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ESAT-6/CFP-10 Fusion Protein and Peptides for Optimal Diagnosis of *Mycobacterium tuberculosis* Infection by Ex Vivo Enzyme-Linked Immunospot Assay in The Gambia

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Overlapping peptides of *Mycobacterium tuberculosis* antigens ESAT-6 and CFP-10 offer increased specificity over the purified protein derivative skin test when they were used in an ex vivo enzyme-linked immunospot (ELISPOT) assay for gamma interferon detection for the diagnosis of *M. tuberculosis* infection from recent exposure. We assessed whether equivalent results could be obtained for a fusion protein of the two antigens and whether a combined readout would offer increased sensitivity in The Gambia. We studied the ELISPOT assay results for 488 household contacts of 88 sputum smear-positive tuberculosis (TB) cases. The proportions of subjects positive by each test and by the tests combined were assessed across an exposure gradient, defined according to sleeping proximity to a TB case. Eighty-eight (18%) subjects were positive for CFP-10 peptides, 148 (30%) were positive for ESAT-6 peptides, 161 (33%) were positive for both peptides, and 168 (34%) were positive for the fusion protein; 188 (39%) subjects had either a positive result for a peptide or a positive result for the fusion protein. There was reasonable agreement between the peptide and the protein results (kappa statistic = 0.78) and no significant discordance ($P = 0.38$). There was a strong correlation between the fusion protein and combined peptide spot counts ($r = 0.9$), and responses to the peptide and the proteins all increased significantly according to *M. tuberculosis* exposure. The proportion of subjects positive for either the pool of peptides or the fusion protein offered maximum sensitivity, being significantly higher than the proportion of subjects positive for ESAT-6 peptides alone ($P = 0.007$). A fusion protein of ESAT-6 and CFP-10 is equivalent to overlapping peptides for the diagnosis of latent *M. tuberculosis* infection. Use of a combination of peptides and fusion protein offers improved sensitivity.

There is an urgent need for reproducible and rigorous frameworks to assess new tests for the diagnosis of *Mycobacterium tuberculosis* infection (13). We have recently presented a reproducible model which uses an established gradient of recent *M. tuberculosis* exposure according to sleeping proximity to an index tuberculosis (TB) case. Using this model, we showed that an ex vivo enzyme-linked immunospot (ELISPOT) assay for gamma interferon (IFN-γ) detection with secreted antigens of *M. tuberculosis*, ESAT-6 and CFP-10, offers improved specificity over assays with the purified protein derivative (PPD) for the diagnosis of latent *M. tuberculosis* infection in The Gambia, a tropical setting where TB is endemic (9). However, when the results were compared to those of the PPD skin test, it appeared that the specificity gain was at the cost of some sensitivity, which supports the assertion that two *M. tuberculosis*-specific antigens may not be sufficient for the diagnosis of infection (14). Longitudinal follow-up studies to find secondary cases will shed more light on this issue.

It is not known whether it is preferable to conduct the ELISPOT assay with recombinant protein or pools of peptides or whether increased sensitivity could be achieved through measurement of a combined peptide and protein response (3). In addition, a fusion protein of antigens is likely to offer a far cheaper and more realistic alternative for large-scale production and field testing. Therefore, we engineered a fusion protein of ESAT-6 and CFP-10 and formally evaluated its diagnostic value against and in addition to those of the respective peptides of the single antigens for the ability to detect *M. tuberculosis* infection in The Gambia.

MATERIALS AND METHODS

Participants. TB index cases over 15 years of age were recruited from the major government health center in greater Banjul and the outpatient clinic at the Medical Research Council Laboratories in The Gambia. The cases included in the study had two sputum smear samples positive for acid-fast bacilli, and *M. tuberculosis* was isolated upon culture. Household contacts were eligible for inclusion in the study if they were at least 6 months of age and lived the majority of the time in the same compound as the case patient. They were not invited to participate in the study if they had been treated for TB in the past year or if they were recruited 60 days after recruitment of the case patient. Contacts were excluded from further study if they had been diagnosed with TB within 1 month of recruitment and had a duration of coughing longer than that of the case patient. Written informed consent was obtained from the adults as well as from the parents or legal representatives of participating minors. The subjects were interviewed and examined, and a blood sample was taken for the ELISPOT assay and a test for human immunodeficiency virus (HIV). Fresh samples from all participants were processed on-site.

The study was approved by The Gambia Government-Medical Research Council Joint Ethics Committee.

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Laboratory procedures. Sputum smears were prepared and stained with auramine-phenol (8), and the results were confirmed by Ziehl-Neelsen staining. Decontaminated specimens were inoculated into Lowenstein-Jensen medium and BACTEC 9000 MB liquid medium for the isolation and identification of *M. tuberculosis*, as described previously (9).

Testing for HIV type 1 (HIV-1) or HIV-2 infection was by competitive enzyme-linked immunosorbent assays (Wellcome Laboratories, Kent, United Kingdom) and Western blotting (Diagnostics Pasteur, Marnes-la-Coquette, France).

Synthetic, sequential peptides spanning the lengths of ESAT-6 and CFP-10 (Advanced Biotech Centre, Imperial College, London, United Kingdom) were used. Each peptide was 15 amino acids long and overlapped its adjacent peptide by 10 residues. ESAT-6 and CFP-10 peptides, used at 5 μg/ml, were used in one pool each of 17 peptides. The positive control was phytohemagglutinin (Sigma-Aldrich, United Kingdom). All antigens were tested in duplicate wells.

The fusion protein of ESAT-6 (Rv3875) and CFP10 (Rv3874) was engineered as follows. The individual genes were amplified from *M. tuberculosis* H37Rv genomic DNA by PCR. During the amplification steps the genes were fused with a linker encoding asparagine-valine-alanine. The product was subsequently cloned by Gateway technology into bacterial expression vector pDEST17 (Invitrogen, San Diego, CA) containing an N-terminal hexahistidine tag. Sequencing was performed to confirm the identity of the cloned DNA fragment. The recombinant fusion protein was overexpressed in *Escherichia coli* BL21(DE3) and was purified as described previously (6). Recombinant protein batches were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by Coomassie brilliant blue staining and Western blotting with an anti-His antibody (Invitrogen), to confirm the size and purity of the protein. Residual endotoxin levels were determined by a *Limulus* amebocyte lysate assay (Cambrex) and were found to be less than 50 IU/mg recombinant protein. Protein batches were subsequently tested for nonspecific T-cell stimulation and for potential cellular toxicity in IFN-γ release assays with peripheral blood mononuclear cells of *M. tuberculosis*-unexposed and *M. bovis* BCG-unvaccinated healthy Mantoux-negative donors. The fusion protein was used at a concentration of 10 μg/ml in the ELISPOT assays (see below).

The ex vivo ELISPOT assays for IFN-γ were performed in duplicate, as described previously (10). Assays were scored with an ELISPOT counter (AID-GmbH, Strassberg, Germany). The number of spot-forming units (SFU) in each well was automatically entered into a database by using Matlab software (MathWorks). Supplementary details were added by double data entry by two immunologists blinded to subject details. Positive test wells were predefined as containing at least 10 SFU more than and at least twice as many as SFU as the negative control wells. For a positive ESAT-6/CFP-10 result, it was necessary for one or both pools of overlapping peptides to be positive. Phytohemagglutinin-positive control wells were set to at least 150 SFU/well/2×10^5 cells above the values for the negative control wells. Negative control wells were required to have less than 30 SFU.

Data management and statistical analysis. All data were entered by using double entry of the data into a Microsoft Access database, and the data were checked for errors. Agreement between the qualitative test results was assessed by use of the kappa statistic, and the significance of the discordance was assessed by McNemar's test. For analyses by exposure, contacts were stratified according to their proximity to the index case, as defined previously (9). The following categories were used: sleeping in the same bedroom as the index case, sleeping in a different bedroom in the same house as the index case, or sleeping in a different house in the same compound as the index case. A random-effects logistic regression model, which takes into account household clustering, was used to assess the relationship between exposure and test results. All statistical analyses were conducted by using Stata software (version 8; Stata Corp., College Station, TX).

RESULTS

From 20 May 2003 to 29 April 2004, 603 consenting TB case contacts were selected for evaluation by the ex vivo ELISPOT assay for IFN-γ, and an adequate specimen was taken. Of these, 488 (81%) contacts of 88 cases had both fusion protein and peptide ELISPOT assay results that met the inclusion criteria (Table 1). Overall, 88 (18%) subjects were positive for CFP-10 peptides, 148 (30%) subjects were positive for ESAT-6 peptides, 161 (33%) subjects were positive for either or both the CFF-10 and the ESAT-6 peptides, and 168 (34%) subjects were positive for the fusion protein. A total of 188 (39%) subjects had a positive result for one or more of the peptides or the fusion protein.

TABLE 1. Characteristics of 488 contacts of 88 cases

Characteristic	Value
Demographic	
Mean (median, range) age (yr)	20 (17, 0–80)
No. (%) of subjects	
Male	242 (49.6)
Ethnic group	
Mandinka	144 (30)
Jola	147 (30)
Wolof	44 (9)
Fula	62 (13)
Other	91 (19)
Proximity to case	
Same room	142 (29)
Different room	217 (45)
Different house	129 (26)
Clinical (no. [%] of subjects)	
BCG scar present	200 (42)[a]
HIV positive	8 (2)[b]

[a] Seventy-one of the 409 (15%) subjects assessed had uncertain BCG scar status.
[b] HIV serostatus was determined in 480 subjects.

There was reasonable agreement between the qualitative results for the peptides combined and the fusion protein, with a kappa statistic of 0.78 (95% confidence interval [CI], 0.69 to 0.87; $P < 0.0001$) and no significant discordance ($P = 0.38$). Twenty subjects were peptide positive and fusion protein negative, and 27 were fusion protein positive and peptide negative (Fig. 1). When the results of the tests were combined, the proportion of subjects positive for the ESAT-6 peptides or the fusion protein was significantly higher than the proportion of subjects positive for the ESAT-6 peptides alone ($P = 0.036$),

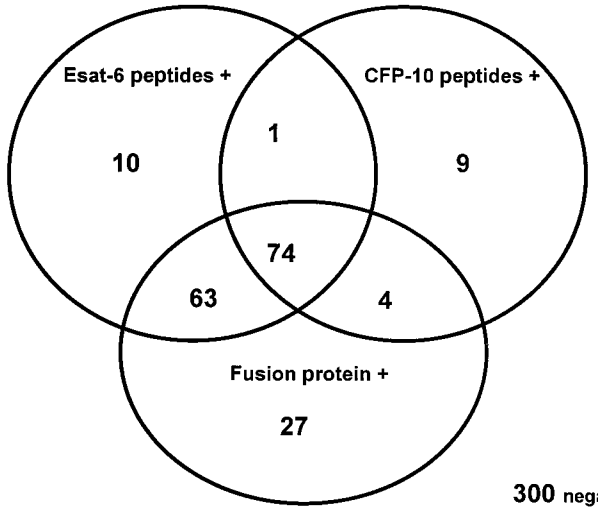

FIG. 1. Venn diagram of the number of subjects with each combination of fusion protein and peptide results ($n = 488$).

TABLE 2. Univariable and multivariable ORs by logistic regression (household as a random effect) according to sleeping proximity to a case

Antigen	Sleeping proximity	% (no.) positive	OR (95% CI)	Adjusted OR (95% CI)	P^a
ESAT-6 peptides	Different house	17 (22)	1.0	1.0	0.0010
	Different room	32 (70)	2.5 (1.3, 4.7)	2.3 (1.2, 4.2)	
	Same room	39 (56)	3.3 (1.7, 6.4)	3.4 (1.8, 6.6)	
CFP-10 peptides	Different house	11 (14)	1.0	1.0	0.015
	Different room	18 (38)	1.7 (0.8, 3.7)	1.7 (0.8, 3.5)	
	Same room	25 (36)	3.0 (1.4, 6.6)	3.5 (1.6, 7.8)	
Combined peptides	Different house	20 (27)	1.0	1.0	0.0024
	Different room	35 (75)	2.1 (1.2, 3.8)	2.0 (1.1, 3.4)	
	Same room	42 (59)	2.9 (1.5, 5.3)	3.1 (1.7, 5.7)	
Fusion protein	Different house	24 (31)	1.0	1.0	0.0055
	Different room	35 (76)	2.0 (1.1, 3.6)	1.8 (1.0, 3.2)	
	Same room	43 (61)	2.7 (1.4, 5.0)	2.9 (1.5, 5.3)	
All combined	Different house	28 (36)	1.0	1.0	0.0064
	Different room	39 (84)	1.9 (1.0, 3.3)	1.7 (1.0, 3.0)	
	Same room	48 (68)	2.6 (1.4, 4.8)	2.9 (1.6, 5.4)	

[a] *P* value for linear trend.

and the proportion of subjects positive for either the combined ESAT-6 and CFP-10 peptides or the fusion protein was significantly higher than the proportion positive for the ESAT-6 peptides alone ($P = 0.007$). Therefore, use of the results for the peptide and the protein combined suggested some additional benefit in terms of sensitivity.

The univariable odds of a result being positive for exposure to *M. tuberculosis* by each test, according to sleeping proximity, are shown in Table 2. All the test results were significantly more likely to be positive for contacts with a closer sleeping proximity to the case. In the multivariable analysis, the odds of being positive for all the antigens and combinations with a closer proximity to the case, adjusted for age, sex, and ethnicity, remained significantly increased. The odds of test positivity decreased, but not significantly, with the presence of a BCG scar for the ESAT-6/CFP-10 peptide result (odds ratio [OR], 0.74; 95% CI, 0.5 to 1.25) and for the fusion protein (OR, 0.9; 95% CI, 0.6 to 1.4).

The median combined peptide count when the result was positive was 48.5 SFU (mean, 81.2 SFU; range, 10 to 352.5 SFU), which was significantly higher than the median count for the fusion protein of 38.5 SFU (mean, 70 SFU; range, 10 to 347.5 SFU) ($P = 0.0001$). The quantitative test results are presented against each other in four scatter graphs in Fig. 2. There was a strong correlation between the fusion protein and the combined peptide SFU counts ($r = 0.9$). The correlation between the counts of the fusion protein and ESAT-6 alone was also strong ($r = 0.87$). However, the significantly lower rate of positivity for CFP-10 overall led to a low correlation with the fusion protein and ESAT-6 peptide responses. These findings confirmed that ESAT-6 is the immunodominant antigen in The Gambia.

DISCUSSION

We report on a large-scale evaluation of a *M. tuberculosis*-derived ESAT-6/CFP-10 fusion protein against and in addition to the corresponding peptides by an ex vivo ELISPOT assay for IFN-γ in 488 TB contacts above 6 months of age in a tropical setting where TB is endemic.

As far as we are aware, this is the first formal assessment of the use of a fusion protein of ESAT-6 and CFP-10 as a reagent to monitor natural active *M. tuberculosis* infection in humans. The ESAT-6 and CFP-10 antigens, whether they are used as peptides or a fusion protein, all induced responses with increasing positivity according to the closeness of the contact to a TB index case. The changes in positivity across the TB exposure gradient are similar to those reported in our recent assessment of the ESAT-6 and CFP-10 peptides by the ELISPOT assay compared with the PPD skin test and the PPD ELISPOT assay (9). We therefore reconfirm with the fusion protein the potential for the use of a TB case-contact study design as a model for the study of natural infection with sensitivity across a gradient of exposure. We show the model to be reproducible, and we believe that it is ready to be applied for the testing of new interventions, including drugs and vaccines, in efficacy trials.

There was good agreement overall between the results obtained with the fusion protein and those obtained with the peptides in both the qualitative comparisons and the quantitative correlations. In effect, the fusion protein and peptide responses are mutual, and each serves as a control for the other. Interestingly, our data show that the response to the CFP-10 peptide antigen is significantly less than that to ESAT-6; in other studies in Zambia (5), India (11), Denmark (2), and The Netherlands (1), the converse was found. Both the ESAT-6 and the CFP-10 antigens are similar in size, are encoded in one operon, and are expressed simultaneously at similar ratios (4, 12). Differences in immune responses to individual ESAT-6 and CFP-10 antigens may be explained by polymorphism in the HLA type in the population and to the actual amino acid compositions of the individual proteins. An alternative explanation might relate to differences of *M. tuberculosis* strains in Africa.

By combining the data obtained with the peptide and the fusion protein, we found a small but significant gain in sensi-

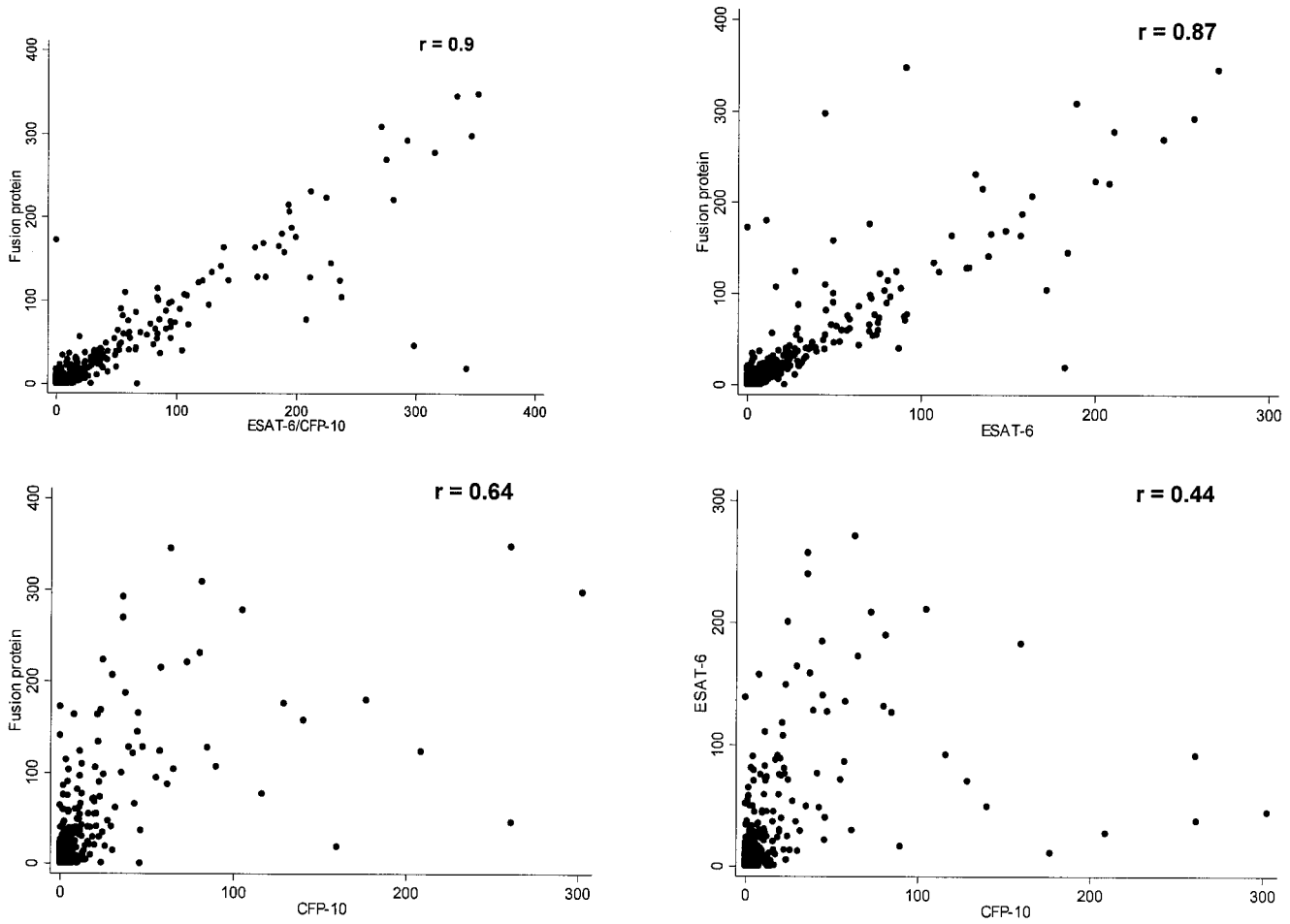

FIG. 2. Scatter graphs showing correlations between quantitative ELISPOT test results.

tivity. While peptides might be expected to stimulate mixed CD4 and CD8 T-cell responses, a response to the peptide in the absence of a response to the fusion protein implies a response that is exclusively mediated by CD8 T cells and representative of about 12% of the response.

Currently, there is no clear explanation for an exclusive protein response in the absence of a peptide response, although there may be several theoretical possibilities. Since the molar concentration of peptide antigen is at least fivefold higher than that of the protein, a lack of a peptide response is unlikely to be a concentration effect. Although the peptides used were 15 amino acids that overlapped by 10 amino acids, there is still a small chance that the peptides do not represent every conceivable sequential T-cell epitope. However, it would seem unlikely that so much of the response is due to sequentially unrepresentative epitopes. There is a possibility that "neoepitopes" are formed across the ESAT-6/CFP-10 junction and are therefore absent from the peptide pools. Yet, it is difficult to see how these might occur naturally. A more likely explanation comes from recent publications that have shown that $CD8^+$ T-cell epitopes are spliced to form nonsequential sequences (7) within the proteosome (15). Our data, which showed differences between the unprocessed (peptide) and the processed (protein) antigen, support the possibility that spliced epitopes might also occur through a CD4 pathway. While confirmation is needed, the possibility remains that as much as 14% (protein positive, peptide negative) of the response to a recent *M. tuberculosis* infection could be due to spliced epitopes. We are conducting longitudinal follow-up of the subjects to assess the changes in responses to the peptides and proteins over time. Finally, since the readout deals only with IFN-γ, discordance in the responses between the peptide and the protein could relate to the secretion of different cytokines.

Of note, since this study was nested in a large case-contact study, 464 of the 488 subjects also had a PPD skin test with a change in positivity across the exposure gradient of 20.8% to 61% (data not shown), which is very similar to the data that we reported previously (9). It is likely, therefore, that the gain in sensitivity in these studies is not enough to counter the sensitivity loss incurred by using only two antigens. We are exploring other ways to improve the sensitivity of the ELISPOT assay in our setting, including the use of mathematical modeling to access a lower cutoff for positivity and searching for new immunogenic, but *M. tuberculosis*-specific, diagnostic antigens.

In terms of practicality, the fusion protein will be much cheaper to manufacture than bulk peptides and costs half of what the individual proteins cost. However, the use of a combination of fusion protein with peptides is advantageous. First,

the combination allows optimal detection of *M. tuberculosis* infection, at least by the ex vivo ELISPOT assay; and second, protein and peptide antigens act as quality controls for one another. The quality control issue becomes a lot more relevant when the assay is used in large field studies, as we did.

In conclusion, the present study has shown good agreement between the measurement of the immune response by ELISPOT assay to a fusion protein of ESAT-6 and CFP-10 and measurement of the immune response to the respective overlapping peptides. Improved sensitivity was gained through the addition of CFP-10 to ESAT-6 peptides and through the combined assessment of the peptide and the fusion protein responses. The fusion protein could be used as an alternative to pools of overlapping peptides or in addition to pools of overlapping peptides in settings where maximal sensitivity is a priority. Further studies will be required to explore the significance, if any, of exclusive responses to either the protein or the peptides, especially in relation to the time from exposure and the development of secondary TB disease.

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