In Silico Prediction of Peptides Binding to Multiple HLA-DR Molecules Accurately Identifies Immunodominant Epitopes from gp43 of *Paracoccidioides brasiliensis* Frequently Recognized in Primary Peripheral Blood Mononuclear Cell Responses from Sensitized Individuals

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One of the major drawbacks limiting the use of synthetic peptide vaccines in genetically distinct populations is the fact that different epitopes are recognized by T cells from individuals displaying distinct major histocompatibility complex molecules. Immunization of mice with peptide (181-195) from the immunodominant 43 kDa glycoprotein of *Paracoccidioides brasiliensis* (gp43), the causative agent of Paracoccidioidomycosis (PCM), conferred protection against infectious challenge by the fungus. To identify immunodominant and potentially protective human T-cell epitopes in gp43, we used the TEPITOPE algorithm to select peptide sequences that would most likely bind multiple HLA-DR molecules and tested their recognition by T cells from sensitized individuals. The 5 most promiscuous peptides were selected from the gp43 sequence and the actual promiscuity of HLA binding was assessed by direct binding assays to 9 prevalent HLA-DR molecules. Synthetic peptides were tested in proliferation assays with peripheral blood mononuclear cells (PBMC) from PCM patients after chemotherapy and healthy controls. PBMC from 14 of 19 patients recognized at least one of the promiscuous peptides, whereas none of the healthy controls recognized the gp43 promiscuous peptides. Peptide gp43(180-194) was recognized by 53% of patients, whereas the other promiscuous gp43 peptides were recognized by 32% to 47% of patients. The frequency of peptide binding and peptide recognition correlated with the promiscuity of HLA-DR binding, as determined by TEPITOPE analysis. In silico prediction of promiscuous epitopes led to the identification of naturally immunodominant epitopes recognized by PBMC from a significant proportion of a genetically heterogeneous patient population exposed to *P. brasiliensis*. The combination of several such epitopes may increase the frequency of positive responses and allow the immunization of genetically distinct populations.

INTRODUCTION

Recent advances in peptide biochemistry and immunochemistry have led to the development of protective antimicrobial vaccines based on synthetic peptides and defined epitopes. Immunization with synthetic peptides has been widely used in animal models (1–3), and the relative ease, low cost of preparation, safety, prolonged shelf-life, and the ability to focus on defined epitopes (4–7) encouraged the use of synthetic peptide vaccines in animal (8–14) and human diseases (15,16).

The central event in the adaptive immune response to invasive microorganisms is the specific recognition of intracellularly processed foreign antigenic peptides bound to the peptide-binding region of the human leukocyte antigen (HLA) class II molecules on the surface of antigen-presenting cells by the T-cell receptor of CD4+ T cells. This is followed by activation, proliferation,

and differentiation of specific CD4+ T cells to effector cells that are fully capable of interacting with other inflammatory cells and thus inducing specialized effector immune responses. Pathogen-induced activation of CD4+ T cells can generate memory T cells, which will be ready to respond more rapidly in case of a later contact with the same pathogen.

To induce protective immunity, epitopes contained in synthetic peptide vaccines must match epitopes naturally presented to the immune system during infection, be recognized by the entire human population targeted for vaccination, and induce an effector immune response capable of effectively eliminating the pathogen. Single epitope-based vaccines may, however, have some drawbacks. On the pathogen side, the monospecificity of the induced immune response favors the emergence of sequence mutants that can escape from the vaccine's protective effect (7). On the host side, it is unlikely that T cells from large proportions

of genetically distinct populations can recognize—and therefore be protected—by the same single peptide epitope. This is secondary to the wide polymorphism of HLA molecules that present antigenic peptides to T cells. Because the antigen-binding groove of each of the hundreds of allelic HLA molecules has distinct peptide-binding preferences, a distinct set of epitopes from a given protein antigen will be presented to T cells in each individual bearing a different HLA molecule. Additionally, some HLA molecules may not be able to bind at all to any of the peptides derived from a given protein (17,18).

Such interindividual variation of antigen recognition can be a problem for selecting immunogens for peptide vaccines, because they must contain immunodominant epitopes recognized by individuals with a wide range of different HLA molecules. The identification of single peptides that can bind to multiple HLA types, the so-called "promiscuous" epitopes, could lead to effective coverage of the human population by a peptide-based vaccine. Thus, the major challenge of a peptide-based vaccine is the identification of 1 or several "promiscuous" epitopes that could bind to many HLA alleles and thus cover close to 100% of a genetically diverse human population (19).

Until very recently, the search for immunodominant peptides relied on the direct testing of substantial numbers of overlapping peptides or peptide libraries. The identification of major histocompatibility complex (MHC)-binding motifs allowed the prediction of potential T cell epitopes (20,21), and such motifs were found to cluster in certain protein regions (22). The TEPITOPE algorithm, that predicts binding to 25 distinct HLA-DR molecules based on quantitative matrices established from HLA-DR binding assays (18,23), leads to the selection of high affinity-binding peptides, those with the highest chance of eliciting effective T-cell responses against immunogens (24). Additionally, TEPITOPE also allows detection of sequences predicted to bind to several HLA-DR molecules simultaneously, opening the possibility of selection of promiscuous T-cell epitopes. This approach has been used to successfully identify allele-specific and promiscuous T-cell epitopes (25–35). However, no study has addressed PBMC recognition of TEPITOPE-predicted promiscuous peptides in a genetically heterogeneous group previously exposed to an infectious agent.

PCM is a prevalent human systemic mycosis in Latin America where 90 million people live in endemic areas and almost 10 million may be infected with the fungus *Paracoccidioides brasiliensis* (36–38). Cellular immunity seems to be the major defense mechanism in both experimental and human PCM (39,40). Patients with active disease have high levels of anti–*P. brasiliensis* antibodies (41–45) as well as transient antigen-specific cell-mediated immunosupression (43), whereas conversion to positive delayed-type hypersensitivity (DTH) skin test and reduction in the antibody levels are useful parameters for successful chemotherapy and cure.

The immunodominant 43 kDa glycoprotein gp43 is the major diagnostic antigen from *P. brasiliensis* (46–48), being recognized by sera from patients with active PCM (49,50). Treated and healed PCM patients display positive DTH to *P. brasiliensis* gp43 (51). Gp43-immunized Balb/c (H-2^d), A/Sn (H-2^a), and C57bl/6

(H-2^b) mice induce lymph node cells to proliferate and secrete interferon-γ and interleukin (IL)-2 but not IL-4, IL-5 or IL-10, when stimulated by the immunogen, indicating a T helper cell (Th)1 response (2). Epitope mapping of the entire gp43 identified peptide P10 (gp43[181-195]) as that carrying the immuno-dominant epitope in lymphocyte proliferation assays. Immunization of Balb/c mice with either purified gp43 or P10 was protective against subsequent intratracheal challenge by virulent *P. brasiliensis* (2). Furthermore, peptide P10 (gp43 [181-195]) does not elicit an antibody response, which is not protective, and may down-regulate the cellular immune response (2).

To identify the immunodominant epitopes in the gp43 of *P. brasiliensis* for immune response in humans, we used the TEPITOPE algorithm to select several different sequences that were predicted to bind to multiple HLA-DR molecules (promiscuous epitopes) and tested their binding to multiple HLA-DR molecules in peptide-binding assays and recognition in primary PBMC responses. Additionally, we assessed the specificity and sensitivity of the prediction by TEPITOPE, selecting peptides that were predicted not to bind to any of the HLA-DR molecules. Peptides were synthesized and tested in proliferation assays with PBMC from nonanergic treated and healed, genetically heterogeneous PCM patients. The antigen-induced primary in vitro PBMC proliferation assay detects the "central" memory T cells expanded by previous exposure to the pathogen (52). In the proliferation assay, one measures the DNA synthesis in lymphocytes after incubation with the antigen, which is presented by monocytic cells expressing MHC class II molecules. Presentation occurs after endocytosis and processing of proteins like gp43, or by direct binding of a synthetic peptide antigen to HLA-DR, -DP, or -DQ molecules on the cell surface of human antigen presenting cells. Peptide-induced assays with PBMC from sensitized individuals can lead to the identification of naturally presented epitopes. With the aid of the TEPITOPE algorithm, we successfully identified multiple promiscuous, naturally presented immunodominant CD4$^+$ T cell epitopes in the gp43 glycoprotein from *P. brasiliensis*.

MATERIALS AND METHODS

Patients and Healthy Individuals

Heparin-treated venous blood for PBMC isolation and EDTA-treated venous blood for DNA extraction were obtained from clinically healed PCM patients represented by low or negative anti–*P. brasiliensis* antibody titers with positive cutaneous test for paracoccidiodin. Patients originally diagnosed after direct fungal identification on lesion samples underwent chemotherapy with either ketoconazole or itraconazole or a combination of trimethoprim with sulfadiazine or sulfamethoxazole prior to the current study. All patients were followed at the Department of Infectious and Parasitic Diseases, University Hospital, University of São Paulo Medical School. Healthy volunteers, paired by age and gender to the patients, were also tested. All subjects gave their written informed consent to participate in this study, which was approved by the Internal Review Board of the University of São Paulo Medical School.

Table 1. Synthetic peptides from gp43 of *P. brasiliensis* used in this study

Number[a]	Peptide	Sequence
1	gp43 (45-59)	IGGWLLLEPWISPSV-NH$_2$
2	gp43 (94-108)	TEDDFKNIAAAGLNHV-NH$_2$
3	gp43 (106-120)	LNHVRIPIGYWAVNP-NH$_2$
4	gp43 (283-298)	IDQHVKLACSLPHGRL-NH$_2$
5	gp43 (180-194)	KQTLIAIHTLAIRYA-NH$_2$
6	gp43 (183-197)	LIAIHTLAIRYANRT-NH$_2$
7	gp43 (179-199)	IKQTLIAIHTLAIRYANRTDV-NH$_2$
8	gp43 (181-195)[b]	QTLIAIHTLAIRYAN-NH$_2$
9	gp43 (118-132)	VNPIEGEPYVQGGQLD-NH$_2$
10	gp43 (161-175)	GHRGAINWQHGDTIK-NH$_2$
11	gp43 (244-258)	DASLPPRTWNGFLAP-NH$_2$
12	gp43 (259-271)	KTYKNVYIDTYHN-NH$_2$
13	gp43 (347-360)	SKGSSSELSAQQKK-NH$_2$

[a]1-5: selected peptides that were predicted to bind to a large number of HLA-DR molecules. 6-8: peptides neighboring peptide gp43(181-195), which is immunodominant and conferred protection in mice infected intratracheally with a virulent strain of *P. brasiliensis*. 9-13: peptides predicted not to bind to any of the 25 HLA-DR molecules. [b]Corresponds to the P10 peptide of Taborda and others (2).

Peptide Selection

The amino acid sequence of *P. brasiliensis* gp43 glycoprotein (Genbank accession number AY005437) was scanned by a TEPITOPE algorithm that can predict, after scanning all 9-mer windows starting with a hydrophobic residue on a protein sequence, those sequences that have a potential ability to bind to 1 or more of 25 different HLA-DR molecules by use of 25 virtual matrices that cover most of the HLA class II peptide binding specificities in the Caucasian population (18). Briefly, the algorithm incorporates, for each of the 25 HLA-DR molecules, a matrix of values for each amino acid residue at each position, p2 to p9. Position- and residue-specific matrix values were assembled from empirical HLA-DR peptide binding assays (53). The algorithm provides a score—the algebraic sum of the matrix values for each peptide position—to each of the 9-mer windows along the scanned sequence. Nonamers attaining a score above the threshold for a given HLA-DR molecule (for example, a 3% threshold selects sequences with HLA-binding scores equal to or higher than those of the 3% sequences with highest scores in the TEPITOPE database) are selected by the software. The algorithm also allows the selection of sequences predicted to bind simultaneously and thus, promiscuously, to several HLA-DR molecules (23). Peptides predicted to bind to the largest number of HLA molecules at the highest threshold (1%) in addition to displaying binding to an increased number of distinct HLA-DR molecules (3% threshold) were selected (Table 1). We synthesized peptides that correspond to an inner nonamer core selected by TEPITOPE as the HLA-binding motif with flanking amino acids added at both N- and C-terminal ends, to increase the efficiency of in vitro peptide presentation to CD4$^+$ T cells (23).

Synthetic Peptides

All peptides (see Table 1) derived from gp43 antigen were synthesized by solid phase technology using 9-fluorenylmethoxycar-bonyl (Fmoc) strategy (54) on an automated benchtop simultaneous multiple solid-phase peptide synthesizer PSSM8 (Shimadzu, Tokyo, Japan) with Fmoc protected amino acid residues and TGR resin (Novabiochem, San Diego, CA, USA). Therefore, all peptides were obtained with the C-terminal carboxyl group in amide form. All peptides were deprotected and cleaved from the resins by treatment with K reagent composed by 80% trifluoroacetic acid, 2.5% triisopropylsilane, 2.5% ethanedithiol, 5.0% anisol, 5.0% water, and 5.0% phenol (55). The resulting peptides were analyzed by reverse-phase high performance liquid chromatography (Shimadzu, Tokyo, Japan) on a C18 column eluted at 1 mL/min using 5% to 95% gradient of acetonitrile 90% in 0.1% TFA over 30 min. Peptide quality was assessed by Matrix Assisted Laser Desorption and Ionization—Time of Flight instrument (Micromass, Manchester, UK) using a-cyano-4 hydroxy cinnamic acid as the matrix.

Proliferation Assay

PBMC were isolated from peripheral blood by density gradient centrifugation (d = 1.077). Cells were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% normal human serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μg/mL gentamicin, and 10 mM HEPES buffer, in triplicate 96-well U-bottom culture plates (10^5 cells/well; final volume 0.2 mL) with gp43 (1 and 10 μg/mL), and synthetic peptides (0.1; 1.0 and 10.0 μM). Plates containing phytohaemagglutinin (PHA; 2.5 mg/mL) and complete culture medium were used as positive and negative controls, respectively. In some assays, we tested the proliferative responses of whole or CD4$^+$ T-cell–depleted PBMC from patients to gp43 and derived peptides. Plates were incubated in 5% CO$_2$ at 37 °C for 5 d, and cultures were pulsed with 1 μCi/well (H^{3+})-thymidine (Amersham, Buckinghamshire, UK) for the next 18 h (H^{3+})-thymidine incorporation was determined with a Betaplate β counter (Wallac, Turku, Finland). Data are represented as mean counts per min (cpm) of triplicate cultures and the stimulation index (SI) defined as mean cpm response value with antigen/mean cpm of culture medium control. SI values ≥ 2.0 were considered positive.

CD4$^+$ T Lymphocyte Depletion and Flow Cytometry Analysis

CD4$^+$ T cells were depleted from PBMC with Dynabeads M-450 (Dynal, Oslo, Norway) with shaking at 4 °C for 30 min and washed with 0.1% phosphate buffered saline–bovine serum albumin. The efficiency of T-CD4$^+$ depletion was evaluated by flow cytometry analysis with a FACScan (Becton Dickinson, San Jose, CA, USA). Antibody conjugates used included anti–CD4-PE, anti–CD8-FITC, and anti–CD3-FITC (Dakopatts, Glostrup, Denmark), with anti-β2 microglobulin-FITC (56) and anti–HBS-FITC (57) used as positive and negative controls, respectively, as previously described (58). Purity of cell populations is expressed as percentage of stained cells.

HLA Class II Typing

DNA was extracted alternatively by DTAB/CTAB (59) or salting out (60) methods and HLA-DR typing was performed by low resolution PCR-SSP (61).
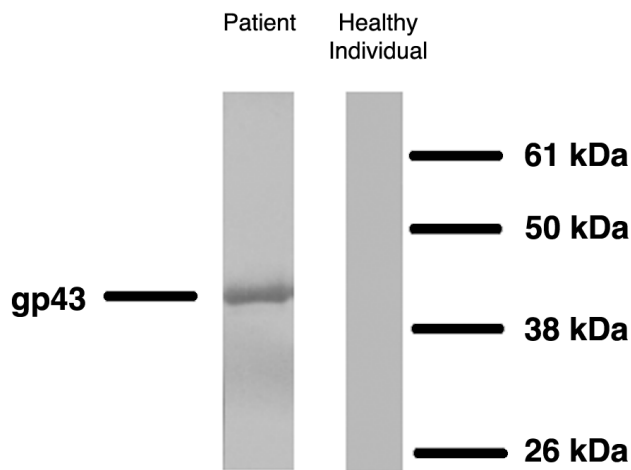
Figure 1. Western blotting of PCM patients' sera and a healthy individual against gp43 of *P. brasiliensis*. Serum dilution 1:40; gp43 concentration used: 2.5 μg/mL.

Immunoblotting

Heparin-treated plasma from PCM patients or healthy controls was tested for anti-gp43 IgG responses. Briefly, purified *P. brasiliensis* gp43 (2) was resolved in 10% acrylamide mini-gels (15 mA, constant amperage) using a Hoefer mini-VE Electrophoresis Unit (Pharmacia Biotech, Uppsala, Sweden), and electroblotted onto nitrocellulose membranes. Blots were blocked for 2 h at room temperature with 5% nonfat dry milk in TBS-Tween (Tris-buffered saline; 10 mM Tris, pH 7.5; 100 mM NaCl; 0.1% Tween-20) prior to incubation for 2 h at room temperature with plasma from PCM patients and healthy individuals diluted at 1:40. After serum incubation, membranes were extensively washed with several changes of TBS-Tween-5% milk and then probed for 2 h at room temperature with anti-human IgG conjugate with alkaline phosphatase at a dilution of 1:1000. Membranes were washed with several changes of T-TBS and processed for detection by o-phenylenediamine substrate. In all blots, bands corresponding to the protein of interest were identified by reference to molecular weight protein standards (markers) run in parallel and were scanned using a conventional scanner.

ELISA Analysis

Anti-gp43 antibodies were measured using ELISA with gp43 in the solid phase. Briefly, Corning polypropylene 96-well microtiter ELISA plates (Corning, New York, USA) were sensitized with gp43 (12.5 ng/well), overnight at 4 °C. After blocking with 2% bovine serum albumin, serum samples (diluted 1:100 in phosphate-buffered saline) from 16 PCM patients and 6 healthy individuals were incubated for 2 h at 37 °C in duplicate wells. The reaction was developed using an anti-human IgG-peroxidase conjugated with o-phenylenediamine as chromogenic substrate. The absorbance was subsequently measured at 490 nm.

Class II Peptide-Binding Assays

HLA class II molecules were purified from Epstein-Barr virus transformed homozygous B lymphoblastoid cell lines or trans-

fected fibroblasts by affinity chromatography, as previously described (14). Peptide binding assays were performed by incubating purified human class II molecules (5 to 500 nM) with various concentrations of unlabeled peptide inhibitors and 1 to 10 nM ^{125}I-radiolabeled probe peptides for 48 h in phosphate buffered saline containing 0.05% to 0.15% Nonidet P-40 in the presence of a protease inhibitor cocktail (4,14). Class II peptide complexes were separated from free peptide by gel filtration on TSK200 columns (part number 16215; Tosoh Biosciences LLC, Montgomeryville, PA, USA) and the fraction of bound peptide calculated. Alternatively, the percent of MHC bound radioactivity was determined by capturing MHC/peptide complexes on LB3.1 antibody coated Lumitrac 600 plates (Greiner Bio-one, Frickenhausen, Germany), and determining bound cpm using the TopCount (Packard Instrument Co., Meriden, CT, USA) microscintillation counter.

The radiolabeled probe peptides utilized were HA Y307-319 (sequence YPKYVKQNTLKLAT; DRB1*0101), an analog of TT Y828-843 (sequence YATFFIKANSKFIGITE; DRB5*0101, DRB1*1101, DRB1*0701), MBP Y85-100 (sequence PVVHF-FKNIVTPRTPPY; DRB1*1501, DRB1*0401, DRB1*0405), and an analog of TT 830-843 (sequence QYIKANAKFIGITE; DRB1*1302).

Statistical Analysis

Comparison of SI and absorbance values between clinical groups was performed with nonparametric Mann-Whitney's U test. Correlations between TEPITOPE prediction, binding assays, and proliferation assays were performed with the nonparametric Spearman correlation test with 95% confidence intervals.

RESULTS

Anti-gp43 IgG Antibody Responses in Patients and Controls

Nineteen patients with PCM (21 to 75 y old) and 6 healthy controls (30 to 59 y old) were studied. All tested PCM patients, but none of the control individuals, displayed IgG antibodies against *P. brasiliensis* gp43 on immunoblotting assay (Figure 1). The median absorbance of anti gp43 IgG detected by ELISA was 0.850 in patients (Abs$_{490}$ = 0.224 to 1.711) and 0.059 in healthy individuals (Abs$_{490}$ = 0.027 to 0.176) ($P < 0.0002$).

Peptide Selection, Binding Analysis, and T Lymphocyte Response to *P. brasiliensis* gp43 and Synthetic Peptides

The entire sequence of *P. brasiliensis* gp43 (416 residues) was scanned by the TEPITOPE algorithm at 3% threshold, which led to the identification of regions predicted to bind with high affinity to several different HLA-DR molecules (TEPITOPE promiscuous region scanning profile for gp43 in Figure 2). Peptides predicted to bind to the largest number of HLA molecules at the highest threshold (1%) in addition to displaying binding to an increased number of distinct HLA-DR molecules (3% threshold) were selected (see Table 1): gp43(45-59), gp43(94-108), gp43(106-120), gp43(283-298), and gp43(180-194) that bound to 18, 12, 5, 15, and 21 HLA-DR molecules, respectively. The gp43(180-194) peptide was predicted to be the most promiscuous ligand, binding to
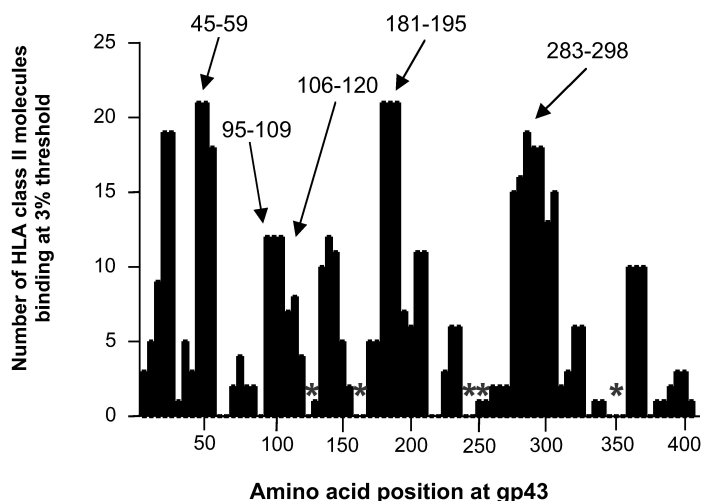
Figure 2. Scanning of gp43 of *P. brasiliensis* by TEPITOPE algorithm at 3% threshold. Five peptides marked with arrows, predicted to bind to at least 10 of the 25 HLA-DR molecules included in the algorithm, were selected as promiscuous epitopes. Peptides marked with asterisks were predicted not to bind significantly to any HLA-DR molecules and were used as peptide controls.
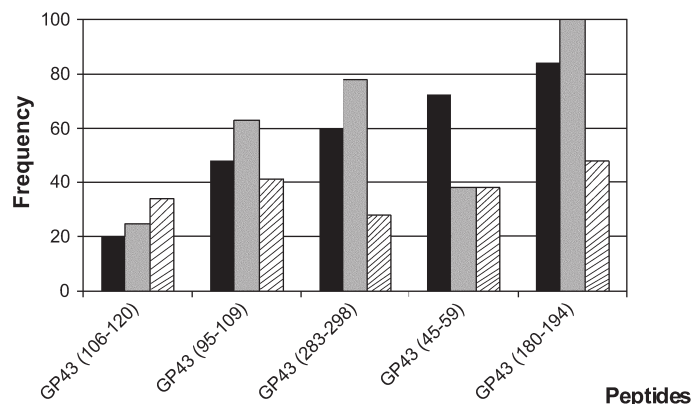

Figure 3. Predicted promiscuity of peptides for binding with high affinity to different HLA-DR molecules at 3% threshold (■); frequency of peptides binding to 9 different HLA-DR molecules (▨); frequency of responders to peptides tested in proliferation assay with PBMC of 19 treated and healed nonanergic PCM patients (▨).

84% (21/25) of the HLA-DR molecules at 3% and 36% (12/25) of the HLA-DR molecules at 1% threshold (data not shown).

Binding assays of these peptides with the 9 most prevalent HLA-DR molecules in the general population, showed that gp43(180-194) bound to 100% (9/9) of the HLA-DR molecules followed by gp43(283-298), gp43(94-108), gp43(45-59), and gp43(106-120) that bound to 89% (8/9), 56% (5/9), 33% (3/9), and 22% (2/9) of the tested molecules, respectively (Table 2).

From the PBMC samples of the 19 patients tested, 14 responded to 1 to 5 peptides, whereas 5 failed to respond to any peptide (Table 3); PBMC from healthy controls who did not respond to the gp43 failed to respond to any of the peptides (Table 4). The distribution of HLA-DR molecules predicted by TEPITOPE to bind to the gp43 peptides was similar between the peptide responder and non-responder PCM patient groups (Table 5). We observed that depletion of CD4^{+} T cells from PBMC from

patients p24 and p25 completely abrogated the proliferative response against native gp43 protein and all gp43-derived peptides tested (data not shown). Interestingly enough, PBMC samples from the PCM patients that failed to proliferate to gp43 peptides also showed significantly lower proliferative responses against purified gp43 (median SI = 9.8) than gp43 peptide-reactive PCM patients (median SI = 78.3; P = 0.0026). However, median PHA responses did not differ significantly between groups.

Considering positive responses to any of the 3 peptide concentrations tested, gp43(180-194) was the most frequently recognized peptide (53%), followed by gp43(94-108), gp43(45-59), gp43(283-298), and gp43(106-120) at frequencies of 42%, 37%, 32%, and 32%, respectively. We observed that the frequency of responders to each peptide was commensurate with the promiscuity of HLA-DR binding predicted by TEPITOPE (Figure 3). Moreover, we observed that the frequency of peptide responses accurately predicted by TEPITOPE, taking into account the HLA-DR molecules carried by each patient, varied from 40% to 80% of all observed responses. On the other hand, the proportion of

Table 2. Peptide binding analysis of the 5 peptides selected by TEPITOPE with 9 most prevalent HLA-DR molecules in the population[a]

	Binding capacity/IC50% (nM)										
Peptides	DR1 DRB1 *0101	DR3 DRB1 *0301	DR4w4 DRB1 *0401	DR4w15 DRB1 *0405	DR7 DRB1 *0701	DR5w11 DRB1 *1101	DR6w19 DRB1 *1302	DR2w2 B1 DRB1 *1501	DR2w2 B2 DRB5 *0101	Molecules bound	Freq[b]
gp43(45-59)	**0.87**	–	1337	**122**	1795	1349	40767	**3.8**	1509	3	33%
gp43(95-108)	**0.49**	–	6449	**241**	**51**	17790	**80**	7662	**11**	5	56%
gp43(106-121)	**25**	–	5775	5248	2347	1852	33333	**31**	5466	2	22%
gp43(180-194)	**0.27**	**93**	**37**	**33**	**8.8**	**4.8**	**2.7**	**6.3**	**2.6**	9	100%
gp43(283-298)	**470**	3427	**135**	**59**	**92**	**106**	1275	**506**	**23**	7	89%

[a]The results are expressed in IC50% (nM). Peptide binding assays were performed by incubating purified human class II molecules (5 to 500 nM) with various concentrations of unlabeled test peptides and ^{125}I-radiolabeled probe peptides. Bold numbers = significant affinity threshold below 1000; dash (–) means IC50% > 50000.
[b]Freq = frequency.

Table 3. Proliferative response of PBMC from PCM patients to 5 gp43 peptides selected by TEPITOPE program[a]

Patients[b]	gp43 (45-59)[c]			gp43 (94-108)[c]			gp43 (106-120)[c]			gp43 (283-298)[c]			gp43 (180-194)[c]			gp43 (1 µg)	gp43 (10 µg)	PHA
	0.1	1.0	10.0	0.1	1.0	10.0	0.1	1.0	10.0	0.1	1.0	10.0	0.1	1.0	10.0			
p1	3.2	17.5	14.9	–	2.6	2.7	4.5	3.9	4.5	3.0	2.5	8.4	5.0	12.0	8.1	210.6	189.4	2368.6
p2	–	–	–	2.8	5.2	3.9	2.0	2.1	–	2.8	8.3	2.9	3.2	5.3	4.3	26.9	48.9	16.9
p3	5.7	3.8	–	3.4	4.6	–	2.9	2.3	–	3.3	2.9	–	3.1	9.2	–	5.0	13.3	243.0
p4	2.0	–	–	–	2.1	–	2.0	–	–	–	2.1	3.0	2.6	3.5	2.2	40.8	61.5	1181.7
p5	–	–	6.8	–	2.4	2.6	–	–	–	–	–	–	–	2.3	3.5	25.6	81.5	121.6
p6	4.2	4.5	–	–	–	–	–	–	–	–	–	–	10.6	4.5	–	88.3	123.1	710.7
p7	–	–	–	3.9	–	–	12.0	2.6	–	–	–	–	–	–	–	19.4	31.7	761.3
p8	–	–	–	–	–	2.0	–	–	–	–	–	–	–	2.3	6.8	30.6	185.8	977.9
p9	–	–	–	–	–	–	–	–	–	–	2.2	5.4	–	–	–	23.4	51.7	293.1
p10	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2.6	11.6	102.7	261.6
p11	–	–	–	–	2.2	–	–	–	2.3	–	–	–	–	3.4	–	29.8	71.3	340.1
p12	2.0	–	–	–	–	–	–	–	–	–	–	–	–	2.8	–	29.8	99.5	803.8
p13	–	–	–	–	–	–	–	–	–	–	–	2.1	–	–	–	3.8	22.0	236.7
p14	–	–	3.4	–	–	–	–	–	–	–	–	–	–	–	–	8.8	13.9	2205.8
p15	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	19.9	6.9	261.4
p16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3.2	7.2	53.4
p17	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	15.0	15.5	162.4
p18	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2.3	7.0	106.5
p19	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	18.1	12.6	740.0

[a]Only positive stimulation index values (SI ≥ 2) are shown. Dash (–) corresponds to SI < 2.
[b]p = patients.
[c]PBMC (10^5 cells/well) were stimulated with peptides at 0.1, 1.0, and 10.0 µM as described.

observed responses not predicted by TEPITOPE varied from 20% to 60% (see Table 3 and data not shown).

Furthermore, we observed that for most of the TEPITOPE-selected peptides, at least 50% of the responder patients recognized the peptide at the lowest concentration (0.1 mM): peptides gp43(106-120) by 5 of 6 responding patients, gp43(45-59) by 5 of 7, gp43(180-194) by 5 of 10, gp43(283-298) by 3 of 6, and gp43(94-108) by 3 of 8, consistent with high avidity T-cell recognition. Peptide gp43(180-194) was the peptide with the highest average SI at 0.1 mM concentration (4.9) followed by gp43(106-120), gp43(45-59), gp43(94-108), and gp43(283-298) with 4.7, 3.4, 3.3, and 3.0 average SI, respectively. Analysis of the HLA-DR profile from patients that responded to any peptide indicated that a wide diversity of HLA-DR molecules was represented among the responders for each peptide and few HLA-DR molecules were only expressed among non-responders (see Table 5).

In addition, we selected 5 gp43 peptides that were predicted not to bind at 3% threshold to any HLA-DR molecule: gp43(118-132), gp43(161-175), gp43(244-258), gp43(259-271), and gp43(347-360) (marked with asterisks in Figure 2 and shown as peptides 9 to 13 in Table 1). The binding assays of these peptides showed that peptides gp43(244-258) and gp43(347-360) failed to bind to any of the 9 HLA-DR molecules tested, peptide gp43(259-271) bound to 11% (1/9) and peptides gp43(118-132) and gp43(161-175) showed significant binding to 33% (3/9) of the HLA-DR molecules tested (Table 6). Four of 5 peptides were recognized by 10% to 20% of the

Table 4. Proliferation response of PBMC from healthy individuals to 5 gp43 peptides selected by TEPITOPE program[a]

Controls[b]	gp43 (45-59)[c]			gp43 (94-108[c]			gp43 (106-120)[c]			gp43 (283-298)[c]			gp43 (180-194)[c]			gp43 1 µg	PHA
	0.1	1.0	10.0	0.1	1.0	10.0	0.1	1.0	10.0	0.1	1.0	10.0	0.1	1.0	10.0		
c1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	234.1
c2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	439.2
c3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	232.1
c4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	216.4
c5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	353.2
c6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	545.3

[a]Only positive stimulation index values (SI ≥ 2) are shown. Dash (–) corresponds to SI < 2.
[b]c = healthy control individual.
[c]PBMC (10^5 cells/well) were stimulated with peptides at 0.1, 1.0, and 10.0 µM as described.

Table 5. HLA-DR molecules carried by responders and nonresponders to each gp43 peptide selected by TEPITOPE[a]

| | | HLA-DR | | | | | | | | | | | | Response[b] |
		4	7	8	9	10	11	13	14	15	16	17	Blank	
gp43(45-59)	Responder	2	2	1	1		1	2		1	2	2		10/11
	Nonresponder			1				4	1			2	2	
gp43(94-108)	Responder	2	1	2	1		1	4		1	1	2	1	10/11
	Nonresponder		3		1		3	1		1	2	1		
gp43(106-120)	Responder	2		2	1		1	3				2	1	7/11
	Nonresponder		4		1		3	1	1	2	2	1		
gp43(180-194)	Responder	2	3	2			1	4		1	2	3		10/11
	Nonresponder						3	1			1	2		
Gp43(283-298)	Responder	2		1			1	3	1			3	1	7/11
	Nonresponder		4	1	2		4		1	2	1	1		

[a]Results are among the 14 PCM patients that responded to at least 1 peptide.
[b]Response = number of HLA-DR molecules associated with response.

10 patients tested in PBMC proliferation assay, however, peptide gp43(161-175) was recognized by 5 of 10 patients tested (Table 7), a frequency of recognition similar to TEPITOPE-selected immunodominant peptide gp43(180-194) (see Table 3).

T-cell Recognition of Epitopes Neighboring the Immunodominant gp43(180-194)

Peptide gp43(180-194), the most frequently recognized sequence (see Figure 1, Table 3), contains 14 of 15 residues of the P10 peptide shown to be immunodominant and protective in mice (2). TEPITOPE analysis at 3% threshold of the region 180-200, which comprises the immunodominant epitopes, showed the presence of 2 additional nonamer core sequences 186-194 (IHTLAIRYA) and 191-199 (IRYANRTDV), in addition to the major HLA-binding nonamer core sequence 183-191 (LIAIHTLAI), predicted to bind to 5 of 25, 6 of 25, and 20 of 25 HLA-DR molecules, respectively. To finely map the peptide epitopes within the immunodominant region, we synthesized and tested for HLA-DR binding assays as well as proliferation of PBMC from PCM patients and healthy controls 3 additional peptides positioned in different registers in relation to the nonamer core sequences contained within the positions 180-200 sequence of gp43. They were gp43(181-195), equivalent to peptide P10 (2), gp43(183-197), the center piece of which had the 183-191(LIAIHTLAI) nonamer core sequence, and a 21-mer containing both nonamers and additional flanking residues, gp43(179-199) (see Table 1). The binding assays of these peptides with 9 different HLA-DR molecules showed that all peptides bound to 89% to 100% of the HLA-DR molecules similarly to the peptide gp43(180-194) (see Table 8). Also, these peptides were tested in proliferation assays of PBMC from 19 patients and 6 healthy individuals. Taking into account responses to any peptide concentration, gp43(181-195) and the 21-mer peptide gp43(179-199) were recognized by 47% and peptide gp43(183-195) by 37% of the patients tested (Figure 4A), compared with 53% of gp43(180-194), whereas none of the gp43-negative healthy individuals responded to any of the peptides (data not shown). We observed that peptides gp43(181-195), gp43(183-197), and gp43(179-199) were recognized at the lowest concentration (0.1 mM) by 56%, 57%, and 67% of peptide responders, respectively, similar to the 50% of the original peptide gp43(180-194). Average SI values of peptides gp43(181-195), gp43(183-197), and gp43(179-199) at 0.1 mM were 8.3, 8.2, and 7.9, respectively, higher than the 4.9 SI of gp43(180-194) (Figures 4B to 4D).

Table 6. Peptide binding analysis with 9 most prevalent HLA-DR molecules with 5 peptides predicted by TEPITOPE not to bind promiscuously[a]

| | Binding capacity/IC50% (nM) | | | | | | | | | | |
Peptides	DR1 DRB1 *0101	DR3 DRB1 *0301	DR4w4 DRB1 *0401	DR4w15 DRB1 *0405	DR7 DRB1 *0701	DR5w11 DRB1 *1101	DR6w19 DRB1 *1302	DR2w2 B1 DRB1 *1501	DR2w2 B2 DRB5 *0101	Bound molecules	Freq[b]
gp43(118-132)	3952	4420	1885	4728	**767**	**724**	**171**	1432	1228	3	33%
gp43(161-175)	**68**	–	8540	7163	9787	12477	**165**	**405**	1690	3	33%
gp43(244-258)	4401	–	–	–	21540	–	16254	5170	22074	0	0%
gp43(259-271)	20724	5044	16989	1854	7530	16257	5621	**429**	19765	1	11%
gp43(347-360)	–	–	–	21038	–	12983	22876	–	7758	0	33%

[a]The results are expressed in IC50% (nM). Peptide binding assays performed as described in Table 2. Bold numbers = significant affinity threshold below 1000. Dash (–) means IC50% > 30000.
[b]Freq = frequency.

Table 7. Proliferation response of PBMC from 10 PCM patients to 5 gp43 peptides selected by TEPITOPE program not predicted to bind to any of the HLA-DR molecules[a]

Patients[b]	gp43 (118-132)[c]			gp43 (161-175)[c]			gp43 (244-258)[c]			gp43 (259-271)[c]			gp43 (347-360)[c]			gp43	
	0.1	1.0	10.0	0.1	1.0	10.0	0.1	1.0	10.0	0.1	1.0	10.0	0.1	1.0	10.0	10 μg	PHA
p2	5.4	4.5	2.8	2.0	–	2.0	–	2.0	–	–	–	–	–	–	–	43.5	111.4
p3	–	–	–	2.4	–	–	–	–	–	–	–	–	–	–	–	57.6	288.6
p4	–	–	–	10.5	10.2	7.1	–	–	–	–	–	–	3.0	–	–	51.3	294.8
p10	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	14.3	216.6
p11	–	2.4	2.0	6.9	6.8	6.9	–	–	–	–	–	–	–	–	–	26.7	130.9
p12	–	–	–	–	–	–	–	–	–	3.0	5.3	4.8	–	–	–	28.4	421.1
p20	–	–	–	–	2.4	2.8	–	–	–	–	–	–	–	–	–	95.5	521.7
p21	–	–	–	–	–	–	–	–	–	–	–	2.0	–	–	–	346.1	409.3
p22	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	84.9	452.7
p23	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	5.2	114.1

[a]Only positive stimulation index values (SI ≥ 2) are shown. Dash (–) corresponds to SI < 2.
[b]p = patient.
[c]PBMC (10^5 cells/well) were stimulated by peptides at 0.1, 1.0, and 10.0 μM as described.

DISCUSSION

In silico analysis of *P. brasiliensis* gp43 glycoprotein in search of promiscuous peptides binding to different HLA-DR alleles led us to the identification of several immunodominant epitopes recognized by primary CD4^{+} T cells from treated and healed PCM patients. Furthermore, we observed that the frequency of peptide binding and recognition was commensurate with the promiscuity of HLA binding as determined by TEPITOPE analysis.

The observation that sera from all treated and healed PCM patients, but none of the healthy individuals, had a significant IgG antibody response against gp43 (see Figure 1 and ELISA results, data not shown) showed that a T-helper anti-gp43 response was induced among PCM patients. This is in agreement with the fact that T-cell response against gp43 and its peptides in treated-healed PCM patients is restricted to the CD4^{+} T-cell subset (data not shown).

The fact that all TEPITOPE-selected gp43 peptides were recognized by a significant proportion of PCM patients tested (see Table 3) indicates that the algorithm was able to successfully identify promiscuous epitopes in gp43. Furthermore, the observation that the frequency of peptide binding to multiple HLA-DR molecules and the frequency of responders to each peptide was commensurate with the promiscuity of HLA-DR binding predicted by

TEPITOPE (see Figure 3) indicates that the algorithm can correlate with actual peptide binding results and identify promiscuous epitopes in a semi-quantitative manner. Analyzed as a whole, the 5 TEPITOPE selected peptides showed significant binding in 27 of 45 assays (60%), and 37 of 95 proliferation assays (39%), whereas the 5 peptides predicted not to bind only showed binding in 7 of 45 assays (16%) and 11 of 50 proliferation assays (22%), emphasizing the accuracy of the algorithm for predicting promiscuous HLA-DR binding. The observation that all TEPITOPE-selected peptides were recognized by 32% to 53% of PCM patients, while 4 of 5 peptides not predicted to bind to any HLA-DR molecules were recognized by 20% or less patients, emphasizes the usefulness of the algorithm. This can be observed in patients p3 and p4, whose PBMC recognized 5 of 5 TEPITOPE-selected peptides, but only 1 and 2 of 5 peptides that were predicted not to bind to HLA-DR molecules, respectively. Additionally, there was significant correlation between the promiscuity of TEPITOPE prediction and HLA-DR binding assay ($P < 0.001$, $r^2 = 0.78$), or proliferation assay ($P = 0.01$, $r^2 = 0.48$); significant correlation of promiscuity was also observed between HLA-DR binding assay versus proliferation assay ($P = 0.001$, $r^2 = 0.60$). However, the observation that peptide gp43(161-175)—predicted not to bind to any of the HLA-DR molecules—bound to 33% of the HLA-DR molecules tested and was recognized by 50% of patients indicates that TEPITOPE predic-

Table 8. Peptide binding analysis of peptides neighboring gp43(180-195) with 9 most prevalent HLA-DR[a]

Peptides	Binding capacity/IC50% (nM)									Bound Molecules	Freq[b]
	DR1 DRB1 *0101	DR3 DRB1 *0301	DR4w4 DRB1 *0401	DR4w15 DRB1 *0405	DR7 DRB1 *0701	DR5w11 DRB1 *1101	DR6w19 DRB1 *1302	DR2w2 B1 DRB1 *1501	DR2w2 B2 DRB5 *0101		
gp43(183-197)	**13**	1447	**464**	190	**11**	**5.1**	146	**59**	**51**	8	89%
gp43(179-199)	**1.8**	**116**	**92**	**11**	**18**	**12**	**9.1**	**5.9**	**6.2**	9	100%
gp43(181-195)	**0.080**	736	**68**	**23**	**2.0**	**11**	**1.3**	**4.9**	**1.0**	9	100%
gp43(180-194)	**0.27**	**93**	**37**	**33**	**8.8**	**4.8**	**2.7**	**6.3**	**2.6**	9	100%

[a]The results are expressed in IC50% (nM). Peptide binding assays were performed as described in Table 2. Bold numbers = significant affinity threshold below 1000.
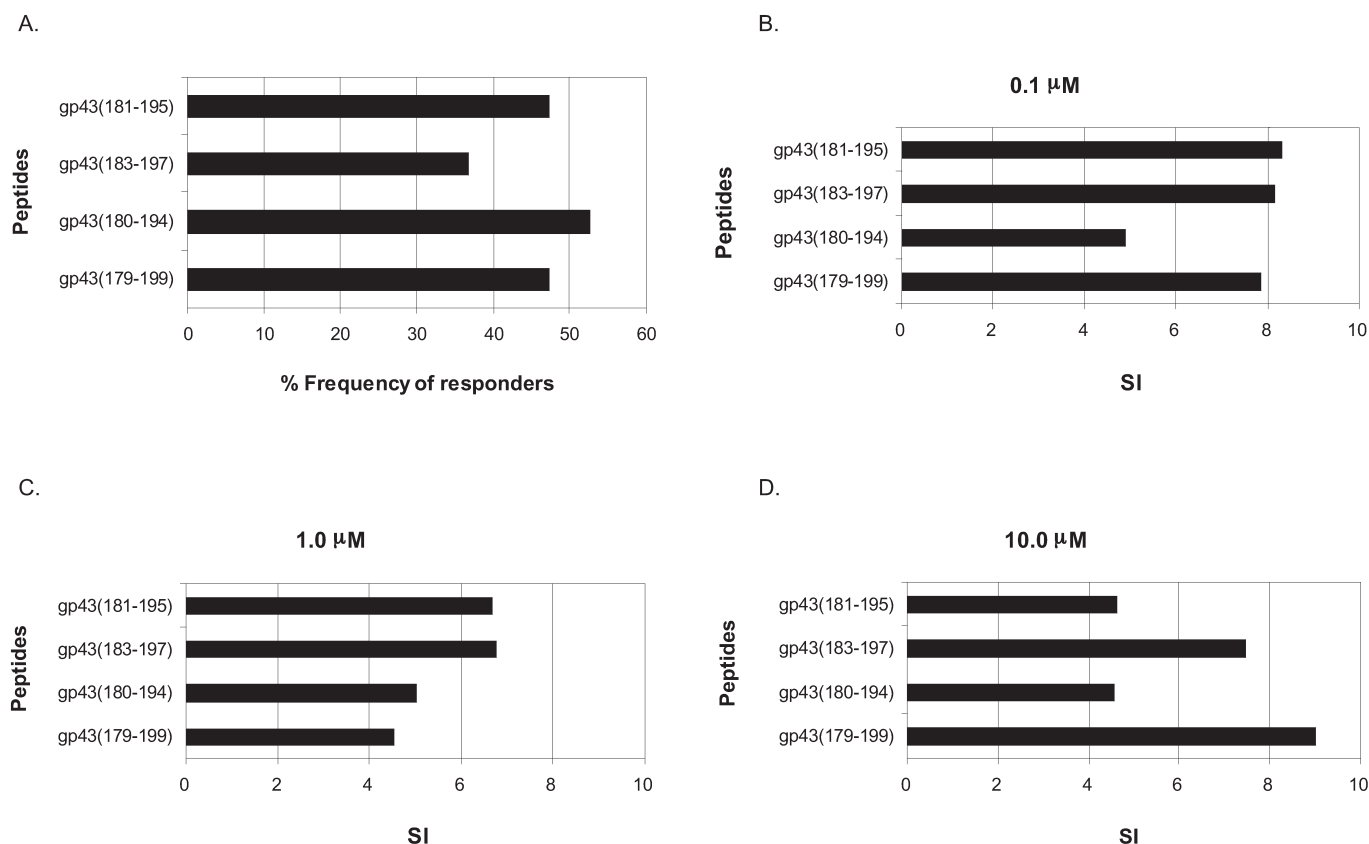[b]Freq = frequency.

A.

B.

0.1 μM

C.

1.0 μM

D.

10.0 μM

Figure 4. A: Frequency of responders to P10 peptide (gp43[181-195]) and its neighboring peptides gp43(183-197), gp43(180-194), and gp43(179-199). B–D: Average SI of responders to peptides P10 and its neighboring peptides at 0.1 μM (B), 1.0 μM (C), and 10.0 μM (D).

tion misses some immunodominant epitopes. By virtue of the built-in strategy of the TEPITOPE algorithm, it is only able to identify HLA-DR binding sequences starting with hydrophobic residues (17,23). This means that it was not built to identify every single potential epitope, but to be able to reliably identify those with this structural feature. One has to bear in mind that epitopes predicted by TEPITOPE not to bind to any of the 25 build in HLA-DR molecules may still be presented by other HLA-DR, -DP, or -DQ molecules.

The observation that a wide diversity of HLA-DR molecules was associated with recognition of each peptide (see Table 5) suggests that peptides predicted to be promiscuous by TEPITOPE were indeed capable of being presented by multiple HLA-DR molecules. In addition, the frequency of allelic variation in the HLA-DR distribution of the patients tested is similar to the spectrum of the Brazilian mixed population; thus, the lack of some particular haplotypes reflects the low frequency of the same haplotypes in the Brazilian population (62). The slight differences of frequency of DR1, DR10, and DR16 observed between the patients tested and the Brazilian population can be attributed to the low number of individuals tested. The observation that TEPITOPE-selected promiscuous peptides could often be recognized by individuals carrying HLA molecules to which they were predicted not to bind (data not shown) may indicate that peptides were even more promiscuous than predicted. It is known that the

HLA-DR binding motifs from different HLA molecules are relatively similar (20,22,63), and it is conceivable that epitopes predicted to bind to several HLA-DR molecules included in the TEPITOPE repertoire may also bind to other HLA class II molecules. Recently we observed that a peptide from Plasmodium vivax antigen identified as promiscuous by TEPITOPE was recognized by mice bearing distinct H-2 molecules (data not shown). This is in agreement with the observation that peptides derived from the herpes simplex virus selected with the TEPITOPE algorithm to bind to multiple HLA-DR molecules were capable to eliciting potent T-cell responses in several MHC-disparate strains of mice (34). On the other hand, the lack of response to a given peptide by some individuals whose HLA profile is predicted by TEPITOPE to respond underlines the fact that TEPITOPE predicts HLA binding but not necessarily T-cell recognition. The observation that PCM patients sharing the same HLA-DR molecules (DR7, DR13: p8, p10, and p14) recognized different peptides, suggests that the individual-specific peptide recognition may also depend on the T-cell receptor repertoire.

The fact that PBMC from some patients failed to respond to the promiscuous gp43 peptides tested was probably related to the significantly lower stimulation indexes observed in the same patients when their PBMC were stimulated with gp43 (see Table 3). This may suggest that these patients had comparatively weak T-cell responses against gp43, which could be secondary to

incomplete recovery from the nonresponsiveness/anergy observed during active disease (43).

A fine analysis of the immunodominant region of gp43 (positions 180-200) indicated that the 3 additional peptides were significantly antigenic, although not more than the original TEPITOPE-predicted gp43 (180-194). The fact that their overall frequency of recognition was lower, but the relative recognition at 0.1 µM was more frequent and attaining higher SI values than gp43 (180-194) indicated that the presence of additional HLA binding registers may not increase promiscuity but can affect the avidity of peptide recognition. Importantly, this study demonstrated that peptide P10, which is immunodominant and protective in murine models of *P. brasiliensis* infection (2), is also an immunodominant and promiscuous epitope in PCM patients.

The analysis of responsiveness and avidity among TEPITOPE-selected promiscuous peptides showed that the peptides gp43(180-194) and neighbors, including P10, are the most immunodominant and promiscuous epitopes of gp43, being recognized by more than 50% of all tested patients followed by TEPITOPE-nonselected peptide gp43(161-175). Panigada and others (30) also identified TEPITOPE-derived peptides capable of being recognized by peptide-primed T-cell lines from PBMC of *Mycobacterium tuberculosis*-infected patients (30). Given the fact that in vitro immunization can elicit antigen-specific T-cell lines even in naive donors, the reported epitope may not have been naturally processed upon infection (64). The fact that we showed primary PBMC recognition of TEPITOPE-derived promiscuous gp43 peptides among PCM patients is evidence that such epitopes were actually presented during infection, thus being prime candidates for vaccinal epitopes, because immunity triggered by such epitopes generated memory responses that can be boosted by contact with the same epitope or infectious agent. Recent data have suggested that single epitope-based vaccines are not powerful enough to induce full protective immunity. The combination of multiple B-cell and T-cell epitopes and conjugates as a pool or as a multiepitope polypeptide was shown to increase the immunogenicity (65,66). In the present study, we observed that 74% of the patients recognized the combination of the 5 TEPITOPE selected promiscuous gp43 peptides, gp43(180-194), gp43(45-59), gp43(283-298), gp43(94-108), and gp43(106-120). Preliminary results in a different set of PCM patients indicated that the response to the 5 pooled peptides reached 75% (data not shown). It is conceivable that inclusion of additional TEPITOPE-derived peptides to the above mentioned peptide combination may cover close to 100% of a genetically distinct population.

ACKNOWLEDGMENTS

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