Attempts at a peptide vaccine against paracoccidioidomycosis, adjuvant to chemotherapy

Luiz R. Travassos · Elaine G. Rodrigues · Leo K. Iwai · Carlos P. Taborda

Abstract Chemotherapy is the basis of treatment of paracoccidioidomycosis in its various forms. Depending on the *Paracoccidioides brasiliensis* virulence, the status of host immunity, the degree of tissue involvement and fungal dissemination, treatment can be extended for long periods with an alarming frequency of relapses. Association of chemotherapy with a vaccine to boost the cellular immune response seemed a relevant project not only to reduce the time of treatment but also to prevent relapses and improve the prognosis of anergic cases. The candidate immunogen is the gp43 major diagnostic antigen of *P. brasiliensis* and more specifically its derived peptide P10, carrying the CD4$^+$ T-cell epitope. Both gp43 and P10 protected Balb/c mice against intratracheal infections with virulent *P. brasiliensis* strain. P10 as single peptide or in a multiple-antigen-peptide (MAP) tetravalent construction was protective without adjuvant either by preimmunization and intratracheal challenge or as a therapeutic agent in mice with installed infection. P10 showed additive protective effects in drug-treated mice stimulating a Th-1 type immune response with high IFN-γ and IL-12. P10 and few other peptides in the gp43 were selected by Tepitope algorithm and actually shown to promiscuously bind several prominent HLA-DR molecules suggesting that a peptide vaccine could be devised for a genetically heterogenous population. P10 was protective in animals turned anergic, was effective in a DNA minigene vaccine, and increased the protection by monoclonal antibodies in Balb/c mice. DNA vaccines and peptide vaccines are promising therapeutic tools to be explored in the control of systemic mycoses.

Keywords Paracoccidioidomycosis · *Paracoccidioides brasiliensis* · Peptide vaccine · gp43 · P10 · Chemotherapy

L. R. Travassos (✉) · E. G. Rodrigues
Department of Microbiology, Immunology and Parasitology, Experimental Oncology Unit, Federal University of São Paulo, Rua Botucatu 862, 8 andar, São Paulo, SP 04023-062, Brazil
e-mail: travassos@unifesp.br

L. K. Iwai
Section on Immunology and Immunogenetics, Joslin Diabetes Center, Harvard Medical School, Boston, MA, USA

C. P. Taborda
Department of Microbiology, University of São Paulo, São Paulo, SP, Brazil

Introduction

Vaccines have been devised with live attenuated or inactivated microorganisms that are injected in the recipient to elicit a protective immune response. Pathogenic organisms may, however, contain products in the complex mixture of immunogenic molecules that cause untoward host responses.

Prophylactic mild infections may not be acceptable in immune compromised hosts. Isolated protein antigens mainly in the form of recombinant vaccines have alternatively been used as reliable and safer immunogens. In the case of tumor cells, the selection of tumor-specific antigens is important considering the great majority of self nonmutated proteins which do not induce an immune response within the limits of immunological tolerance. Even in autologous tumor cell preparations, there is the possible inclusion, however, of components that may aggravate malignancy. Recombinant proteins, that can be adequately purified for vaccination purposes are still very complex and carry a mixture of epitopes that induce B cell and T cell responses. While in many cases a combined response of Th-2 and T-dependent B cell lymphocytes with that of Th-1 lymphocytes is most effective for a balanced immune response, there are many cases of immunosuppression or immunological imbalance in which a predominant proinflammatory Th-1 response should be stimulated for effective host protection against infectious agents and tumor cells. Antibodies are beneficial in most cases and constitute the basis of the current human vaccines, but the cells involved in their production synthesize a set of cytokines that may negatively regulate the generally immunoprotective IFN-γ-mediated Th-1 response. The idea of reducing even further the size of the immunogen so as to have unique peptide units able to specifically direct the immune response involving T-CD$_4^+$, T-CD$_8^+$, and B lymphocytes with the perspective of having available timely useful protocols, is a growing concept, although there are no peptide-based vaccines so far approved for human use.

Generally peptides are regarded as unstable, readily degradable molecules with delivery problems to be solved and, in the case of MHC presentation, being restricted by the genetic background of the human recipient. A new comprehension of this area is, however, emerging with new biotechnological perspectives. While each peptide has its clearance *in vivo* defined by many factors it is also evident that peptides can be modified for increased stability and immunogenicity. Furthermore, the degree of circulation of intradermally or subcutaneously injected peptides can be minimal in the case of dendritic cell uptake and presentation. For distal targets peptides can be administered in liposomes, ISCOMs (immunostimulatory complexes), virosomes, microspheres

or in many other associations. Peptides can carry B and T cell epitopes and target proteins, receptors, tumor cells, virus and microbial cells. As to immunogenetic restrictions peptides can be selected for promiscuity in MHC presentation so that they can still be used in vaccines in a genetically diverse population. Otherwise, a combination of peptides can be used to cover both MHC class I and II restrictions with the aim of fostering immunoprotective responses. From the cautious remarks by Rothbard [1] to the updated views of Purcell et al. [2] it is clear that peptides "display more drug-like properties than recombinant proteins or whole pathogen vaccines". The authors listed 31 peptide-based vaccines at various stages of development against virus and bacterial infections, cancer, malaria, diabetes, Alzheimer's, and other diseases.

In the present review we focus on vaccination attempts against paracoccidioidomycosis with the recent proposition of a peptide vaccine as adjuvant to chemotherapy.

Attempts at devising vaccines against fungal diseases

Both the innate immune response and the adaptive immunity are important for an antifungal protective effect. Fungi or fungal antigens react with recognition receptors at the surface of macrophages (Toll-like receptors, dectin-1, Fc receptors, the mannose receptor, integrins, and other not fully characterized receptors) and activate them to produce cytokines (IL-12, IL-10, IL-18) and chemokines (chemokine receptors CCR2 and CX(3)CR1). Dendritic cells (DCs) which are nondividing terminally differentiated cells link innate and adaptive immunity and activate antigen-specific T cells. The differentiation of monocytes into DCs demonstrated in human cells has become the essential methodology for the production of DCs to be used in cancer immunotherapy protocols. In the mouse, monocytes generate DCs not only on entry into the lymph and migration to the lymph nodes but also on extravasation from blood and homing to the spleen [3]. Both peripheral blood monocyte subsets with phenotypes Gr1(high)CX(3)-CR1(int) and GR1(low)CX(3)CR1(high) can give rise to pulmonary DCs whereas only the latter had the potential to differentiate into lung macrophages [4].

Upon maturation, DCs become key antigen-presenting cells (APCs) with increased expression of MHC molecules. Fungal antigens are processed and peptide epitopes presented for recognition by T-lymphocyte receptors. In the case of IL-12-producing DCs Th-1 cells are induced to proliferate and produce IFN-γ and TNF-α. IL-12 and IL-18 also stimulate IFN-γ production by natural killer (NK) cells. IFN-γ-activated macrophages have increased fungistatic and fungicidal activities. DCs can also be induced to produce IL-4 which leads to the proliferation of Th-2 lymphocytes that inhibit the protective cell immunity. Nevertheless, Th-2 lymphocytes recognize B-cell-presented antigens and produce cytokines that stimulate B cell maturation and proliferation. Activated B cells produce antibodies that opsonize fungal elements for increased phagocytosis. Opsonization is also achieved with complement and collectins. B cells can also be activated by T cell-independent antigens generally represented by carbohydrate structures. In a comprehensive review on the development of vaccines against fungal infections, Cutler et al. [5] listed a number of immunogens from pathogenic species that could elicit antibodies and cellular immune responses.

Antibodies were the main mediators of immune protection particularly against *Candida* spp., *Aspergillus* spp., *Cryptococcus neoformans*, and *Pneumocystis carinii*. Immunogens comprised carbohydrate structures such as β-1,2-linked mannobiose and mannotriose [6, 7] and also β-1,3-glucan, a candidate for a universal fungal vaccine [8, 9]. Further data on *Candida* β-1,2-oligomannosides and specific antibodies to them can be found in Travassos et al. [10]. An interesting aspect was the identification of peptides from a phage library able to mimic β-1,2-linked mannoside epitopes [11]. Peptide FHENWPS was specifically recognized by anti-β-1,2-oligomannoside monoclonal antibodies (mAb). It inhibited binding of mAb to both live yeast cells and phosphopeptidomannan (PPM). A keyhole limpet hemocyanin (KHL)-peptide conjugate elicited an antibody response to yeast PPM which was inhibited by β-1,2-mannotetraose.

Idiotypic vaccination with mAb KT4 that neutralizes the antimicrobial activity of *Pichia anomala* killer toxin (KT) elicited antiidiotypic Abs that were microbicidal and showed *in vitro* therapeutic effects [12]. Several linear fragments from the mAb variable region showed activities similar to the antibody, and a killer decapeptide (KP) was generated by alanine substitution of the first amino acid of an active 10-amino acid fragment. The KP had increased candidacidal activity *in vitro* and exerted therapeutic effect in established murine models of vaginal and systemic candidiasis, cryptococcosis and paracoccidioidomycosis [13–15]. Noticeable is the fact that laminarin inhibited in a dose dependent manner the cytotoxic activity of KP suggesting that it may react with β-1,3-glucan molecules at the surface of the yeast forms. Secreted aspartyl proteinase and mannoprotein 65 are two other *Candida* immunogens that elicit protective antibodies [16].

The mAb (C7), raised against a *C. albicans* cell wall stress mannoprotein, is an IgM that cross-reacted with cell wall proteins Als3 and enolase, as well as with the nuclear pore complex Nup88 [17, 18]. MAb C7 is the first Ab able to exert three different antifungal activities against *C. albicans*, namely inhibition of germination and adhesion and a direct fungicidal effect which was extensive to *C. lusitaniae*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, and *Scedosporium prolificans* [19]. Recently, mAb C7 proved to be protective in a murine model of systemic candidiasis [20].

The most well studied antibody protective effect is that directed to glucuronoxylomannan (GXM) from *Cryptococcus neoformans* [9]. Antibodies to GXM and to oligosaccharides therefrom are protective in animal models of infection and have a potential to be used in the clinical treatment of human cryptococcosis [21]. A heptasaccharide corresponding to the immunodominant motif of serotype A GXM has been produced and it strongly reacted with two different IgM mAbs to GXM. Conjugation of the heptasaccharide to human serum albumin rendered a complex that elicited high-titered IgG responses in mice administered with complete Freund's adjuvant. Also in this case a peptide mimetic of a GXM epitope, GMDGTQLDRW, was isolated using a protective human mAb. This peptide linked to either BSA or tetanus toxoid elicited a protective antibody response [22].

The major surface glycoprotein (gp120), the p55 and Kexin are *Pneumocystis carinii* immunogens with protection mechanisms based on antibodies and T-cell dependent and independent but CD40L-dependent, immune responses. In this last case,

CD4-deficient mice receiving DNA vaccines encoding Kexin and CD40L showed significantly higher anti-*P. carinii* IgG titers and opsonic killing of *P. carinii* compared with those vaccinated with Kexin alone [23].

Immune protection involving CD4+T-cells, CD4+T-, and CD8+T cells and IFN-γ has been described for immunogens from *C. neoformans*, *Coccidioides* spp., and *Histoplasma capsulatum* (Hsp60) (reviewed in [5]). Generally, a Th-1 response based on IL-12 and IFN-γ production is effective against fungal infections. The induction of IL-10 and other type-2 cytokines is regarded as a limitation to vaccine success. However, depending on the degree of inflammation of the Th-1 response, the Th-2 cytokines are essential for a balanced immune protective response. This balance can be achieved by incorporation of Th-1 and Th-2-stimulating epitopes in the vaccine formulation to achieve protective immunity while keeping the inflammatory response within a tolerable limit. In the immune response against HSP60 from *Histoplasma capsulatum* or a polypeptide fraction from this protein (F3), production of IL-10 and IFN-γ was essential for therapy efficacy. Apparently in this particular case, the cytokine milieu determines the conformation of TCRs making $V\beta 8.1/8.2^+$ T cells essential for the protective effect of HSP60 [24].

A competent CD4+-T cell response producing IFN-γ and TNFα is usually the chief protective mechanism in fungal infections in addition to specific antibodies as described before. In the course of CD4+-T cell deficiency as in AIDS patients, however, CD8+-T cells could have a protective role and this was examined in *Histoplasma*-infected mice. In MHC class II-deficient mice infected with *H. capsulatum*, CD8+-T cells were able to limit fungal growth. These cells expressed both IFN-γ and granzyme B and were cytotoxic to macrophages containing *Histoplasma* [25].

Vaccination against *Blastomyces dermatitidis* using recombinant BAD1 was not much effective even when combined with IL-12 used as adjuvant [26]. Therefore, a genetically engineered *BAD1* null mutant was used as a vaccine. Live yeast of the null mutant injected subcutaneously protected mice against a lethal challenge with evidence of sterilizing immunity [27]. Vaccination in IFN-γ or TNF-α knock-out mice was still effective requiring either the reciprocal cytokine or GM-CSF. As with *H. capsulatum*, antigens from live attenuated yeast can be cross-presented to DCs hence cross-priming CD8+ T-cells which can function without either TNF-α or IFN-γ. CD4+T-cell independent CD8+T-cell memory in these fungi appeared long lasting (at least 8 weeks post-vaccination). Above all, immunogens from live attenuated fungi seemed to recruit multiple arms of the immune response and be effective even in immunodeficient hosts that can compensate the lack of certain immune mediators (TNF-α or IFN-γ) by substituting elements (GM-CSF or TNF-α and GM-CSF, respectively) to render protective immunity [5].

Immunoprophylaxis of paracoccidioidomycosis (PCM)

Subcutaneous infection with *Paracoccidioides brasiliensis* was shown to be self-healing inducing strong DTH reactions. The high T-cell reactivity observed in susceptible mice could result in immunoprotection or disease exacerbation depending on the route of a secondary infection [28]. Immunoprotection was obtained with a secondary intraperitoneal challenge associated with persistent cell-mediated immunity of mixed type-1/type-2. In contrast, exacerbated disease followed a secondary intravenous challenge which was associated with anergy and prevalent type-2 immunity. High and low virulence *P. brasiliensis* strains were used to induce immunoprotection in susceptible mice. Immunoprotection was also achieved against a secondary intratracheal infection by virulent *P. brasiliensis* characterized by a combination of CD4+T cells and CD8+ T cells and the production of endogenous IFN-γ and IL-12 as well as increased levels of anti-*P. brasiliensis* specific IgG1 and IgG2a antibodies [29].

Although the protection by primary subcutaneous infection with high and low virulence *P. brasiliensis* seemed quite effective against a secondary infection, this model refers to prophylactic vaccination as in other fungal species [5] but not to therapeutic vaccination against the already installed mycosis that will be controlled primarily by chemotherapy. It could, nevertheless, explain the dynamics of multiple infections in endemic areas with few individuals developing progressive disease.

Gp43 the major diagnostic antigen of *P. brasiliensis* protects mice against intratracheal challenge by virulent yeast forms

The 43 kDa glycoprotein (gp43) was isolated from *P. brasiliensis* culture supernatant fluids in 1986 [30]. Virtually 100% of patients with paracoccidioidomycosis had antibodies reacting with gp43. Anti-gp43 antibodies from patients submitted to chemotherapy had lower titers compared with patients with relapsing disease who in turn had antibody titers higher than patients with installed disease. A decrease of anti-gp43 IgG, IgA, and IgM was associated with clinical improvement [31]. Epitopes in gp43 that elicited a strong antibody response were peptidic in nature [32] and different isoforms of gp43 including point mutations and distinct isoelectric points varied in their reactivity with patients' sera. The gp43 gene cloning, sequencing, expression, and its polymorphism have been reviewed elsewhere [33].

Gp43 being secreted in the fungal environment or reaching the blood stream is processed and presented by dendritic cells or B cells inducing a preferential Th-1 or Th-2- type immune response respectively [34, 35].

Immunization with gp43 elicits a vigorous IFN-γ-mediated T-CD4$^+$ response that is protective against the intratracheal challenge by virulent yeasts of this fungus. The first report showing that gp43 carried an immunodominant epitope eliciting DTH reactions was that of Rodrigues and Travassos [36]. The T cell epitope responsible for DTH reactions, and CD4$^+$ T-cell proliferation, has been mapped to a peptide called P10 with the sequence: QTLIAIHTLAIRYAN [37]. The hexapeptide HTLAIR has been shown to be essential for priming the cellular immune response. Various 12-amino acid peptides derived from P10 with different flanking sequences were able to induce lymphoproliferation in sensitized animals. Both P10 as compared to gp43 was equally protective against the intratracheal challenge of mice with virulent *P. brasiliensis*. Gp43 shares 50% identity in the amino acid sequence with the exo-β-1, 3-D-glucanases from *Aspergillus oryzae*, *Blumeria graminis*, *Schizosaccharomyces pombe*, *Pichia augusta*, *Saccharomyces cerevisae*, and *Candida albicans*. Gp43 has a mutated catalytic site essential for glucanase activity (NKP instead of NEP) and in consequence is not an active enzyme. The nucleotide sequence encoding P10 was conserved which is an important attribute for a vaccine candidate [33, 38]. No epitope for CD8$^+$T cells was detected in the gp43 but this is by no means a closed matter.

Immunization of Balb/c mice with a mammalian expression vector (VR-gp43) carrying the full gene of gp43 with CMV promoter induced B and T cell-mediated immune responses protective against the intratracheal challenge by virulent *P. brasiliensis* yeast forms [39]. The amount of IFN-γ produced by gp43-stimulated lymphoid cells from plasmid immunized mice was comparable to that using a mitogen, therefore indicating a powerful immune response. The cellular immune response in mice immunized with VR-gp43 was maintained for at least 6 months although reduced to half of the stimulation index obtained 15 days after immunization. After 3 months of prime intratracheal infection with 2×10^5 yeast cells of *P. brasiliensis* isolate 1914 of high virulence, mice immunized with VR-gp43 had a significant reduction in CFUs from lungs, spleen and liver, and the histopathological image showed lungs with very few and small granulomas with no detectable fungal cells. One of the advantages of using DNA vaccines is the long-term expression of constituent genes [40] compared to the shorter half-life of recombinant proteins. The VR-gp43 vaccination induced anti-gp43 antibodies of subclasses IgG1 > IgG2a = IgG2b with little IgE, confirming a mixed Th-1/Th-2 immune response.

Historically, protective vaccination with plasmid cDNA encoding a fungal antigen (antigen 2) was reported for the first time against *Coccidioides immitis* [41] followed by the gp43-DNA vaccine in *P. brasiliensis* one year later [39].

Attempts at formulating a peptide vaccine against paracoccidioidomycosis

Peptide P10 derived from *P. brasiliensis* gp43 contains the T cell epitope that is presented by MHC class II molecules from three different mouse haplotypes [37]. The promiscuous nature of P10 was also extended to the HLA-DR alleles since this peptide and the analogous peptide at position gp43 (180–194) without the C-terminal asparagine residue (N-glycosylation site in the original gp43) and with N-terminal lysine, bound to the 9 prevalent Caucasian HLA-DR molecules [42].

By using the TEPITOPE algorithm we also looked for additional peptides in the gp43 that could also be promiscuous with respect to HLA-DR binding. This property is essential for a vaccine candidate considering the genetic diversity of the target population. In terms of lymphoproliferation, gp43 (180–194) was recognized by 53% of patients with treated PCM and the other promiscuous peptides were recognized by 32–47% of patients; 74% of patients recognized the combination of 5 promiscuous gp43 peptides [42]. An expansion of this study with 10 more patients (total of 29) showed that 79% of PCM patients recognized at least one peptide, and by pooling peptides gp43 (45–59), gp43 (94–108), gp43 (106–120), gp43 (181–195) or P10, and gp43 (283–298) the recognition frequency increased to 86% [43]. The TEPITOPE algorithm scanned 25 Caucasian HLA-DRs and P10 and neighboring peptides were predicted to bind to 90% or more of these molecules.

A reduced number of healthy individuals had PBMC proliferating with gp43 and even fewer with gp43 derived peptides. Possibly, these individuals may have been exposed to *P. brasiliensis* on a trip to reserve areas of the fungus or cross-reacted with related fungal antigens, possibly also exo-β-1,3-D-glucanases. Looking for sequences homologous to that of P10 in data banks we found indeed similar sequences in β-1,3-glucanases from *Aspergillus nidulans*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Lacazia loboi* (a gp43-like protein). However, similar sequences were also found in different proteins from a series of bacteria, protozoa, and even fowl (*Gallus gallus*). Some of these peptide sequences were predicted by the TEPITOPE algorithm to bind to HLA-DR molecules (at 3% threshold) in a way similar to P10. While P10 from *P. brasiliensis* was recognized by 21 of 24 HLA-DR alleles, the peptide from *A. nidulans* would bind to 18/24 in great contrast with peptides from *H. capsulatum* and *B. dermatitidis* that showed a very poor recognition, 5/24 and 3/24 respectively (Table 1). It seems then that the P10 homologous peptides from related proteins of *H. capsulatum* and *B. dermatitidis* are not vaccine candidates for a MHC class II-restricted T-cell activation. Remarkably, completely unrelated species as *Plasmodium yoelli* and *Streptococcus gordonii* had hypothetical proteins with homologous peptides being recognized by a high number of HLA-DR molecules (21/24).

Strategic use of peptide vaccine

Stimulation of an effective IFN-γ-producing T-helper response can simultaneously trigger the production of potentially protective antibodies and the activation of CD8$^+$ T cells in addition to activation of phagocytic cells. In the presence of several immunogenic molecules of the fungal agent, stimulation of one arm of the immune system may alter a state of early or installed immunosuppression. It is possible then that, once complying with the MHC class II restrictions, a vaccine could be effective primarily with a peptide inducing a robust Th-1 immune response. But fungal infections are regularly treated with drugs, therefore a peptide vaccine can function as an adjuvant to reduce the time of treatment, avoid frequent relapses, improve treatment of anergic cases, and cope with fungal drug resistance. To expect that a vaccine made of a polysaccharide, a glycoprotein or peptide (s) may alone sterilize a massive experimental infection or an acute or progressive mycosis in patients without the concomitant use of fungistatic/fungicidal drugs is not realistic. One should, therefore, test the prospective vaccine to be introduced as an adjuvant to chemotherapy. Successful drug treatment can lyse a great number of fungal cells and the liberated cell contents may enhance a Th-2/B cell response with nonprotective antibodies. As the fungal load is reduced specific antibody titers also wane. Endogenous or vaccine stimulated specific Th-1 response should help to balance the immune response, increase the fungal clearance and shorten the recovery period.

Experiments with Balb/c mice that respond quite well to P10 immunization in terms of IFN-γ production and anti-fungal protection effects, served to confirm some of these expected responses.

P10 immunization was coupled to chemotherapy in intratracheally infected Balb/c mice using two protocols. Infected mice with yeast cells of highly virulent *P. brasiliensis* underwent P10 and/or drug treatment starting after 48 h of infection. In the second protocol, P10 and/or drug treatment started after 30 days infection. The treatment continued for 30 days, during which groups of mice received intraperitoneal doses of itraconazole, fluconazole, ketoconazole, sulfamethoxazole or trimethoprim-sulfamethoxazole every 24 h. Amphotericin B was administered every 48 h. Immunization with P10

Table 1 In silico prediction of HLA-DR binding of various peptides, homologous to *P. brasiliensis* P10, from proteins of different species

Species	Protein description	Position from N-t[a] methionine	Sequence(mostly from BLAST/NCBI data base)	Number of HLA-DR molecules recognized (at 3% threshold)
P. brasiliensis	gp43	181–195	Q T L I A I H T L A I R Y A N	21
Aspergillus nidulans	glucanase-like protein	251–266	E Q T I L A F E T L A Q R Y L	18
Histoplasma capsulatum[b]	β-glucanase	180–194	A Q T L N A L R A L A E R Y A	5
Blastomyces dermatitidis[b]	β-1,3-glucanase	185–199	A Q T L N A V R A L A E R Y A	3
Bacillus thurigiensis Bacillus cereus	transcriptional regulator Ara C family	236–252	N N G I I A I - T L A I R Y A I D G	4
Lacazia loboi	gp43-like protein	AAW21849[c] 21–37	K Q T L A A I R A L A N R Y A K R	13
Gallus gallus	ortholog of rat PB-cadherin	545–563	K G T L L A V H T L A V S Y T A G V D	13
Herpetosiphon auranticus	UDP-N-acetylmuramoyl alanyl-D-glutamyl-2,6-dipimelate—D-alanyl-D-alanyl ligase	114–131	V D Q T L T A M H R L A T R Y R Q Q	12
Desulfotalea psychrophila	Similar to dolichyl phosphate mannose synthase	284–300	A L P L I A C H T L A I I Y F C C	18
Plasmodium yoelli yoelli	Hypothetical protein	XP727303[c] 786–803	I E L L I S I L T L S I R Y I L R N	21
Streptococcus gordoni	Hypothetical protein	477–491	G L S M I H T L A I I Y A V V	21
Gibberella zeae	Hypothetical protein	27–42	L N S Q T I A I H T L A H Q D S	3
Rhodococcus erythropolis	Hypothetical protein	65–81	F G G L I A S G I H T L A I L Q R	10

[a] N-t, N-terminal

[b] Data base for *H. capsulatum*, Hc, and *B. dermatitidis*, Bd (http://www.genome.wustl.edu/)

[c] Assession number in the BLAST/NCBI data base, for incomplete sequence indicating the corresponding peptide fragment

was carried out weekly for 4 weeks, once in complete Freund's adjuvant and three times in incomplete Freund's adjuvant [44].

A significant reduction in the fungal load (measured as CFUs) was observed in both groups, either immunized with P10 only or treated with antifungal drugs, with an additive protective effect obtained with the combination of both. Unexpectedly, animals treated with sulfamethoxazole, showed early protection followed by relapse, but the association of sulfamethoxazole and P10 vaccination successfully controlled the infection. With the second protocol that aimed to reproduce the patient's condition of established infection, the fungal burden was examined after 60 and 120 days of infection. Also in this case, an additive protective effect of P10 immunization and drug treatment was observed, with 60 to 80% reduction in lung CFUs. As examined in lung homogenates chemotherapy led to a predominant Th2 response with increased production of IL-4 and IL-10 as hypothesized above. P10 vaccination stimulated a Th1 response, rich in IFN-γ and IL-12

without suppressing the Th-2 elements of the immune response [44]. It should be pointed out that these experiments could be further optimized with prolonged treatment aiming complete infection control.

In acute or subacute forms of PCM patients may have high antibody titers accompanied by severe depression of cell-immunity [45]. In an attempt to experimentally reproduce these conditions, Balb/c mice were treated with dexamethasone-21 phosphate added to drinking water. After 30 days animals showed negative DTH. Infected animals, treated with 0.15 mg/kg of dexamethazone and injected with *P. brasiliensis* crude antigen also showed negative DTH after the same period. Mice were then infected with a virulent *P. brasiliensis* isolate and after fifteen days were submitted to chemotherapy and/or P10 immunization. The association of drugs and P10 immunization conferred additive protection. A significant increase in IL-12 and IFN-γ and decrease of IL-4 and IL-10 was observed in mice immunized with P10 alone or in association with antifungal drugs [Marques et al., unpublished results]. These results suggest that P10 immunization can be effective in the case of anergic patients.

Peptide delivery and adjuvants

Peptide delivery using formulations that did not include CFA has also been a concern of our group. Immunization with the P10 minigene in plasmid DNA associated or not with a plasmid carrying a murine IL-12 insert, was tested in Balb/c mice intratracheally infected with a virulent isolate (Pb18) of *P. brasiliensis*. Such immunization elicited significant reduction of fungal burden in lung, spleen, and liver. A significant production of IL-12 and IFN-γ and reduction of IL-4 levels in lung homogenates were observed with the DNA vaccination [Rittner et al., unpublished results].

Another attempt involved a MAP (multiple antigen peptide) construction. MAP-10 or M10 had four equal LIAIHTLAIRYAN (N-terminal QT-less P10) chains synthesized on a branched lysine core containing glycine at the C-terminal position. Proliferation of lymph node cells from P10 or M10-sensitized mice was identical when stimulated *in vitro* with either P10 or M10. Mice immunized with a single dose of M10 without adjuvant and challenged

i.t. with *P. brasiliensis* showed fewer lung, spleen and liver CFUs and few or no yeasts in lung histopathological sections [46].

In Balb/c mice with established 30-day intratracheal infection, the therapeutic effect of P10 was tested alone or mixed with adjuvants: alum, monophosphoryl lipid A or complete Freund's adjuvant (Fig. 1). Surprisingly, P10 administered in PBS was the most effective preparation with a very significant reduction in the CFUs from the lungs with no fungi detected in spleens and livers. Modifications of N and C termini of a peptide are known to prevent its degradation by exopeptidases [47]. Such modifications usually involve N-acetylation and C-amidation. In the experiments described, P10 was always used in the C-amidated form.

Association of P10 and protective anti-gp43 monoclonal antibodies

An association of P10 with protective anti-gp43 mAbs has also being explored. Recently, it has been reported that anti-gp70 mAbs were protective against experimental PCM [48]. Both gp43 and gp70 are markers for monitoring the regression of PCM through their decreased antigenemia and specific antibody response in patients submitted to chemotherapy [49, 50].

The protective effect of anti-gp43 mAbs injected i.p. on the intratracheal infection with *P. brasiliensis* was examined in Balb/c mice (unpublished results). The reactivity of mAb 3E, the most efficient mAb in the reduction of fungal burden and phagocytosis *in vitro*, against a panel of gp43 derived peptides suggested that the recognized epitope could lie within the sequence NHVRIPIGYWAV shared with *Aspergillus fumigatus*, *A. oryzae* and *B. graminis* internal sequences of β-1,3-glucanases. This peptide would represent with P10, another candidate for a peptide vaccine against PCM.

In a P10-preimmunization protocol, which is not the most effective in the absence of a simultaneous infection and chemotherapy, mAbs were tested as protective agents. The association of P10-preimmunization and mAb 3E administered 24 h before intratracheal challenge with virulent *P. brasiliensis* yeasts resulted in the best protection using short-term protocols in comparison with a nonprotective mAb.
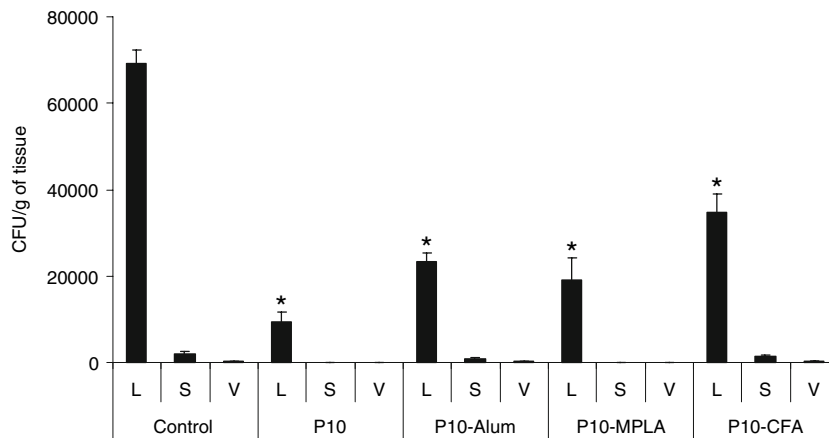
Fig. 1 Therapeutic effect of P10 administered alone or with adjuvants. Balb/c mice (five per group) were infected with 3×10^5 cells of *Paracoccidioides brasiliensis* Pb18 and after 30 days vaccination with P10 alone or mixed with adjuvants was initiated. P10 was diluted in 20% DMSO and PBS and administered at 20 µg per animal. Alum and complete Freund's adjuvant (CFA) were used in the same volume (50 µl) as for P10 solution. Monophosphoryl lipid A (MPLA) was used at 1 µg per animal. Thirty days after therapy mice were sacrificed totaling 90 days of infection. Colony forming units (CFUs) were determined in the lung (L), spleen (S) and liver (V) of mice. Values are means with standard deviations

Table 2 Association of monoclonal antibody against gp43 and immunization with P10 in Balb/c mice infected i.t. for 45 days (short period) with *P. brasiliensis* isolate Pb18

Balb/c mice[a]	Lung CFUs[e]	Cytokine (ng/g/ml)			
Treatment[b]	CFU/g tissue	IL-4	IL-10	IL-12	IFN-γ
Sham infection	-	0.81 ± 0.38	1.30 ± 0.60	5.56 ± 2.41	0.33 ± 0.39
Only infected[c]	26,185 ± 2,914	1.44 ± 0.16	2.87 ± 1.27	11.02 ± 0.564	0.78 ± 0.57
Immunized P10	13,291 ± 1,551	1.26 ± 0.16	7.54 ± 1.01	15.30 ± 2.39	7.42 ± 1.53
mAb 32H (nonprotective)[d]	31,523 ± 7,991	1.20 ± 0.25	13.40 ± 2.17	4.28 ± 6.52	3.20 ± 1.35
mAb 3E (protective)[d]	19,220 ± 2,279	1.23 ± 0.17	11.12 ± 0.14	13.50 ± 1.52	6.35 ± 0.68
P10 and mAb 32H	20,924 ± 4,359	1.39 ± 0.01	9.15 ± 1.53	10.78 ± 2.51	5.06 ± 0.86
P10 and mAb 3E	8,800 ± 1,886	0.75 ± 0.17	6.89 ± 1.88	22.24 ± 1.77	8.87 ± 1.44

[a] Male, 6–8 week-old Balb/c mice, groups of 10

[b] Animals were preimmunized with P10 (4 doses of 20 µg, the first in CFA and the others in IFA) before infection

[c] Intratracheal infection with 3×10^5 Pb18 yeast forms

[d] Antibodies purified in protein A and LPS-removing detoxigel injected i.p. 24 h before i.t. challenge

[e] CFUs, colony forming units

Significantly, this association yielded the highest IFN-γ and IL-12 levels and fewest lung CFUs (Table 2).

Short-term protocols (30 to 45 days) have the advantage of allowing repeated experiments to define a certain response. However, longer periods of treatment and observation may lead to even more effective results, aiming at sterilization in experimental models with massive infection loads.

Concluding remarks

Chemotherapy is the basic treatment of systemic mycoses. In cases of immunodepression and even of anergy the fungal elements, usually yeast forms, proliferate intensely because of deficient innate and adaptive immune response. In these conditions, chemotherapy is less efficient and requires long periods of treatment with the risk of relapsing

disease. Therapeutic vaccination with fungal antigens may boost the cell immune response and add to the protective effect of chemotherapy even counteracting the possibility of relapsing disease.

Of all prospective vaccines, DNA-based vaccines and peptides appear as the most promising because they can be obtained in large quantities at high degrees of purity for human immunization. The results with P10 show that a single peptide had a remarkable therapeutic effect even without adjuvant. It also showed that protection can be achieved in mice with established disease, undergoing chemotherapy, by stimulating one arm of the immune response, that of IFN-γ-mediated CD4$^+$ T-cells. The present results also show that monoclonal antibodies can be protective against paracoccidioidomycosis and that a new peptide, carrying a B cell epitope can also be tested in a vaccine to foster a balanced immune response.

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