# Regions of High Antigenicity within the Hypothetical PPE Major Polymorphic Tandem Repeat Open-Reading Frame, Rv2608, Show a Differential Humoral Response and a Low T Cell Response in Various Categories of Patients with Tuberculosis

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**The function of the PE/PPE families of proteins, which represent ~10% of the coding capacity of the *Mycobacterium tuberculosis* genome, has remained relatively unknown. We earlier described a PPE family member, Rv2430c, as an immunodominant antigen. We now report another PPE family gene, Rv2608, a member of the major polymorphic tandem repeat subfamily, for its ability to elicit a high humoral and a low T cell response. Rv2608 was also found to be polymorphic in different clinical isolates of *M. tuberculosis,* as determined by polymerase chain reaction–restriction fragment–length polymorphism analysis. A total of 51 clinically confirmed patients with tuberculosis (TB), belonging to 3 different categories—fresh infection ($n = 22$), relapsed infection ($n = 21$), and extrapulmonary infection ($n = 8$)—and 10 healthy control subjects were included in the study. Recombinant Rv2608 protein showed positive reactivity to patients' serum samples. Enzyme-linked immunosorbent assays and T cell–proliferation assays with synthetic peptides corresponding to predicted regions of high antigenicity showed a predominantly humoral response in patients with relapsed TB. We additionally identified the Gly-X-Gly-Asn-X-Gly repeat motifs as being primarily responsible for eliciting a humoral immune response.**

The existence of PE/PPE gene families was evident even before the *Mycobacterium tuberculosis* genome was sequenced, with occasional reports of occurrence of glycine- and alanine-rich multiple repetitive sequences in the genome [1] and identification of a few fibronectin-binding proteins [2]. Sequencing categorized the PE/PPE gene families as 2 large unrelated families of highly acidic glycine-rich proteins that constitute ~10% of the coding capacity of the genome [3]. Comparative genome sequencing in various mycobacterial species revealed that, by and large, PE/PPE gene families are unique to *M. tuberculosis,* with few homologues in *M. leprae, M. bovis, M. marinum,* and other species [4]. Among the *M. leprae* homologues, a major serine-rich antigen is expressed in patients with leprosy [5].

It is generally believed that the PE/PPE genes could be a source of antigenic variability. A recombinant PE_PGRS (Rv1759c) protein was shown to possess fibronectin-binding properties and was also recognized by patients' serum samples [6]. The same group also reported immense intrastrain variability in the polymorphic GC-rich sequence (PGRS) domain, with the N-terminal region staying constant. Transposon insertion in the PE_PGRS gene, Rv1818c, was shown to reduce the macrophage-infection ability of *M. tuberculosis* [7]. Surface localization of a PPE protein, Rv1917c, and of many other

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PE_PGRS proteins has been reported [8, 9]. Few PE_PGRS genes have also been shown to be expressed during preclinical infection [10]. Dissection of the PE_PGRS genes into the PE and the PGRS domains, to study their specific immunological responses during infection of mice, revealed that the PE region can elicit an effective cellular immune response and that the humoral response is largely directed against the Gly-Ala–rich PGRS domain [11]. Involvement of PE/PPE genes in the virulence of the pathogen has also been reported [12]. We recently described the biophysical and biochemical properties of a PPE gene, Rv2430c, and further showed that it is an immunodominant antigen of *M. tuberculosis* [13–15].

In the present study, we used an in silico approach to identify probable antigens from the PPE major polymorphic tandem repeat (MPTR) subfamily and studied the humoral and cellular immune response to the same, using well-characterized serum samples from patients. Synthetic peptides corresponding to regions of high antigenic index of the protein were used to map the antigenic domains and assess the antigenic potential of the Gly-X-Gly-Asn-X-Gly repeat motif in eliciting a differential immune response. Our results suggest that the PPE_MPTR open-reading frame (ORF), Rv2608, could be involved in directing the host toward development of a more humoral type of immune response.

## SUBJECTS, MATERIALS, AND METHODS

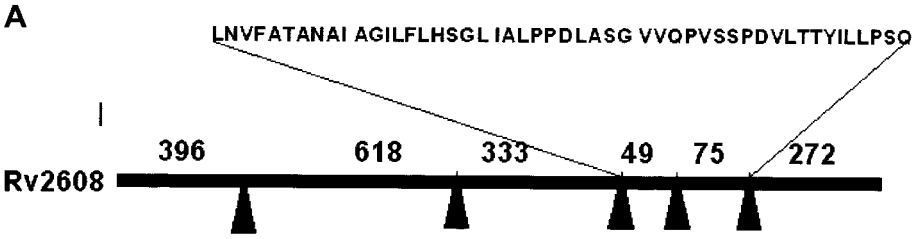
***Subjects.*** Fifty-one patients with tuberculosis (TB) confirmed by tuberculin skin test, radiographic examination, and observation of acid-fast bacilli in sputum (for pulmonary infections) and at the site of presumed TB (for extrapulmonary infections) were selected for the present study. These patients were reporting to the outpatient department of the Mahavir Hospital and Research Centre, Hyderabad, India. All patients with confirmed diagnosis of TB were culture positive as well. We categorized the patients as follows: category I, patients diagnosed with TB for the first time ($n = 22$); category II, patients with relapsed TB ($n = 21$); and category III, patients with extrapulmonary TB ($n = 8$). Serum samples were collected from all the subjects during the early stage of infection when chemotherapy had just started. Healthy control serum samples ($n = 10$) were collected from the laboratory staff of the Centre for DNA Fingerprinting and Diagnostics (CDFD). These were individuals who had not had prolonged, direct contact with a patient with TB. Since this study was performed on a PPE gene family member of *M. tuberculosis,* members of which are unique to mycobacteria [3], cross-reactivity to this protein would not be expected; therefore, control subjects with other bacterial infections were not considered to be necessary for inclusion in the present study. The bioethics committee of CDFD approved the present study, and informed consent was obtained from all subjects.

***Polymerase chain reaction (PCR)–restriction fragment–length polymorphism (RFLP) analysis of Rv2608.*** PCR-RFLP analysis was performed to examine whether Rv2608 exhibited polymorphism in different clinical isolates of *M. tuberculosis.* In brief, Rv2608 was amplified by PCR, from ~30 different clinical isolates, and the amplified product was digested with *Sau*3AI enzyme. The digested product was separated on a 10% polyacrylamide gel and visualized under UV, after ethidium bromide staining.

***Cloning, overexpression, and purification of Rv2608, a PPE_MPTR subfamily member of* M. tuberculosis.** Rv2608 was amplified from *M. tuberculosis* H37Rv genomic DNA by use of primers carrying specific restriction enzyme sites, to enable directional cloning. The amplified gene was first cloned in pGEMT easy vector, followed by subcloning in pRSETa expression vector. Expression of the 59.6-kDa recombinant (r) Rv2608 protein in *Escherichia coli* BL21 cells was achieved as described earlier [14]. The recombinant protein was purified to homogeneity on a nickel nitrilotriacetic acid affinity column (Qiagen).

***Synthetic peptides.*** Rv2608 was scanned to identify regions of high antigenic index, by use of the Protean software of Lasergene Navigator (DNA STAR). Ten synthetic peptides of varying lengths, corresponding to in silico–predicted regions of high antigenic index, were commercially obtained as lyophilized powders. Peptide stocks (concentration, 0.1 mg/mL) were prepared in carbonate bicarbonate buffer and stored in aliquots at −70°C.

***ELISA and lymphocyte proliferation assay.*** All 51 patients were tested against each of the 10 peptide antigens, to evaluate for a B cell, as well as a T cell, response. ELISAs with 2 μg/mL rHsp10/rRv2608 protein/synthetic peptide were performed as described elsewhere [14]. The lymphocyte proliferation assays were performed essentially in accordance with the method described elsewhere, with a few modifications [16]. Heparinized blood was drawn and diluted with an equal volume of RPMI 1640 medium without serum. Diluted blood was layered on Ficoll gradient, in a 1:3 proportion. After a low-speed (800 *g*) centrifugation for 30 min, the peripheral blood mononuclear cells (PBMCs) were isolated and washed twice for 10 min at 800 *g,* to remove the cell debris and platelets. Cell concentration was adjusted to $10^6$ cells/mL. Viability of the cells was checked by use of trypan blue. To each well of the microtiter plates, 0.1 mL of cell suspension and 0.1 mL of antigen (2 μg/mL) were added. Concanavalin A was used as a positive control antigen. Control and experimental cultures were run in triplicate. Plates were incubated for 72 h at 37°C with 5% $CO_2$. At the end of the third day, 15 μL of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (2 mg/mL) was added, and plates were incubated for another 4 h. The culture was terminated, and the MTT crystals were dissolved in 100 μL of acidified isopropanol. After 1 h, the optical density was recorded by use of an ELISA plate reader at a dual




B

| Location of *Sau*3AI sites | Expected bands (bp) | Additional bands (bp) | 25 isolates | 2 isolates | 3 isolates |
|---|---|---|---|---|---|
| 396 | 396 | | + | + | + |
| 1014 | 618 | | + | + | + |
| 1347 | 333 | | + | + | + |
| 1396 | 49 | | + | + | - |
| 1471 | 75 | | + | - | - |
| 1753 | 272 | | + | + | + |
| | | ~120 | - | + | + |
| | | ~100 | - | + | + |

**Figure 1.** *Sau*3AI restriction map of the PPE open-reading frame (ORF), Rv2608. Arrowheads point to the *Sau*3A sites in the 1743-bp ORF. Nos. above the line indicate the size of the restriction fragments (in base pairs) generated after *Sau*3AI digestion. *B,* Summary of *Sau*3AI polymerase chain reaction–restriction fragment–length polymorphism pattern of 30 different clinical isolates of *Mycobacterium tuberculosis.*

wavelength of 570 nm and 620 nm reference filter. Data were expressed as stimulation index (SI)—that is, as the ratio of the mean optical density of experimental cultures (with test antigen) to the mean optical density of control cultures (without antigen). SI $\geq 2$ was considered to be positive.

***Statistical methods.*** Analysis of variance (ANOVA), as a test of statistical significance, was performed by use of online software (available at: http://www.physics.csbsju.edu/stats/anova.html) to calculate the *P* values and determine whether there was any difference between different categories of patients, with respect to each antigen tested. The 95% confidence intervals (CIs) for means were also determined for each set of data. Differences between groups were considered to be statistically significant if the 95% CI limits did not overlap. To ascertain the results obtained by ANOVA, we also performed the Kruskal-Wallis nonparametric test (available at: http://department.obg.cuhk.edu.hk/ResearchSupport/KruskallWallis.asp). In addition, we performed *t* tests for paired comparison of means. $P<.05$ was considered to be statistically significant.

## RESULTS

***Genetic variation in Rv2608.*** PE/PPE genes are predicted to be a source of antigenic variability of *M. tuberculosis,* and, in a few of them, polymorphism based on variation in the number of repeat sequences has already been reported [6]. We analyzed the PPE gene, Rv2608, of the MPTR subclass, using PCR-RFLP to score for the presence of genetic variation in different clinical isolates. The 1.7-kb amplicon was digested with *Sau*3AI, and the digest was electrophoresed on a 10% polyacrylamide gel. A total of 16% of the clinical isolates showed a deviation from the normal band pattern. Figure 1 gives the complete summary of the polymorphisms obtained in 30 different clinical isolates. The disappearance of restriction fragments was restricted to the C-terminus of the ORF, which is the predicted variable region of the PPE ORFs. It was therefore important to further evaluate Rv2608 in terms of its ability to elicit B and T cell responses, to study its role as a possible antigen for immune surveillance.

***Expression and purification of the rPPE protein.*** To evaluate the antigenic ability of Rv2608, the corresponding gene was expressed in *E. coli* BL21 cells and purified as a 6× His-tag fusion protein. Purified rRv2608 was fractionated by elec-

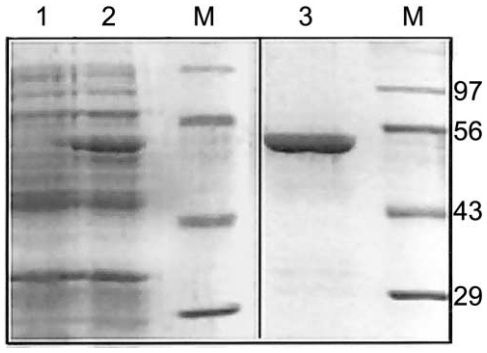



**Figure 2.** Expression and purification of *Mycobacterium tuberculosis* protein corresponding to the PPE open-reading frame, Rv2608. *Left panel,* Uninduced and induced cell lysates and protein molecular size marker *(lanes 1* and *2* and *M). Right panel,* Purified recombinant protein *(lane 3)* and protein molecular size marker *(M).*

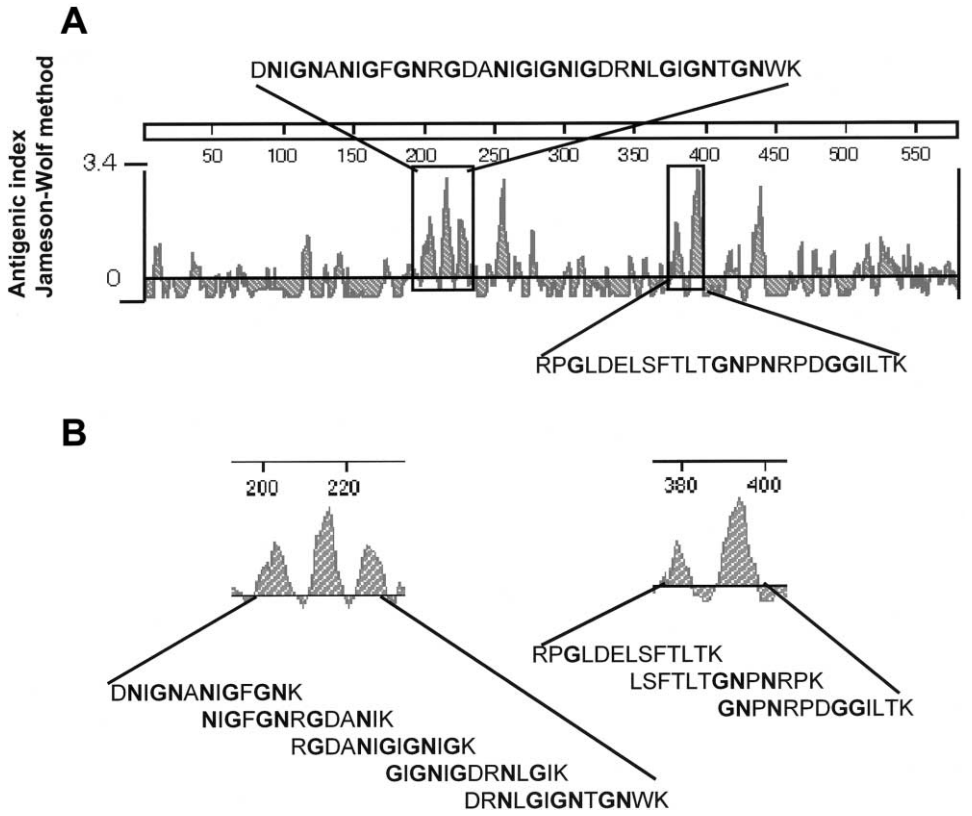



**Figure 3.** In silico analysis of Rv2608, revealing regions of high antigenic index (potential antigenic determinants). The overall antigenic index of the protein was calculated by use of the Jameson-Wolf method of the Protean software of Lasergene Navigator (DNA STAR). The boxed areas indicate the regions selected for designing synthetic peptides to map the region that was actually eliciting a variable immune response. As can be seen, one of the peptides (37 mer) is largely composed of Gly-Asn repeats, of which fewer are in the other peptide (23 mer). *B,* Stretches of overlapping peptides used for ELISA and T cell–proliferation assay. These peptides were used to further map the region that was antigenic.

trophoresis on a 12% polyacrylamide gel. A single band corresponding to the 59.6-kDa protein was observed on staining the gel with Coomassie brilliant blue dye (figure 2). The expression of the gene was confirmed by probing, with antihistidine antibody, the membrane containing the total cellular protein of *E. coli* BL21 cells harboring the Rv2608 construct (data not shown). There was no leaky expression of the protein in uninduced cells. The recombinant protein was largely present in the insoluble fraction and was therefore purified in the presence of 8 mol/L urea (figure 2, *lane E*). The yield of the protein was 6 mg/L culture. The recombinant protein was dialyzed overnight and used for immunoreactivity analysis.

***Design of synthetic peptides based on antigenicity prediction of Rv2608.*** In silico analysis of Rv2608 revealed the presence of 2 regions of high antigenicity: 2 amino acid stretches (37 and 25 aa) corresponding to important antigenic epitopes within Rv2608 were selected for peptide synthesis (figure 3*A*). An additional 8 overlapping regions (figure 3*B*), which were essentially the subsets of the 2 main peptides, were also selected for peptide synthesis. These peptides were used to map the antigenic domains of the protein. Table 1 shows the amino acid sequences of all 10 synthetic peptides used in the present study. The peptides were part of the C-terminal region of Rv2608 and, apart from the high antigenic index, also possessed the repeat motif Gly-X-Gly-Asn-X-Gly, which is characteristic of the PPE_MPTR gene family.

***Positive reactivity of rPPE protein with serum samples from different categories of patients.*** The humoral response to the

**Table 1. Amino acid sequence of the synthetic peptides.**

| Peptide antigen | Amino acid sequence (no. of residues) |
|---|---|
| P1 | D**N**I**GN**A**N**I**G**F**GN**R**G**DA**N**I**G**I**GN**I**G**DR**N**L**G**I**GN**T**GN**WK (37) |
| P2 | RP**G**LDELSFTLT**GN**P**N**RPD**GG**ILTK (25) |
| P1a | D**N**I**GN**A**N**I**G**F**GN**K (13) |
| P1b | **N**I**G**F**GN**R**G**DA**N**IK (13) |
| P1c | R**G**DA**N**I**G**I**GN**I**G**K (13) |
| P1d | **G**I**GN**I**G**DR**N**L**G**IK(13) |
| P1e | DR**N**L**G**I**GN**T**GN**WK (13) |
| P2a | RP**G**LDELSFTLTK (13) |
| P2b | LSFTLT**GN**PNRPK (13) |
| P2c | **G**NPNRPD**GG**ILTK (13) |

**NOTE.** Residues in bold type represent the glycine-asparagine repeat motifs.

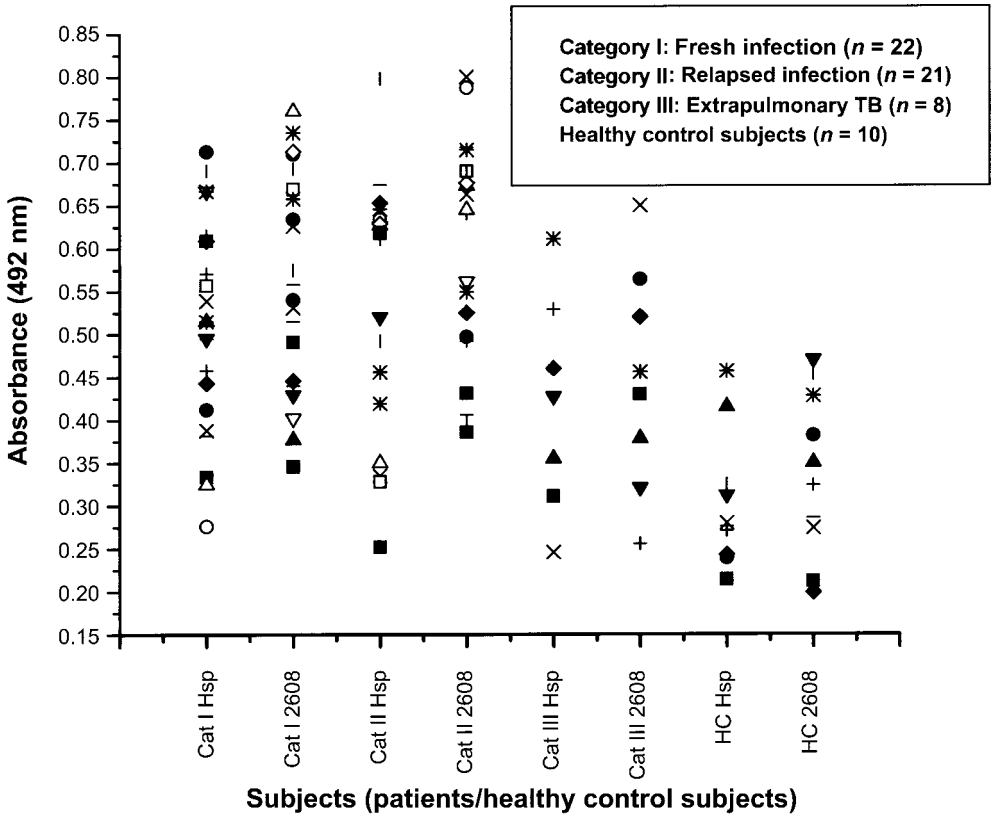



**Figure 4.** Antibody response of different categories of patients with tuberculosis (TB) to recombinant (r) Rv2608, which is equivalent to the response to rHsp10, a well-documented antigen of *Mycobacterium tuberculosis*. Serum reactivity was measured by ELISA, and the graph was plotted as the patient's response (optical density at 492 nm) to rHsp10 and rRv2608. The difference between the response to Hsp10 and that to rRv2608 was not significant for all categories of patients ($P > .05$, paired *t* test). However, the responses of healthy control subjects (HCs) were lower and differed significantly from those of the patients ($P = .0002$, paired *t* test). Cat, category.

rPPE protein was characterized by measuring serum IgG antibodies to the protein by use of ELISA. Antibody response was analyzed as a function of mean absorbance at 492 nm. rHsp10, a major antigen of *M. tuberculosis,* was used for comparison of the response to the rPPE protein. It was observed that, for all categories of patients, serum reactivity to rRv2608 was equal to or higher than the response to Hsp10 ($P > .05$, indicating no difference between the response to Hsp10 and that to Rv2608) (figure 4). Serum samples from healthy control subjects also showed some reactivity to the recombinant protein; however, the response was significantly less, compared with that of patients' serum samples ($P = .0002$, for paired comparison of means between the patients and the healthy control subjects [Student's *t* test]).

***Strong humoral immune response in category II patients elicited by synthetic peptides corresponding to regions of high antigenicity.*** Having shown that the recombinant protein coded by Rv2608 elicited an antibody response that, in all the categories of patients selected for the present study, was equal to or higher than that elicited by Hsp10 antigen, we tried to dissect differential responses, if any, as a function of category of patients. For this, synthetic peptides spanning the 2 major antigenic regions within Rv2608 (P1 and P2) were used in ELISA (table 1). The results suggest that these peptides strongly react with patients' serum samples (figure 5), and, hence, the protein must be generating a strong humoral response in the host. Since a positive response was obtained with peptides 1 and 2, patients' serum samples were also tested for reactivity to the short overlapping peptide sequences 1a, 1b, 1c, 1d, and 1e, which were all components of peptide 1 and 2a, 2b, and 2c, which were a part of peptide 2. The results obtained indicate that even these overlapping peptide stretches react equally well with patients' serum samples. Exact mapping of the antigenic region was not possible, since most of the peptides showed a similar response. This was a reflection of the fact that the Gly-X-Gly-Asn-X-Gly repeat motifs were present in all the peptides. Very interestingly, there was a significantly varied response to the peptides in different categories of patients with TB, which was not seen when the complete rRv2608 protein was used. The peptides could clearly distinguish between the categories of patients ($P < .001$, ANOVA for each peptide antigen) (table 2). Whereas the humoral response observed in category I pa-

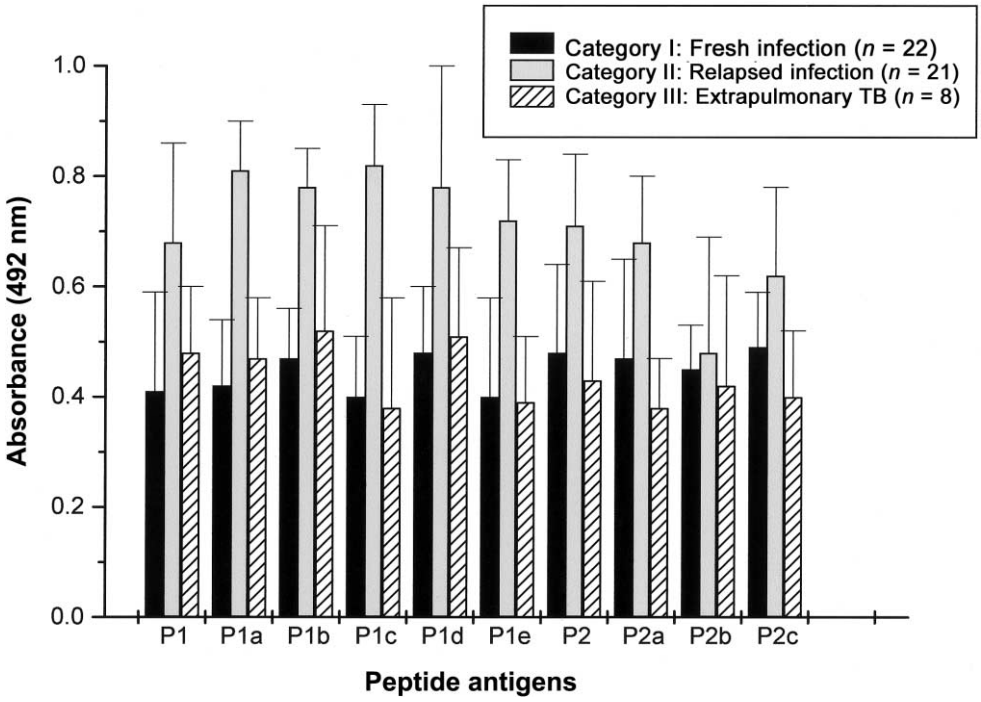



**Figure 5.** Antibody response of different categories of patients with tuberculosis (TB) to different synthetic peptides (regions of high antigenicity within Rv2608), as determined by ELISA. Response to all the peptides was plotted as absorbance at 492 nm (mean ± SD). The response of category II patients was significantly higher than that of category I or III patients ($P < .001$ for both, paired $t$ tests), with respect to each peptide antigen.

tients was similar to that seen in category III patients, category II patients showed an unusually high antibody response to all the peptides. The response of category II patients was significantly higher than that of category I or III patients ($P < .001$, for both [$t$ test]).

***Low T cell response of patients with TB to Rv2608 peptide antigens and lack of difference between various categories of patients.*** T cell proliferation assays were performed to evaluate the response to different synthetic peptides. The overall T cell response of patients to these peptides was very low (SI < 2), and the response could not distinguish between categories of patients ($P > .05$, ANOVA and Kruskal-Wallis test), at least for peptide 1 and its derivatives (data not shown). Peptide 2 and its derivatives exhibited a higher response in category I patients, compared with those in category II and III patients ($P < .05$ for both, for paired comparison of means [$t$ test]). As can be noted from the amino acid sequence of the peptides (table 1), peptide 2 has fewer glycine-asparagine repeats and shows a higher T cell proliferative response in category I patients.

## DISCUSSION

The ORF Rv2608 selected for the present study is a member of the PPE_MPTR class, which is characterized by the presence of a conserved N-terminal region and a C-terminal region with MPTRs of Gly-X-Gly-Asn-X-Gly residues. Apart from this, the ORF also possesses regions of high antigenic index, which is a measure of overall hydrophilicity and surface probability. To test whether polymorphism of the C-terminal region of this ORF exists in different clinical isolates of *M. tuberculosis,* PCR-amplified Rv2608 was subjected to PCR-RFLP analysis. The observed variation in the band pattern lends weight to the hypothesis that PE/PPE genes, notably Rv2608, are a source of antigenic variability in the otherwise conserved genome of *M. tuberculosis.*

The rRv2608 protein was used in ELISAs to determine its reactivity to patients' serum samples. The primordial observation that the recombinant protein reacted with patients' serum samples indicates that this protein is definitely expressed during infection. Serum responses of patients, as well as those of healthy control subjects, to rRv2608 were equivalent to or greater than the response to Hsp10, a well-documented antigen of *M. tuberculosis* [17]. Although category-wise differentiation of serum reactivity to the full-length recombinant protein was not very apparent, it was significant that serum from category III patients showed less reactivity to rRv2608 protein than that from category I or II patients ($P = .048$). It will be worthwhile to explore whether Rv2608 represents a protein required by the bacterium to establish a pulmonary infection.

Since the serum response to the rPPE protein was equal to or greater than that to Hsp10, it was decided to attempt to map the antigenic domains of the probable PPE antigen by use of a synthetic-peptide approach [18–20]. Peptides correspond-

**Table 2. Summary of the results of statistical analyses to estimate differences in humoral immune response to different peptide antigens.**

| Peptide antigen, category of patients | OD at 492 nm (95% CI), mean | *df* | *F* | *P* | Difference between categories |
|---|---|---|---|---|---|
| P1 | | 2 | 12.69 | <.0001 | S |
| I | 0.412 (0.338–0.486) | | | | |
| II | 0.675 (0.598–0.750) | | | | |
| III | 0.483 (0.359–0.606) | | | | |
| P1a | | 2 | 29.69 | <.0001 | S |
| I | 0.426 (0.360–0.492) | | | | |
| II | 0.770 (0.704–0.836) | | | | |
| III | 0.473 (0.363–0.582) | | | | |
| P1b | | 2 | 35.47 | <.0001 | S |
| I | 0.469 (0.416–0.521) | | | | |
| II | 0.775 (0.720–0.828) | | | | |
| III | 0.520 (0.432–0.607) | | | | |
| P1c | | 2 | 70.48 | <.0001 | S |
| I | 0.416 (0.364–0.467) | | | | |
| II | 0.810 (0.756–0.862) | | | | |
| III | 0.380 (0.293–0.464) | | | | |
| P1d | | 2 | 33.47 | <.0001 | S |
| I | 0.482 (0.427–0.535) | | | | |
| II | 0.787 (0.731–0.842) | | | | |
| III | 0.527 (0.437–0.617) | | | | |
| P1e | | 2 | 31.26 | <.0001 | S |
| I | 0.407 (0.348–0.464) | | | | |
| II | 0.711 (0.651–0.770) | | | | |
| III | 0.405 (0.308–0.501) | | | | |
| P2 | | 2 | 14.51 | <.0001 | S |
| I | 0.480 (0.413–0.546) | | | | |
| II | 0.706 (0.637–0.774) | | | | |
| III | 0.441 (0.331–0.551) | | | | |
| P2a | | 2 | 18.63 | <.0001 | S |
| I | 0.473 (0.412–0.533) | | | | |
| II | 0.689 (0.627–0.750) | | | | |
| III | 0.392 (0.291–0.491) | | | | |
| P2b | | 2 | 0.7688 | .4 | NS |
| I | 0.450 (0.379–0.521) | | | | |
| II | 0.498 (0.425–0.570) | | | | |
| III | 0.422 (0.304–0.540) | | | | |
| P2c | | 2 | 10.05 | .0002 | S |
| I | 0.491 (0.433–0.548) | | | | |
| II | 0.632 (0.572–0.690) | | | | |
| III | 0.410 (0.314–0.505) | | | | |

**NOTE.** NS, not significant; OD, optical density; S, significant.

ing to regions of high antigenic index were accordingly designed. Our analyses of the comparative humoral immune responses indicate that the serum responses of patients to all 10 peptides are similar. This could be explained by the fact that all the peptides have a common repeat motif, thereby eliciting similar responses. Although this negated our efforts to map the immunodominant epitope required for eliciting a strong humoral immune response, a difference in the response of patients categorized according to different states of infection was surprisingly evident. Category II patients demonstrated the highest B cell responses to the peptides.

The synthetic peptides were also used for T cell–proliferation assays with PBMCs from patients in all categories. It has been shown elsewhere that, in ~90% of patients with active TB, there is a significant antibody response and/or T cell–proliferative response to peptide-specific single antigens of *M. tuberculosis* [21]. The 38-kDa antigenic protein of *M. tuberculosis* is a potent stimulus for both T cell and B cell responses in humans [22, 23]. The T cell–proliferative response to the synthetic peptides was of the order of the following: category I > category II > category III, at least for peptide 2 and its derivatives. However, the observed SI with all the peptides was very low in all categories of patients with TB (SI < 2). A high humoral response and a low cellular immune response to the peptides

in category II patients points to an important possible function of the PE/PPE gene families. It is likely that these antigens play a role in evading the host immune response and prevent the establishment of an effective cellular response, which is required to contain the disease. The positive T cell response in some cases could be explained by the fact that IgG antibody responses require the involvement of helper T cells.

Antibody levels usually decrease in patients cured of TB but dramatically increase in patients showing poor compliance [24]. High antibody response to the peptides and a low T cell response, hence, explain the relapse of infection in category II patients. In vivo, it is possible that the responsive T cells are not able to expand, since the glycine-asparagine repeat motifs somehow prevent antigen processing. The situation can be equated with the Epstein-Barr virus nuclear antigen, in which, again, the Gly-Ala repeat regions play an important role in preventing antigen processing [25]. Peptides 2 and 2c, which have fewer Gly-Asn repeats, show a comparatively higher T cell response.

In conclusion, we have been able to establish a relationship between immune responses to the PPE antigen and the status of the disease (fresh infection or relapsed infection). The present study is the first report that has demonstrated, in a clinical setting, that the repeat sequences present within Rv2608 elicit a high humoral immune response and a low T cell response. Since PPE_MPTR is a gene family of *M. tuberculosis,* of which Rv2608 is a member sharing the MPTR motif, it is likely that other members of the same family also serve the same function in the bacterium. Our data contribute toward a better understanding of humoral, as well as cellular, immune responses elicited by PPE antigens. The practical utility of using these peptides for differentiating fresh infections from relapsed or reactivated infections is another interesting proposition.

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