Original Articles

Experimental paracoccidioidomycosis: alternative therapy with ajoene, compound from Allium sativum, associated with sulfamethoxazole/trimethoprim

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Ajoene has been described as an antithrombotic, anti-tumour, antifungal, antiparasitic and antibacterial agent. This study deals with the efficacy of ajoene to treat mice intratracheally infected with Paracoccidioides brasiliensis. The results indicate that ajoene therapy is effective in association with antifungal drugs (sulfamethoxazol/trimethoprim), showing a positive additive effect. Ajoene-treated mice developed Th1-type cytokine responses producing higher levels of IFN-γ and IL-12 when compared to the infected but untreated members of the control group. Antifungal activity of ajoene involves a direct effect on fungi and a protective pro-inflammatory immune response. Reduction of fungal load is additive to chemotherapy and therefore the combined treatment is mostly effective against experimental paracoccidioidomycosis.

Keywords Paracoccidioides brasiliensis, chemotherapy, ajoene, Allium sativum, sulfamethoxazole/trimethoprim

Introduction

Paracoccidioidomycosis (PCM) is a systemic granulomatous disease caused by Paracoccidioides brasiliensis, a thermally dimorphic fungus. This mycosis is widespread in Latin America, mainly affecting rural workers. Inhalation of conidia is the probable route of infection. Approximately 10 million people may be infected with this fungus and up to 2% of them may develop the disease [1]. The incidence may increase with the deforestation and development of new areas, as well as in immunodeficient individuals [2]. In fact, in the period of 1980-1995 there were 3,181 lethal cases of PCM in Brazil [3]. The acute and sub-acute forms of PCM are found in both genders and primarily involve the reticuloendothelial/lymphatic system. Adult males present with a predominantly pulmonary and/or mucocutaneous involvement, characterizing the chronic form of PCM [4]. Activation of cellular immunity is the most effective mechanism to control experimental and human PCM [5,6], and a correlation has been found between the severity of the disease and impaired delayed-type hypersensitivity (DTH) response [7].

Antifungal chemotherapy is routinely used in cases of PCM, with initial treatment lasting from 2 to 6 months and involving sulfonamides, amphotericin B, or azoles. The extended periods of treatment are necessary due to the significant frequency of relapsing disease.

The antifungal properties of ajoene have been observed since 1951 when it was shown that aqueous extracts of garlic inhibited growth of fungi recovered a soil sample [reviewed in 8]. The same aqueous extract also inhibited growth of several fungal species of
medical, industrial and agricultural interest [reviewed in 8]. Yamada et al. showed the antifungal activity of allicin against several yeasts, dermatophytes and dimorphic fungi [9]. San-Blas et al. demonstrated through in vitro assays that ajoene inhibits the growth of P. brasiliensis, with yeast cells being more sensitive to its action than the mould form [10]. The dimorphic conversion was also interrupted when the mycelium was set to transform to the yeast form [10].

Even while several authors have shown the antifungal activity of ajoene in vitro, it has been only from 1996 that it has been used in a cream (0.4% w/w) for short-term therapy of Tinea pedis [11]. The authors obtained complete clinical and mycological cure in 79% of the patients after 7 days treatment and the remaining patients achieved complete cure after seven additional days of therapy [11]. All patients were evaluated for recurrence of mycotic infections 90 days after the end of treatment and no fungi were recovered in culture from any of the patients.

Ajoene and 5-fluorouracil were used in the topical treatment of localized lesions of chromoblastomycosis. Complete clinical and mycological remission was achieved in 74% patients treated with ajoene and 78% patients treated with 5-fluorouracil. All 5-fluorouracil treated patients developed a post-treatment scar at the site of lesion, whereas ajoene-treated patients showed only a slight depigmentation of the skin [12].

Allium sativum has been utilized for decades in popular medicine throughout the world. In these studies, we explored the effects of synthetic ajoene alone or combination with sulfamethoxazole/trimethoprim (SMT) in the treatment of mice intratracheally infected with P. brasiliensis.

Material and methods

Animals. Balb/c mice (6- to 8-week-old males) were bred at the University of Sao Paulo animal facility under specific-pathogen-free conditions. Procedures involving animals and their care were conducted in accord with the local Ethics Committee and international rules.

Fungal strain. Yeast cells of the virulent P. brasiliensis Pb18 strain were maintained by weekly subculturing on Sabouraud dextrose agar and incubated at 37°C. Before experimental infection, 7–10 day old cells were grown in modified McVeigh-Morton medium (MMcM) and incubated at 37°C for 5–7 days [13]. Fungal cells were washed in phosphate-buffered saline (PBS, pH 7.2) and counted in a haemocytometer. The viability of inoculum suspensions was determined by staining with Janus B (Merck, Darmstadt, Germany) and found to be higher than 90%.

Antifungal drugs and Ajoene. SMT was obtained from Bac-sulfittin (Ducto, Brazil). Ajoene was supplied by Dr Rafael Apitz-Castro as a solution containing 414 mg/ml in ethylacetate. The preparation of this solution has been previously described [14].

Minimal inhibitory concentration (MIC) of SMT and ajoene. MICs were determined by a modification of the method of Shadomy et al. [15]. Briefly, yeast cells were suspended in sterile saline and their concentration adjusted to 6 × 10⁵ cells/ml. A 0.1 ml suspension of yeast cell was brought up to 1.0 ml through the addition of MMcM [13] containing SMT and ajoene. Final drug concentrations ranged from 0.015 to 640 μg/ml for SMT and 0.19 to 50 μg/ml for ajoene. MICs were recorded after incubation at 37°C for 7–10 days. The MICs were defined as the lowest drug concentration that gave 90% growth inhibition as compared to the growth found in the drug-free control. CFU was determined as described previously by Castañeda et al. [16].

Intratracheal infection of Balb/c mice. Each Balb/c mouse was inoculated intratracheally (IT) with up to 50 μl of 3 × 10⁵ yeast cells of Pb18 which had been grown on MMcM and suspended in sterile saline (0.85% NaCl). Mice were anesthetized intraperitoneally (IP) with 200 μl of a solution of 80 mg/kg ketamina (União Quimica Farmaceutica, Brazil) and 10 mg/kg of xylazina (União Quimica Farmaceutica, Brazil). After approximately 10 minutes, their necks were hyper-extended, the trachea was exposed at the level of the thyroid, and the inoculums suspension injected with a 26-gauge needle. The incisions were sutured with 5-0 silk.

Chemotherapy of infected mice. Drug treatment started after 48 h of IT infection and continued for 45 days. Groups of mice received doses every 24 h of SMT (15 mg and 3 mg/kg) and ajoene (10 mg/kg).

Groups studied. Six groups of mice (with 5 to 10 animals each) were used. The 3 control groups consisted of those with a sham infection, a second infected with Pb 18 only and a third that received only ajoene. The 3 infected groups included those treated with SMT, another with only ajoene and finally a group that received a combination of SMT and ajoene.
Fungal burden in organs of infected mice. Mice were sacrificed 45 days after IT infection, with sections of the lung, liver and spleen removed, weighed, homogenized and then washed 3 times with PBS. The corresponding pellets were re-suspended and each homogenized in 1 ml of PBS. One hundred μl samples of these suspensions were inoculated on to brain-heart infusion (BHI) agar medium supplemented with 4% fetal calf serum (Gibco, NY), 5% spent P. brasiliensis (strain-192) culture medium, streptomycin/penicillin 10 IU/ml (Cultilab, Brazil) and cycloheximide 500 mg/ml (Sigma, St Louis, MO). Colony counts were made visually after 10 days of incubation at 37°C.

Cytokine analysis. Mice were sacrificed at 45 days after infection and sections of the lung (right and left alternately), were homogenized in 2 ml of PBS in the presence of protease inhibitors (Complete Mini; Boehringer Mannheim, Indianapolis, IN). The homogenates were centrifuged and the supernatants frozen at −80°C until tested. The supernatants were assayed for IL-2, IL-4, IL-10, IL-12 and IFN-γ using ELISA kits (BD PharMingen, San Diego, CA). The detection limits of such assays were as follows: 3.1 pg/ml for IL-2; 7.8 pg/ml for IL-4; 31.25 pg/ml for IL-10 and IFN-γ; 15.6 pg/ml for IL-12/p40, as previously determined by the manufacturer.

Histopathology. The lungs were excised, fixed in 10% buffered formalin, and embedded in paraffin for sectioning. The sections were stained with haematoxylin-eosin and examined microscopically at 10× (Optiphot-2; Nikon, Tokyo, Japan).

Statistical analysis. Pairwise comparisons among groups were done by Student’s t test.

Results

Before the in vivo treatment of infected mice, SMT and ajoene were tested in vitro to determine their minimal inhibitory concentrations (MICs) and growth inhibition in terms of colony forming units (CFUs) using the virulent isolate Pb18. MICs for SMT ranged from 64/12.8 to 8/1.6 μg/ml and for ajoene from 0.05 to 0.0125 mg/ml. Determination of CFUs with ajoene showed the fungus to be susceptible in the 0.05 to 0.00625 mg/ml range.

It should be noted that in vitro drug susceptibility as measured under laboratory conditions does not always correlate with clinical efficacy against subacute or chronic mycotic infections. Consequently, the MICs for antifungal drugs may not necessarily predict their successful use in P. brasiliensis infections. Results with the NCCLS method have shown a correlation with clinical outcomes only in C. albicans infections [17–19].

Organ CFUs. To explore the effects of antifungal drug treatment and ajoene in mice infected intratracheally with P. brasiliensis, the CFUs recovered from organs were determined on day 45 post infection (i.e. 43 days after initiation of therapy). The CFUs per gram of tissue showed a significant reduction of fungal cells recovered from animals treated with SMT and ajoene, indicating a possible additive effect. Fungal dissemination to liver and spleen was detected mainly in the SMT treated group but it was virtually undetectable in the group of mice treated with ajoene alone or in combination with the antifungal drugs (Fig. 1).

Cytokine assays. We studied the cytokine levels in the lung homogenates of mice treated with SMT, ajoene and in control animals. The untreated and non-infected animals, non-infected but ajoene treated and infected but untreated groups were used as control groups. Animals that received only ajoene showed significant increase of IL-12 and IFN-γ when compared with untreated and non-infected mice. Animals that were only infected showed an increase of IL-10 and low levels of IL-12 and IFN-γ when compared with untreated and non-infected mice. Animals that received only ajoene showed a significant increase of IL-12 and IFN-γ when compared with untreated and non-infected mice. Animals that were only infected showed an increase of IL-10 and low levels of IL-12 and IFN-γ. The group of mice treated only with SMT had significantly enhanced IFN-γ levels. The most impressive results were observed in the groups that received ajoene. The levels of IFN-γ and IL-12 were significantly higher than in the other groups of mice (Fig. 2).

Fig. 1 Organ colony forming units (CFU) from mice infected intratracheally with 3 × 10⁵ yeast cells and submitted to treatment with ajoene, SMT or association of both. Mice were sacrificed after 45 days of infection. Control mice received phosphate-buffered saline and were infected with the same number of yeast cells. Each bar represents the average counts of fungi in organs and error bars indicate SDs. (●) denote significant differences p < 0.05 relative to control.
Fig. 2 Cytokine levels from lung homogenates obtained from mice untreated and uninfected (PBS-sham), ajoene-treated only (Aj), infected but untreated (Pb), infected and ajoene-treated (Pb+Aj), infected and treated with SMT (Pb+SMT), infected and treated with combined ajoene and SMT (Pb+Aj+SMT). Each bar represents the mean values in lung homogenates and error bars indicate SDs. (*) denote significant differences p < 0.05 comparing with controls.

Lung histopathology: Supporting the observation obtained from the CFU assay and cytokine determination, lungs of infected and untreated control animals showed intense infiltrations composed of mainly macrophages, lymphocytes and epithelioid cells. Multiplying fungal cells were seen (Fig. 3A). A few epithelioid granulomas with giant cells and small numbers of yeast cells were observed in the ajoene-treated group (Fig. 3B). SMT treated mice also showed reduction of viable cells in the histopathology studies with granulomas more organized than those found in the control group (Fig. 3C). Association of both treatments showed reduced infection with large areas of preserved lung tissue and few or undetectable yeast cells (Fig. 3D).

Discussion

Ajoene is a well-established antiplatelet agent and its inhibitory effect on platelet aggregation has been extensively studied and documented in both \textit{in vivo} and \textit{in vitro} experiments [14,20]. The mechanism by which ajoene interferes in fungal growth apparently involves different modes of action including: inhibition of phosphatidylcholine biosynthesis [21,22]; modifications in phospholipid dynamics with an increase in membrane fluidity [23]; inhibition of glutathione reductase resulting in increased concentration of free radicals [24]. The inhibition of fungi by garlic extract or ajoene has been reported [25,26] and specifically with \textit{in vitro} studies of \textit{P. brasiliensis} [10,22].

Among sulfonamides, sulfamethoxazole associated with trimethoprim is the most frequently used treatment for PCM [13]. Despite the introduction of several new procedures in medical technology and novel therapeutic options, treatment of PCM patients usually involves long term therapy. Drug toxicity is always a problem and relapses owing to fungal resistance and/or poorly effective drugs are common.

In the present work we studied the \textit{in vivo} protective effect of ajoene by itself and in association with chemotherapeutic drugs. The experiments using infected mice treated with ajoene showed a significant fungal load reduction in the lungs with virtually no dissemination to other organs. The most impressive results were obtained with the combination of ajoene and SMT which resulted in the absence of fungal cells in the lungs, liver or spleen of infected mice.

Reduction of CFUs from tissue collected from ajoene-treated mice could also be documented by histopathology of the lungs. In non-treated control mice the granulomatous inflammation was exudative and ill-organized, containing numerous viable fungal cells and forming paracoccidiomycosis-like masses in the lungs with dissemination of the fungus to the liver and spleen. In contrast, ajoene and SMT treatment of mice caused a remarkable reduction of the number, size, and nature of the granulomas, with lesions better organized and compact, containing many dead fungal cells, with no dissemination of the infection to other organs. The combined treatment of ajoene and SMT was most efficient, as demonstrated by the presence of very few granulomas in the lungs with no fungal cells inside and the absence of disseminating lesions in the liver and spleen.

Additive effect of \textit{Allium sativum} extract and antifungal drugs was also been found by Davis et al. [27], who showed that the concentrated extract had potent \textit{in vitro} fungistatic and fungicidal activities against 3 different isolates of \textit{Cryptococcus neoformans} with synergistic fungistatic activity when combined with amphotericin B.

The mechanism underlying the synergistic action of ajoene and SMT could involve the reduction of fungal load associated with a stimulated anti-fungal immune
response. When cytokine levels were determined in the lungs, a higher level of IFN-γ and IL-12 was found in the animals that received ajoene treatment in comparison with infected by untreated animals that produced higher levels of IL-10 and low levels of IL-12 and IFN-γ. As discussed before, IFN-γ is a key cytokine in the defence mechanism against paracoccidioidomycosis [28]. These results are comparable to those previously reported which used immunization with P10 alone and in association with chemotherapeutic drugs that resulted in the production of IFN-γ and IL-12 concentrations higher than found in control groups [29]. As with ajoene, immunization with P10 was strongly protective against *P. brasiliensis* intratracheal infection.

The immunomodulatory activity of ajoene towards a protective Th-1 type response seems to occur simultaneously with the direct anti-fungal activity of this compound, which is significantly increased by combined chemotherapy. As presently shown, association of ajoene and SMT was most effective in the treatment of mice that were intratracheally infected with virulent Pb18 strain of *P. brasiliensis*.

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*Fig. 3* Histopathology of lung sections from mice intratracheally infected and submitted to antifungal treatment. (A) Lung section from untreated infected mouse; (B) Lung section from mouse infected and treated with ajoene; (C) Lung section from infected mouse treated with SMT; (D) Lung section from infected mouse treated with SMT and ajoene. Results are representative of groups of 5 to 10 mice for each treatment. HE staining, 10 ×.
References