Cellular Immune Responses to ESAT-6 Discriminate between Patients with Pulmonary Disease Due to \textit{Mycobacterium avium} Complex and Those with Pulmonary Disease Due to \textit{Mycobacterium tuberculosis}

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ESAT-6 (for 6-kDa early secreted antigenic target) is a secreted antigen found almost exclusively in organisms of the \textit{Mycobacterium tuberculosis} complex. We compared in vitro gamma interferon (IFN-\(\gamma\)) responses by peripheral blood mononuclear cells to this antigen in patients with pulmonary disease due to either \textit{Mycobacterium avium} complex (MAC) or \textit{Mycobacterium tuberculosis} with those in healthy, skin test-negative, control subjects. Significant IFN-\(\gamma\) responses to ESAT-6 were detected in 16 (59%) of 27 \textit{M. tuberculosis} pulmonary disease patients, 0 (0%) of 8 MAC disease patients, and 0 (0%) of 8 controls. Significant IFN-\(\gamma\) responses to \textit{M. tuberculosis} purified protein derivative were detected in 23 (85%) of 27 \textit{M. tuberculosis} disease patients, 2 (25%) of 8 MAC disease patients, and 5 (63%) of 8 healthy controls. \textit{M. avium} sensitin was recognized in 24 (89%) of 27 \textit{M. tuberculosis} disease patients, 4 (50%) of 8 MAC disease patients, and 1 (13%) of 8 controls. IFN-\(\gamma\)-responses to ESAT-6 are specific for disease due to \textit{M. tuberculosis} and are not observed in patients with MAC disease or in healthy controls.

The standard method for detecting prior infection with \textit{Mycobacterium tuberculosis} is skin testing with purified protein derivative (PPD). PPD skin testing is associated with multiple problems, including variability in intradermal administration and measurement of responses, the need for a follow-up visit to complete testing, and false-positive results in those individuals with prior \textit{Mycobacterium bovis} BCG immunization or prior infection with nontuberculous mycobacteria (NTM). In countries such as the United States, where BCG immunization is not routine, prior infection with NTM, especially organisms of the \textit{Mycobacterium avium} complex (MAC), is the principal reason for false-positive PPD reactions (5). Dual skin testing with PPD and \textit{M. avium} sensitin (MAS) has been shown to distinguish disease due to MAC from disease due to \textit{M. tuberculosis} (17), but this type of testing requires two patient visits, two intradermal injections, and experience reading skin tests.

In vitro methods for the specific diagnosis of \textit{M. tuberculosis} infection would avoid some of the practical difficulties associated with intradermal skin testing. In vitro methods based on antibody detection have had either limited sensitivity or limited specificity (16). Newer in vitro techniques for the assay of cellular immune responses to specific mycobacterial antigens hold considerable promise for more accurate diagnosis of infection with \textit{M. tuberculosis} (10). ESAT-6 (for 6-kDa early secreted antigenic target) is a secreted antigen of \textit{M. tuberculosis} that is predominantly expressed in mycobacteria belonging to the \textit{M. tuberculosis} complex (7, 11, 13) and provokes a strong T-cell response in mouse models of memory immunity to tuberculosis (1). Importantly, the esat-6 locus is found on a genomic segment which has been deleted in all vaccine strains of \textit{M. bovis} BCG (11). Cellular immune responses to this antigen have been shown to distinguish infection due to \textit{M. bovis} from infection with NTM in cattle (13). We examined whether the in vitro cellular immune response to ESAT-6 could differentiae patients with pulmonary disease due to \textit{M. tuberculosis} from patients with pulmonary disease due to MAC.

MATERIALS AND METHODS

Subjects. Patients with culture-confirmed pulmonary disease due to \textit{M. tuberculosis} were recruited from the outpatient tuberculosis clinic at Grady Memorial Hospital, Atlanta, Ga. Pulmonary tuberculosis had been diagnosed from 6 weeks to 69 weeks prior to enrollment, and all patients had tested negative for human immunodeficiency virus (HIV) at the time of diagnosis. Additionally, all patients infected with \textit{M. tuberculosis} were undergoing or had recently completed chemotherapy. Patients with a history of pulmonary disease due to MAC were recruited from the Dartmouth-Hitchcock Medical Center (DHMC) Infectious Disease Clinic in Lebanon, N.H. All patients had disease due to MAC confirmed by respiratory tract culture that had been performed in the setting of a pulmonary syndrome consistent with pulmonary disease due to MAC, and all had been or were being treated for MAC disease. HIV testing was not performed in this group. Skin test results from some patients infected by \textit{M. tuberculosis} or MAC have been reported previously (17); the patients who were newly skin tested in the present study were no. 18 to 27 (patients with \textit{M. tuberculosis} disease) and no. 6 to 8 (patients with MAC disease). Control subjects were healthy volunteers from Lebanon, N.H., or Atlanta, Ga., with skin tests negative for both PPD and MAS and no known history of exposure to tuberculosis. Skin tests were performed with PPD (Connaught Laboratories Inc., Swiftwater, Pa.) and MAS 10/2 (filling lots 61, 62, and 63; Statens Serum Institute, Copenhagen, Denmark) and read as described previously (17). Blinding was not used for patients who were newly skin tested in the present study. A positive skin test was defined by us as a reaction to either MAS or PPD of \(\geq 5\) mm. A positive skin test was considered MAS dominant if the MAS reaction was \(\geq 5\) mm larger than the PPD reaction; a positive skin test was considered PPD dominant if the PPD response was \(\geq 5\) mm larger than the MAS reaction. A positive skin test was considered nondominant if there was \(< 5\) mm difference between the PPD and MAS reactions.

Specimen processing and peripheral blood mononuclear cell (PBMC) separation. Whole blood was collected in 10-ml lithium heparin vacutainer tubes. Specimens from Atlanta, Ga., were sent in insulated containers by overnight mail.

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to DHMC, where they were processed within 22 to 26 h from the time of collection. Specimens collected from DHMC patients and subjects were processed the same day they were collected.

PBMC were separated from whole blood by density centrifugation over Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden). After washing, PBMC were resuspended in RPMI 1640 (BioWhittaker, Walkersville, Md.) supplemented with 10% human male AB serum (H2520; Sigma, St. Louis, Mo.), 2 mM glutathione, 25 mM HEPES buffer, 50 μg of gentamycin sulfate/ml, and 5 μg of polymyxin B/ml, and then diluted to 2 × 10⁶ cells/ml in complete RPMI.

Antigen stimulation and IFN-γ assays. Using 96-well cell culture plates, 100 μl of PBMC suspension was added to wells containing 100 μl of mycobacterial antigen solutions. Triplicate cultures were performed for each condition. Final antigen concentrations were 5 μg of PPD (PPD RT48; Statens Serum Institute)/ml, 5 μg of MAS (lot 35; Statens Serum Institute)/ml, and 4 μg of recombinant ESAT-6 (Statens Serum Institute)/ml. Control wells contained cell suspensions in complete RPMI alone and with phytohemagglutinin (PHA) (L9132; Sigma) at 4 μg/ml. Cultures were incubated in a 5% CO₂ incubator for 5 days. Supernatants were then harvested and frozen at −70°C until assayed for gamma interferon (IFN-γ) by a sandwich enzyme-linked immunosorbent assay by using paired mouse anti-human IFN-γ monoclonal antibodies (PharMingen, San Diego, Calif.). Values for IFN-γ were expressed as a stimulation index derived from sample optical density (OD) measurements. The stimulation index (OD index, or ODI) was calculated for each subject by dividing the antigen-stimulated OD values by that of the nonantigen control, and values above 2.0 were considered positive. Differences between responses in the three subject groupings were assessed by means of the Wilcoxon rank sum test.

RESULTS

The mean age of the patients with M. tuberculosis disease was 45 years (range, 25 to 65); 2 of 27 were women, and all were African-American. The mean age of the patients with MAC disease was 63 years (range, 43 to 78); 7 of 8 were women, and all were Caucasian. The mean age of the healthy control subjects was 34 years (range, 26 to 42); 2 of 8 were women, and all were Caucasian. All tuberculosis patients were HIV seronegative at the time of their diagnosis with M. tuberculosis disease, and none of the patients with MAC disease were known to be immunodeficient. No patients or controls were known to have received BCG vaccine, and all patients except for one with M. tuberculosis disease were born in the United States. One M. tuberculosis patient was excluded from analysis because his cells did not produce IFN-γ in response to PHA and were therefore presumed nonviable.

Skin test responses for dual skin testing with MAS and PPD and results for IFN-γ production expressed as ODI are given in Table 1. Six of eight patients whose disease was due to MAC had positive skin tests, and all six had MAS-dominant reactions as previously defined. Twenty-six of 27 patients whose disease was due to M. tuberculosis had positive PPD skin tests; 13 were PPD dominant, and 11 were nondominant. Two patients infected with M. tuberculosis (no. 14 and 18) had MAS-dominant reactions; patient 14 had also had two previous sputum cultures positive for MAC, while patient 18 had no known history of prior MAC-positive sputum cultures.

Mean IFN-γ levels and ODI in PBMC culture supernatants were highest for all three antigens in patients with M. tuberculosis disease (Table 1). IFN-γ ODI values were higher for PPD than MAS among patients with disease due to M. tuberculosis; however, this difference was not significant (P = 0.64). Among patients with disease due to MAC, mean IFN-γ ODI values were higher for MAS than for PPD, but again the difference was not significant (P = 0.46). Thus, neither the responses to PPD or MAS alone nor the combination of antigen responses effectively discriminated patients whose disease was due to M. tuberculosis and from those whose disease was due to MAC. Mean PPD-stimulated responses were higher in patients whose disease was due to M. tuberculosis than in the healthy subjects, and MAS-stimulated responses were higher in the patients whose disease was due to MAC than in the healthy subjects; however, this difference was significant only in patients whose disease was due to M. tuberculosis (P = 0.03 and 0.08, respectively.)

In contrast, ESAT-6 responses were found only among patients with M. tuberculosis disease and not among those with...
MAC disease ($P \leq 0.001$) or among healthy controls ($P \leq 0.001$). An IFN-γ OD1 cut off of $\geq 2.0$ for the ESAT-6 responses identified 17 (59%) of 27 M. tuberculosis patients and correctly classified all the MAC patients and the skin test-negative subjects as negative for M. tuberculosis (Table 2). With this cut off, the specificity of a positive ESAT-6 response was 100% and its sensitivity was 59%.

**DISCUSSION**

Using IFN-γ production as a marker for cellular immunity, we have shown that the human cellular immune response to ESAT-6 specifically identifies patients with disease due to M. tuberculosis and not patients with disease due to MAC or healthy, skin test-negative controls. Comparable veterinary trials have demonstrated the specificity of ESAT-6 responses for distinguishing M. bovis disease from infection with NTM (13). The specificity of ESAT-6 responses contrasts with earlier studies of in vitro cellular immune responses to more heterogeneous mycobacterial antigens such as PPD, which have shown limited specificity for the detection of M. tuberculosis infection. Flavey and Frankenburg, using a whole-blood modification of the lymphocyte proliferation assay with PPD as the stimulating antigen, found sensitivity, specificity, and positive predictive values for M. tuberculosis infection of 63.6, 59.5, and 58.3%, respectively (6). Among HIV-negative subjects, Converse et al. found a 100% correlation between positive skin test and in vitro PPD responses with a whole-blood commercial assay but also found that 33% of PPD skin test-negative subjects had a positive in vitro IFN-γ response (4). Although the issue has not been rigorously studied, the limited specificity of tests with PPD appears to be due to either antigen cross-reactivity with prior NTM infections or to nonspecific stimulation by the heterologous mycobacterial proteins of the preparation itself.

Our study demonstrated the same pattern of responses to PPD that has been noted in previously reported studies. PPD provoked an IFN-γ OD1 of 4 in 20 (74%) of 27 patients with M. tuberculosis disease, 1 (13%) of 8 patients with MAC disease, and 2 (25%) of 8 healthy subjects. Based on this small study, the resulting test characteristics for in vitro IFN-γ responses to PPD for the detection of disease due to M. tuberculosis are a sensitivity of 74%, a specificity of 81%, and a positive predictive value of 87%. The false-positive rate for PPD in healthy control subjects, however, was 25%. MAS also produced nonspecific IFN-γ production in some skin test-negative controls and patients with M. tuberculosis disease. In contrast, ESAT-6 responses were reliably absent in patients with pulmonary disease due to MAC and in skin test-negative subjects.

The sensitivity of responses to the recombinant ESAT-6 antigen was lower than its specificity. ESAT-6 failed to give significant IFN-γ responses in 11 of 27 patients whose disease was due to M. tuberculosis. Test sensitivity in patients with disease due to M. tuberculosis may be lower than in subjects infected with M. tuberculosis. We and others have noted lower cellular immune responses to recombinant mycobacterial antigens in tuberculosis patients than in healthy PPD-positive subjects (2, 12, 15a). The immunomodulating cytokine transforming growth factor β (TGF-β) has been shown to be produced in antigen-stimulated PBMC cultures of patients whose disease is due to M. tuberculosis but not in healthy, matched, PPD-positive household contacts (9), and inhibitors of TGF-β can restore much of the diminished in vitro IFN-γ response seen in patients with pulmonary disease due to M. tuberculosis (8). Because healthy PPD-positive subjects have been shown to respond well to some mycobacterial antigens in vitro, it may be that the lack of reactivity to ESAT-6 seen in several of the patients with M. tuberculosis disease represents a recoverable function. We are currently investigating this possibility as a means to improve the sensitivity of diagnostic tests with ESAT-6.

It is also clear that in a genetically heterogeneous population a certain frequency of nonresponding individuals will be found. We have previously found restricted recognition of ESAT-6 in mice; although most strains of inbred mice recognize ESAT-6, one of six tested haplotypes (SJJ/N) did not (3). Our M. tuberculosis-infected population was African-American, while our MAC-infected population and healthy controls were Caucasian. We have recently studied peptide-specific ESAT-6 responses in Danish and Ethiopian cohorts, and the dominant epitopes were found to differ (15). However, the 59% response in the African-American tuberculosis patient population of the present study is in agreement with the results in Caucasian Danish patients with disease due to M. tuberculosis, where 56% were found to respond to ESAT-6, as is the absence of any ESAT-6 response in healthy Danish PPD-negative controls (15). This finding, together with the lack of a gene homologous to the esat-6 locus in the M. avium genome, strongly supports a role for ESAT-6 in distinguishing infection with MAC from infection with M. tuberculosis. ESAT-6 testing may prove a reliable means of determining whether a positive PPD skin test is due to prior immunization with BCG. The apparent deletion in the BCG genome of the M. bovis DNA which carries the

### Table 2. Sensitivity and specificity of in vitro IFN-γ production to mycobacterial antigens based on defined cut offs

| Antigen and cut off | M. tuberculosis disease (n = 27) | MAC disease (n = 8) | Controls (n = 8) | Sensitivity (%) for M. tuberculosis | Specificity (%) for M. tuberculosis |
|--------------------|----------------------------------|--------------------|-----------------|------------------------------------|-----------------------------------|
| rESAT-6* OD1       |                                  |                    |                 |                                    |                                   |
| ≥2.0               | 16                               | 0                  | 0               | 59                                 | 100                               |
| <2.0               | 11                               | 8                  | 8               |                                    |                                   |
| PPD OD1            |                                  |                    |                 |                                    |                                   |
| ≥2.0               | 23                               | 2                  | 5               | 85                                 | 56                                |
| <2.0               | 4                                | 6                  | 3               |                                    |                                   |
| ≥4.0               | 20                               | 1                  | 2               | 74                                 | 81                                |
| <4.0               | 7                                | 7                  | 6               |                                    |                                   |

* rESAT-6, recombinant ESAT-6.
esat-6 locus makes it unlikely that BCG immunization will give
in vitro immune responses to ESAT-6. We have previously
found no in vitro recognition of ESAT-6 in healthy Danish
volunteers following BCG vaccination, despite a pronounced
IFN-γ response to PPD and other crude mycobacterial anti-
gens (14).

The next step would therefore be to improve upon the sen-
sitivity of in vitro testing for tuberculosis by employing combi-
nations of antigens. The same genome segment on which
ESAT-6 is found and which is absent in BCG contains a num-
ber of open reading frames. One of these, another small anti-
gen strongly recognized by immunoreactive T cells from tuber-
culosis patients and under control of the same promoter as
ESAT-6, is currently under study (15a). The potential for in
vitro diagnosis of MAC infection with antigens specific to that
organism remains unexplored. This approach might permit the
diagnosis of mycobacterial infection prior to the onset of dis-
ease in HIV infection and other forms of immune system
compromise, allowing early prophylactic treatment.

The present study demonstrates that a simple index of in
vitro INF-γ production in response to ESAT-6 has a high
positive predictive value for infection with M. tuberculosis. This
study provides further support for the development of newer
and less-cumbersome techniques for measuring in vitro cellu-
lar immune responses to specific mycobacterial antigens. Ap-
lication of simplified in vitro testing to large-scale trials in
rigorously defined subject groups will be necessary to ade-
quately assess the potential of this concept.

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