an important issue.

In this study, we recruited 18 otherwise healthy individuals who were originally diagnosed as having pulmonary TB (patients with TB remission). Fourteen healthy BCG-vaccinated donors, all clinically healthy and confirmed to be free of TB, were recruited as control subjects. Proliferative response and IFN-γ production were used as readouts to evaluate long-lived immune responses in these donors. We found that immune responses to PPD were common both in healthy donors and in patients with TB remission, but that responses to ESAT were almost entirely restricted to the patients with TB remission. It is interesting that the responses to ESAT-6 were still strong in at least 1 TB remission patient for as long as 17 years after treatment. These data indicate that long-lived T cell responses to ESAT-6 can persist in humans.

PATIENTS AND METHODS

Human subjects. Fourteen healthy donors were recruited for this study. They were all vaccinated with BCG as children and were confirmed to be free of TB by radiologic and clinical examinations. Eighteen individuals were recruited for this study from the Chronic Disease Control Bureau and Taipei Municipal Chronic Disease Hospital, on the basis of the inclusion criteria: (1) previously diagnosed to have pulmonary TB by either a positive acid-fast bacilli smear or positive sputum culture [10] and (2) having completed a full course of anti-TB chemotherapy at least 6 months before the time of blood sample collection for this study. All 18 individuals selected were healthy at the time of the study, seronegative for HIV and hepatitis B virus surface antigen, and without history of steroid use, immunoglobulin treatment, chemotherapy, or radiation therapy. The Mantoux test was not performed because of ethical concerns. All individuals, including healthy control subjects and patients with TB remission, were ethnic Chinese.

Proliferation assay. Blood was collected in heparinized tubes. After plasma was removed, PBMC were immediately separated on a Ficoll-Paque plus density gradient (Amersham Pharmacia Biotech) and washed with Hanks’ balanced salt solution. PBMC were adjusted to a final concentration of 2.5 × 10^6 cells/mL in complete RPMI 1640 medium (Biological Industries) containing 2% human AB serum (Atlanta Biologicals), L-glutamine (4 mM, GIBCO Life Technologies), HEPES buffer (0.5 mM, Atlanta Biologicals), and penicillin (200 U/mL), streptomycin (0.2 mg/mL), and amphotericin B (0.5 µg/L; Biological Industries). Cells were cultured (2.5 × 10^3 per well) in medium alone, in the presence of recombinant ESAT-6 (rESAT-6; 5 µg/mL [4]), or in the presence of PPD (1 µg/mL, Statens Serum Institute) in triplicate in round-bottomed 96-well plates at 37°C for 5 days. At 18 h before harvesting, cells were pulsed with 5 µCi/mL of ^3H-labeled thymidine. Cells were harvested onto glass fiber filters for scintillation counting in a Filtermate 196 harvester (Packard Instrument). ^3H-labeled thymidine uptake was measured. Results were expressed as mean counts per minute (cpm) ± SD. A stimulation index (SI = cpm of stimulated cells/cpm of cells in medium alone) of >3.0 was considered positive. A paired t test was used to compare the proliferative responses of PBMC from subjects within the same group to stimulus and to medium-only control.

IFN-γ production. Cultures were set up as for the proliferation assay described above. After 5 days of incubation, culture supernatants were collected, centrifuged, and frozen at −80°C until assays were performed. The concentration of IFN-γ was determined by the OptEIA human IFN-γ ELISA Set (Pharmingen) according to the manufacturer’s instructions. IFN-γ production above the mean concentration in unstimulated wells plus 4 SD (45 pg/mL) was considered “positive.” A Mann-Whitney U test was used to compare IFN-γ production by PBMC in response to the same stimulus by subjects in different groups. A U test result of P < .05 was considered significant.

RESULTS

PBMC from individuals who had recovered from clinical TB recognize ESAT-6. To study immune responses to ESAT-6 in individuals after recovery from clinical disease, we selected 18 subjects as described in “Patients and Methods.” Freshly isolated PBMC from either healthy donors or patients with TB remission were cultured in the presence of PPD or ESAT-6 for 5 days. Figure 1 shows that T cells from healthy donors or patients with TB remission were cultured in the presence of PPD or ESAT-6 for 5 days. Figure 1 shows that T cells from healthy donors proliferated strongly to PPD compared with culture medium alone (P = .0065, paired t test). In contrast, cells from healthy donors did not react to ESAT-6. On the basis of an SI >3 as a cutoff, 11 (78.6%) of 14 healthy donors reacted to PPD, and none of them reacted to ESAT-6. Figure 1 also shows strong proliferative responses of T cells from patients with TB remission to both ESAT-6 (P = .0053, paired t test) and PPD (P = .0001, paired t test), as compared with medium-only control subjects. Ten (55.6%) of 18 patients with TB remission had significant proliferative response (by using SI >3 as the cutoff) to ESAT-6 (table 1), and 17 (94.4%) of 18 of them responded to PPD. There was no difference in the magnitude of response to PPD between patients with TB remission and control subjects (figure 1). These data indicate that ESAT-6 elicits specific immune
In this study, we have shown that ESAT-6–responsive cells are found in PBMC of humans who recovered from TB but not in healthy BCG-vaccinated donors. Ten (55.6%) of 18 patients with TB remission demonstrated positive proliferative responses to in vitro stimulation with rESAT-6. A Th1-type response to ESAT-6, as indicated by production of IFN-γ, was demonstrable in 13 (72.2%) of 18 of these individuals. The time between that after treatment and the experiment ranged from 0.6 to 17.3 years. These data therefore show that T cell responses and potentially contains epitopes recognized by T cells in individuals who had recovered from pulmonary TB.  

**IFN-γ production by ESAT-6–responding cells.** To investigate whether T cells reacting to ESAT-6 are functionally active, we collected PBMC culture supernatants at 5 days after ESAT-6 stimulation and analyzed production of IFN-γ. ESAT-6 production by PBMC from patients with TB remission stimulated with ESAT-6 was significantly higher than that by PBMC from healthy donors (P = .0006, Mann-Whitney U test; figure 2). Whereas PBMC from 13 (72.2%) of 18 individuals who had recovered from TB produced increased levels of IFN-γ (≥45 pg/mL on the basis of the mean concentration in unstimulated cells plus 4 SD) in response to ESAT-6, PBMC from only 1 (7.1%) of 14 healthy donors stimulated with ESAT-6 produced ≥45 pg/mL of IFN-γ (figure 2). Whereas 17 (94.4%) of 18 patients with TB remission produced ≥45 pg/mL IFN-γ in response to PPD, 11 (78.6%) of 14 healthy donors met the cutoff. It is interesting that not all cultures of patients with TB remission that produced significant amounts of IFN-γ proliferated in response to ESAT-6. PBMC from 3 patients with TB remission (subjects 3, 12, and 16) produced increased levels of IFN-γ despite a low proliferative response (table 1). PBMC from 10 other patients with TB remission responded to ESAT-6 both by proliferation and production of IFN-γ. However, there was only a weak correlation (correlation coefficient, r = 0.27) between the magnitude of the proliferative response and the amounts of IFN-γ produced. The correlation between the 2 responses to PPD was stronger in patients with TB remission (r = 0.83) than in healthy donors (r = 0.56).

**Relationship between response to ESAT-6 and patient treatment history.** To determine the longevity of the ESAT-6–specific response, the length of time after recovery from clinical illness and its correlation with the readout was assessed. Table 1 shows that the length of time between the completion of treatment and blood collection for those patients with TB remission who responded to ESAT-6 ranged from 0.7 to 17.3 years, overlapping with that for those who did not respond to ESAT-6 (1.9–20 years). These data indicate no correlation between the length of time after recovery and response to ESAT-6. In addition, there is no obvious correlation (r = 0.009) between the magnitude of response and the length of time elapsed after clinical illness, nor is there an obvious correlation between response to ESAT-6 and age and sex of the patient, or duration of treatment. There was also no good correlation (r = 0.466) between the length of time after recovery from clinical illness did not influence the responsiveness of patients with TB remission to ESAT-6, these data demonstrate that ESAT-6 responses were observed in the majority (13 of 18) of patients who were in remission.

**DISCUSSION**

In this study, we have shown that ESAT-6–responsive cells are found in PBMC of humans who recovered from TB but not in healthy BCG-vaccinated donors. Ten (55.6%) of 18 patients with TB remission demonstrated positive proliferative responses to in vitro stimulation with rESAT-6. A Th1-type response to ESAT-6, as indicated by production of IFN-γ, was demonstrable in 13 (72.2%) of 18 of these individuals. The time between that after treatment and the experiment ranged from 0.6 to 17.3 years. These data therefore show that T cell
responses to ESAT-6 can be maintained in patients with TB remission in an endemic area for extended periods of time. In this context, it is therefore interesting to note that some patients with TB remission maintained strong responses to ESAT-6, whereas others, including some with much more recent infections, remained negative. The cause for this discrepancy remains to be determined. It is unlikely to be explained by genetic polymorphism in the population because almost all human leukocyte antigen types can strongly recognize multiple epitopes on ESAT-6, and the subjects of this study were all ethnic Chinese [4, 6, 7]. However, there are 3 likely explanations. It may reflect the donor’s bacteriologic status (latent infection vs. sterilizing immunity) after treatment, because it is reported that many individuals remain latently infected after chemotherapy and can later reactivate their disease [11]. It is also a possibility that the ESAT-6–specific response is maintained in some individuals by exogenous reinfection, which is now known to occur at high rates in endemic regions [12]. This would suggest that such responses are potentially protective, because it has been shown that ESAT-6–specific CD8 T cells were maintained in an asymptomatic contact for >2 years [8]. Alternatively, it is possible that these responses reflect the survival, in some donors, of a population of memory T cells derived from the original infection. To better understand the correlation between responsiveness to *M. tuberculosis*–specific antigens and protection and the progression from latent infection to active disease, large-scale longitudinal human studies are planned, and some studies are currently under way. The studies include close monitoring of ESAT-6–responsive healthy contacts, comparing ESAT-6 reactivity with natural skin test conversion, and clinical intervention studies that are based on reactivity to ESAT-6.

The specificity and sensitivity of ESAT-6, as demonstrated in our study as well as others, show that it has the potential to be a diagnostic reagent. In fact, ESAT-6–based immuno-diagnostics meet the criteria outlined by the World Health Organization as being an “ideal diagnostic” [13]. As we have also shown, however, ESAT-6 reactivity can be maintained for extended periods of time in individuals with TB remission. The question of whether this reflects the maintenance of memory cells or indicates a latent infection is addressed in the studies outlined above. The outcome of these studies will have a great effect, not just on our understanding of the correlation between ESAT-6 reactivity and disease state, but also on the usefulness of ESAT-6 as an immunodiagnostic.

**Table 1. The relationship between response to early secretory antigenic target (ESAT-6) and patient history.**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Age, years</th>
<th>Stimulation index</th>
<th>IFN-γ production, pg/mL</th>
<th>Duration of treatment, years</th>
<th>Time after treatment, years</th>
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<tr>
<td>1</td>
<td>F</td>
<td>26</td>
<td>3.5</td>
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<td>2</td>
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<td>3</td>
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<tr>
<td>4</td>
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</tr>
<tr>
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</tr>
<tr>
<td>6</td>
<td>M</td>
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</tr>
<tr>
<td>7</td>
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<td>8</td>
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<td>10</td>
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<tr>
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<td>110</td>
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<tr>
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<td>40</td>
<td>27.9</td>
<td>462</td>
<td>0.5</td>
<td>14.0</td>
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</table>

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*a Each TB remission patient is represented by a number. Patients that responded to ESAT-6 in both proliferative response and IFN-γ production are underlined. Those who produced IFN-γ but did not mount a proliferative response are shown in boldface.

*b Stimulation index = mean counts per minute (cpm) in rESAT6 stimulated cultures/mean cpm in cultures with medium only.

*c The detection limit of the OptEIA human IFN-γ kit was 0.4 pg/mL.
Independent studies conducted in Germany and Kuwait showed that 15 (88.2%) of 17 and 18 (51.4%) of 35, respectively, of patients with active TB had strong proliferative responses to ESAT-6 [5, 6]. However, the percentage of patients that can produce IFN-γ is lower and seems to correlate negatively with disease severity. In low-endemic regions, where patients recruited generally had minimal disease, the percentages of patients that produced IFN-γ in response to ESAT-6 stimulation were high (56% in Denmark and 59% in the United States) [4, 6]. In high-endemic regions where most patients recruited had severe clinical disease, the percentages were low (35% in Ethiopia and 46% in Kuwait) [4, 6, 14].

Taiwan is an area of relatively high incidence of TB; the incidence of TB is 71.12 per 100,000 people [15]. Most individuals recruited for our study were at stages II–III by radiology at the time of active infection. PBMC of some individuals (subjects 3, 12, and 16; table 1) did not mount a proliferative response to ESAT-6, yet they still maintained an ESAT-6–responsive, IFN-γ–producing T cell population. The cause for the discrepancy between proliferation and IFN-γ production remains speculative. These results may simply reflect the difference of sensitivity of the different assays. It has recently been reported that enzyme-linked immunospot assay detecting production of IFN-γ is more sensitive than proliferative responses to ESAT-6 [7]. Alternatively, it may reflect differences in epitope recognition by T cell subsets primed for proliferation or IFN-γ production [4].

In mouse models of memory immunity to TB, protection has been shown to be mediated by both CD4+ and CD8+ T cells [9, 16]. Upon receiving a secondary infection, T cells are recruited rapidly to the site of infection and produce IFN-γ to exert their effector function [1]. These results indicate that memory T cell populations recognizing ESAT-6 can be maintained for extended periods of time in previously vaccinated or infected animals. Therefore, it is possible that the ESAT-6–responsive cells in humans can also rapidly become memory effector cells upon reexposure to M. tuberculosis. Alternatively, they may represent a population of effector T cells involved in the active control of latent infection or exogenous reinfec tion [8]. Determining the nature of these ESAT-6–responsive T cells will have important implications for the potential of ESAT-6 as a vaccine candidate. It will also affect the treatment of reactivation disease in TB, given that there is currently no marker for latent infection nor any known indicator that may be used to predict reactivation. Our study showing that immune responses to ESAT-6 are specific and long lived in the majority of individuals who had recovered from TB took us a step closer to an understanding of the overall picture of TB.

Acknowledgment

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References