Detection of rat Pneumocystis carinii proteinases and elastase and antipneumocystis activity of proteinase inhibitors in vitro


Summary:
Proteinases play an important role in survival of microorganisms and in pathogenicity of diseases. By using a modified SDS-gelatin-polyacrylamide gel system, proteinases of rat P. carinii were detected as bands of proteolytic digestion after electrophoresis. P. carinii organisms obtained from dexamethasone immuno-suppressed transtracheally infected rats were cultured in spinner flask suspension cultures to minimize host cell contamination. At pH 8.3, seven P. carinii-specific proteolytic bands were detected in three clusters of different molecular weights clearly different from host cell patterns. By using a range of pH, various preparations of organisms and both infected and uninfected culture media, proteolytic activities have been partially characterized. Elastase secretion has been assessed based on elastin digestion model. Proteinase inhibitors have been tested for their ability to inhibit P. carinii growth in HEL299 short-term monolayer cultures. Results indicate that proteolytic activities are involved in the proliferation of microorganisms since leupeptin exerted in vitro antipneumocystis activity while aprotinin enhanced P. carinii growth.

Key Words: P. carinii proteinases, elastase, proteinase inhibitors.

INTRODUCTION

Pneumocystis carinii remains a major cause of pneumonia in patients with AIDS as well as other immunocompromised patients (Simonds et al., 1995). New treatments for P. carinii pneumonia are needed because adverse reactions to standard therapies are common and adverse reactions to TMP/SMX (trimethoprim/sulfamethoxazole) were associated with a more rapid progression to AIDS and death in HIV-infected individuals (Veenstra et al., 1997). There is concern that P. carinii strains may be developing drug resistance (Lane et al., 1997) and there is also an increasing recognition of a casual relationship between the pathogenicity of a number of lung disorders and disturbances in the regulation of proteinase activities (Stockley et al., 1988). In order to learn more about the biochemistry and pathogenesis of this organism, rat P. carinii was cultured in spinner flasks to produce large quantities of organisms with very few host cells. After electrophoresis of fresh, washed trophozoites in a modified SDS-gelatin-polyacrylamide gel system originally described by Lockwood (Lockwood et al., 1987), proteinases of rat P. carinii were detected as bands of proteolytic digestion. A partial destaining procedure allows detection of protein bands and proteinases in the same gel. It is known that many

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parasites and fungi secrete proteinases that can have a role in the pathogenesis of diseases (Irvine J.W. et al., 1992). Proteinases are classified according to their catalytic mechanisms, regulation of proteolytic activity and major biological function. By these criteria we looked for Pe-specific proteolytic bands clearly different from host cell patterns and we partially characterized them according to the response to specific inhibitors (leupeptin, TLCK and EDTA). Elastase secretion has been assessed based on elastin digestion model.

**MATERIALS AND METHODS**

**P. CARINII EXPERIMENTAL INFECTION**

The *P. carinii* organisms used in this study were obtained from Sprague-Dawley female rats infected with *P. carinii* by transtracheal inoculation as previously reported (Bartlett et al., 1988). Rat-derived *P. carinii* trophozoites were inoculated onto human embryonic lung (HEL 299 [ATCC CCL137]) cells sheeted on microcarrier beads in spinner flasks according to the method published by Lee (Lee et al., 1993). Briefly, micro carrier beads (Cytodex, Sigma) were coated with HEL 299 feeder cells and maintained in a 125 ml slow stirring (about 31 rpm) vessel with minimum essential medium (MEM) (ICN-Flow, Irvin Ayrshire, United Kingdom) supplemented with 2 mM L-glutamine, 10 % fetal calf serum (FCS), 1 % nonessential amino acids (NEAA), 100 U of penicillin per ml and 0.1 mg of streptomycin per ml. When the cells were confluent, *P. carinii* infected tissue was homogenized in MEM, centrifuged slowly to settle large lung pieces and the supernate used for inoculum (scored on Giemsa stained calibrated drops). For testing of the proteinase inhibitors, *P. carinii* organisms prepared as above were added to HEL 299 cells grown to confluency in 24 well plates. These short term culture experiments were done according to the method developed by Bartlett (Bartlett et al., 1985). The cultured organisms were incubated in the presence of various proteinase inhibitors and sampled for counting by washing the monolayer with the medium then counting the number of organisms suspended in the medium.

**GELATIN-POLYACRYLAMIDE GEL ELECTROPHORESIS**

The method used has been modified after Lockwood; briefly, double acrylamide concentrations gel (lower gel 11 %, upper gel 3 %) were used, prepared with 1.5 M Tris hydroxymethylaminomethane (THAM), 0.4 % lauryl sulfate, sodium salt (SDS) (pH 8.8) lower gel buffer and 0.5 % THAM, 0.4 % SDS (pH 6.8) upper gel buffer. Gelatin was added to the lower PAGE gel at the time of casting to a final concentration of 1 %. All solutions were filter-sterilized to minimize bacterial contamination. The gels were run at constant current (12.5 mA) for about five hours in a cold room (4°C) by using 15 × 15 × 0.75 vertical gel unit (Hoefer Scientific). After the run, gels were incubated at 37°C in 2.5 % TritonX 100 for two hours to remove SDS, then incubated overnight in 0.1 M glycine buffer at pH (4.3-9.0) to allow proteinases to digest the gelatin and to develop negative bands. Gels were stained with Coomassie blue in 45 % methanol, 10 % acetic acid for one hour, partially destained with 45 % methanol and 10 % acetic acid and then fixed by a vacuum-heated dryer. Proteinase K (1 μg/ml in deionized water) and Staphylococcal Proteinase V8 (0.15 mg/ml) were used as a high and low molecular weight control proteinases in the system (SIGMA). Protein molecular weight standard was a commercially available mixture of low molecular weight marker (BIORAD).

**SAMPLES FOR ELECTROPHORESIS**

The following samples, always resuspended 1:1 in loading buffer (LB, 1 M sucrose with 0.1 % bromphenol blue), were used (20 μl/well.) for electrophoresis: 1) Feeder Cells (FC): a pellet of uninfected HEL 299 feeder cells (about 5 × 10⁹), washed twice in phosphate buffered citrate then osmotically disrupted in 2 ml deionized water. 2) *P. carinii* organisms: Rat-derived *P. carinii* was cultured in spinner flasks as described above. Four to eight days after inoculation the beads were allowed to settle, the supernatant removed and centrifuged at 4°C to pellet the organisms. The pellet was used to prepare the various samples for electrophoresis: 2a) Whole *P. carinii* (WPc): freshly harvested, cold phosphate buffered saline (PBS) washed *P. carinii* trophozoites (about 5 × 10⁹) were osmotically disrupted in distilled water then resuspended 1:1 in LB. 2b) *P. carinii* Pellet (PPc): cold PBS-washed pellet of WPc after centrifugation at 10,000 rpm for 15 min at 4°C. 2c) Disrupted *P. carinii* supernatant (D PcS): supernatant of centrifuged WPc after osmotic disruption. 2d) Sonicated *P. carinii* freshly harvested, cold PBS washed *P. carinii* trophozoites were sonicated at full power for three pulses 20 seconds each. 3) Culture media: in order to examine for the possible release of *P. carinii* proteinases into culture media, centrifuged uninfected culture medium of HEL 299 feeder cells (CUM) and centrifuged *P. carinii*-infected culture medium of HEL 299 feeder cells (CIM) were also examined by gelatin-PAGE.

**SCREENING METHOD FOR ELASTASE PRODUCTION**

As a screening test to detect elastase activity in *P. carinii* trophozoites, we adapted a method developed by Frosco (Frosco et al., 1992) for Aspergillus.
We tested a 50 μl aliquot of *P. carinii* trophozoites (containing approximately 5 × 10⁶ microorganisms) obtained from spinner flasks as described above. This aliquot was washed twice in cold PBS then put on plates of solid agar containing 0.5 % elastin, 0.05 % yeast carbon base, 0.01 % Rose Bengal in 50 mM sodium borate pH 7.6. The plates were sealed by Parafilm (American National Can) maintained at 37 °C and observed at day one, three and eight to detect a clear zone of elastin digestion around the area of *P. carinii* inoculation. The diameter of clearing was measured in order to roughly estimate elastase production by *P. carinii*. The same preparations of *P. carinii* described above for electrophoresis were also tested in plates.

**IN VITRO STUDY WITH PROTEINASE INHIBITORS**

Confluent monolayers of HEL 299 cells were inoculated with rat *P. carinii* as described and incubated along with various proteinase inhibitors. Pancreatic basic trypsin inhibitor (aprotinin), n-acetyl-N-pro-pionyl-L-leucyl-DLargininal (leupeptin), L-1-tosylamide-2-phenyl-ethyl chloromethylketone (TPCK) and N-alpha-p-tosyl-L-lysine chloromethylketone (TLCK) obtained from SIGMA were dissolved in dimethylsulfoxide (DMSO) if needed and adjusted to a final concentration of 50 μg/ml in MEM. The chelating agent EDTA was used at 5 mM final concentration.

The plates were incubated at 37 °C in 5 % CO₂. At one, three, five, and eight days after inoculation the cultures were agitated, and a 10 μl sample of the culture supernatant from each well was taken for analysis, air dried onto a 1 cm² square etched on a glass slide, fixed with methanol, and stained with Giemsa (Bio-Optica). Each slide was examined microscopically with a 100 x objective to quantitate the organisms. The data are reported as the number of trophozoites per field. The final score for each slide was the mean value of 30 observations; multiplying this number by a factor of 4 × 10⁵ (determined by estimating the number of fields per cm² at a magnification of ×1000), yields the number of organisms per ml of culture supernatant. Each proteinase inhibitor was tested in four wells in each experiment and each trial was carried out three times before the final reported evaluation. Cotrimoxazole treated (54 μg/ml) and untreated *P. carinii* cultures were used respectively as positive and negative controls. To evaluate the effect of proteinase inhibitors on cell monolayers we incubated uninfected confluent cells with each inhibitor at the same concentration used with *P. carinii* infected cells: the toxic effect and signs of cell damage, like detachment, vacuolization, or alteration of cell morphology, were recorded at day one, three, five, and eight.

**VIABILITY TEST**

Fluorescin diacetate and ethidium bromide were used to test viability of microorganisms used for and obtained from spinner flasks and culture plates as previously described (Jackson et al., 1985).

**RESULTS**

The spinner flask culture system for *P. carinii* growth on day five provided about 6-8 × 10⁹ Pc organisms with 95 % viable trophozoites from each harvest. This offered the opportunity of performing the enzymatic studies with live, fresh microorganisms virtually free from contaminating host cells. Preliminary gelatin-SDS-PAGE (without digestion of the substrate) performed with sample of washed HEL cells, whole and sonicated *P. carinii* preparations and culture medium alone clearly showed different electrophoretic patterns for each sample. Figure 1 shows the different patterns of protein separation for the digested gelatin-SDS-PAGE gels along with a molecular weight standard used to evaluate the following digested gels.

**DETECTION OF *P. CARINII* PROTEINASES BY GELATIN-PAGE**

No proteolytic activity could be detected by using increasing amounts of sonicated Pc trophozoites and HEL 299 cells. In the same gel digestion of the gelatin substrate occurred with V8 and Proteinase K controls, indicating the effectiveness of the gelatin-SDS-PAGE model (Fig. 2). Proteinases were repeatedly detected in fresh, unsonicated samples of *P. carinii* trophozoites, obtained from PBS washed pellets of spinner flask cultures osmotically disrupted in distilled water (Fig. 3) as preliminarily shown (Atzori et al., 1991). By incubating the gelatin-PAGE in 1 M glycine buffers at different pHs, ranging from 4.3 to 9.0, we found the optimal pH to be 8.3. At this pH we could visualize up to seven clearly detectable proteolytic bands of different MWs in the *Pc* samples. Three groups of proteinases were observed: 1) Two bands of proteinase activity of a high MW (> 98 kDa) which were present in the washed *P. carinii* that was osmotically disrupted and in its washed pellet but not in the disrupted *P. carinii* supernatant, nor were the bands detected in the *P. carinii* infected or uninfected culture media. 2) A group of proteolytic bands with MWs ranging from 55 to 70 kDa which was present in Pc disrupted in distilled water, supernatant of *Pc*, and the Infected Culture Medium (but not the Uninfected Culture Medium), suggesting a possible secretory role. 3) A final proteinase with a MW of about 40 kDa detected only in the
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Fig. 1. – Picture of Comassie Blue stained gelatin-PAGE gel showing the characteristic patterns of protein separation in the following reference samples: HEL 299 feeder cells (FC), whole and sonicated *P. carinii* (lanes are labeled as WPc and SPc, respectively) and culture infected medium (CIM): clear differences in molecular weight distribution of protein contents in each sample appeared when compared with the molecular weight standard (Std). This gel is used as reference to identify bands in other partially digested gelatin-PAGE gels.

* Standard molecular weights: 97.500 D, 66.200 D, 42.699 D, 31.00 D, 21.500 D, 14.400 D.

Fig. 2. – The picture shows gelatin-SDS-PAGE analysis of sonicated *P. carinii* (SPc) and HEL 299 feeder cells (FC) proteinases: no proteolytic activity is detectable in the first lanes containing samples (SPc and FC); areas of proteolytic digestion with staphylococcal proteinase (V8*) and Proteinase K (PK**) controls confirm the digestion of the substrate after incubation in non-ionic detergent, which removed SDS inhibition, and in glycine buffer at 37°C. Major surface glycoprotein (MW: 110-120 KD) of *P. carinii* is well detected in sonicated samples.

# Standard molecular weights: 97.500 D, 66.200 D, 42.699 D, 31.00 D, 21.500 D, 14.400 D.

Fig. 3. – Gelatin-SDS-PAGE analysis of *P. carinii* proteinases. The gel is 11 % acrylamide containing 0.1 % gelatin. Background is stained with Comassie blue. PK and V8 are control proteinases. Mol wt (Std) is Biorad low molecular weight marker lane. WPc indicates osmotically disrupted *P. carinii* trophozoites resuspended in 1 M sucrose loading buffer, DPcS indicates the centrifuged supernatant of WPc and CIM indicates centrifuged culture medium of *P. carinii*-infected HEL 299 feeder cells. Note the contamination of V8 ($) in the lane of HEL 299 sample (FC), which does not express proteinase activity.

# Standard molecular weights: 97.500 D, 66.200 D, 42.699 D, 31.00 D, 21.500 D, 14.400 D.
**P. CARINII PROTEINASES, ELASTASE AND PROTEINASE INHIBITORS**

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FC = Washed Feeder Cells; SPc = Sonicated WPc; WPc = Whole P. carinii trophozoites grown in cells culture, osmotically disrupted in distilled water; PPc = Washed pellet of WPc; DPcS = supernatant of WPc; CUM = centrifuged uninfected culture medium of HEL 299 feeder cells; CIM = centrifuged P. carinii infected culture medium of HEL 299 feeder cells.

**Table I. - Synoptic table of results.**

WPc. A summary of the results obtained by gelatin-SDS-PAGE is reported in Table I.

By including agar with the elastin, we screened dilutions of washed trophozoites derived from spinner flasks for the capability of generating clear zones of elastin digestion: pale areas of about 0.5 to 1.2 cm of clearing around the drops occurred only with undiluted organisms and 1/2 to 1/4 dilutions, suggesting a weak production of elastase by washed P. carinii derived from spinner flasks (Fig. 4). In order to verify the possible presence of elastase from culture medium we also tested P. carinii infected and uninfected culture medium: no areas of digestion occurred in either case even if a change of agar-elastin color was observed in the area of drop deposition. Contamination of plates by day eight prevented further evaluation.

**IN VITRO SCREENING OF PROTEINASE INHIBITORS**

Incubation of proteinase inhibitors with confluent cells showed that 50 μg/ml TPCK was toxic to the uninfected HEL 299, causing vacuolization, enlargement and detachment of many feeder cells while 5 mM EDTA, as well as leupeptin, TLCK, and aprotinin at 50 μg/ml did not affect the monolayers.

Figure 5 shows growth curves of untreated and proteinase inhibitor-treated P. carinii trophozoites cultured in HEL 299 monolayers: each set of experimental data was interpolated by binomial parabolic plot \( y = ax^2 + bx + c \), after verifying the good fitting of the regression curve \( 0.82 < R < 0.98 \). Aprotinin acted as a growth-factor, boosting the active proliferation of P. carinii trophozoites up to the maximum mean number of 19.78 microorganisms per field, observed on day eight, with a rapid increase during the first three days of cell-culture. Leupeptin was inhibitory at 50 μg/ml; comparable to the Cotrimoxazole (54 μg/ml, Trimethoprim 9 μg/ml + Sulphamethoxazole 45 μg/ml) treatment. Both 50 mg/ml TLCK and 5 mM EDTA were inhibitory. Viability tests with ethidium bromide and fluorescein diacetate on aliquots sampled from each well on day eight showed less than 1 % viability of Cotrimoxazole-treated P. carinii forms, while untreated and aprotinin-treated samples had big clusters of 90-95 % viable trophozoites. Leupeptin and TLCK-treated microorganisms had 43 % and 52 % viable P. carinii respectively.

**DISCUSSION**

The spinner flask culture method provided in a short time (five days) large numbers of 95 % viable P. carinii trophozoites almost completely (less than 1 %) free from feeder cells and host cell debris, that were suitable for enzymatic and biochemical studies. Each harvest recovered 5-8 x 10^8 P. carinii trophozoites, which allowed the preparation of multiple samples from the same harvest. HEL 299 cells, which had never been in contact with P. carinii, gave a
clearly different electrophoretic pattern from sonicated or osmotically disrupted P. carinii trophozoites, confirming that washed P. carinii prepared from spinner flask supplied "clean" microorganisms for testing. The gelatin SDS-PAGE system is a useful technique for the detection of proteinase activities of P. carinii trophozoites grown in spinner flask cultures: seven proteinases with molecular weights ranging from 40 to about 98 kDa were observed in fresh, washed, osmotically disrupted P. carinii trophozoites, while no similar bands could be detected in preparations of washed HEL 299 feeder cells or sonicated P. carinii. Since CUM (Pc uninfected cell culture medium) did not show any protease activity, while CIM (Pc infected culture medium) did, it is likely that proteases detected in WPc are specifically derived from P. carinii microorganisms and not due to cell up-regulation. In our system, the best expression of seven different rat-derived P. carinii proteinases was observed in glycine buffer at the optimum pH of 8.3, suggesting that P. carinii trophozoites may contain multiple proteinases of the cysteine-serine type. In another study, authors have identified seven bands from human-derived P. carinii microorganisms at pH 7.5: the 90 kDa human Pc band was selectively inhibited by EDTA, and may be a metallo proteinase (Massetti et al., 1992). In this study, however, P. carinii sample obtained from lung biopsy of a patient with PCP, was reasonably not entirely decontaminated from host cells. The proteinase activity still present in the pellet after centrifuging osmotically disrupted trophozoites could be important in establishing P. carinii interaction with the monolayers and, in vivo, in the alveolar cell environment of the lung, as suggested for the protozoan Trichomonas, interacting with epithelial cells (Arroyo & Alderete, 1989). The soluble P. carinii proteolytic activity found in infected cell culture medium, but not in uninfected culture medium may result from secretory proteinases, these might be associated with lung damage, as has been observed with the flagellate T. tenax which utilizes secretory proteinases for collagen cleavage (Bozner & Demas, 1991). Our data could explain the correlation between the level of P. carinii infection in the lungs of immunosuppressed rats and the measured cathepsin H-like activity which was greater than that seen with any of the other three proteinase activities normally elevated in lung disorders (Hayes et al., 1991). The patterns of immunoelectrophoresis (IEF) gels supported the idea that at least a part of this proteinase activity derived from P. carinii and the reported absence of lysosomes in the microorganism suggested that the proteinases were secreted into the surfactant lining of the alveoli. These results confirmed the observations of Hayes (Hayes et al., 1991) who detected an increased cysteine-proteinase in rat lung associated with development of P. carinii infection, suggesting that the increased proteinase activity was partially due to isoenzymes from P. carinii. The proteinase may be of importance in roles similar to those described for other parasites such as the breakdown of host proteins for nutritional purpose or in the destruction of immunosystem by digestion antibody or cytokine. The screening test for elastase demonstrated for the first time that P. carinii produces at least small amounts of elastase, thus also offering a possible explanation for the lung alveolar damage observed during P. carinii pneumonia. Study of the fungus-derived Aspergillus elastase, demonstrated that elastase is inhibited by 0.21 mM leupeptin, suggesting that the enzyme is a cysteine proteinase. However, the enzyme is also inhibited by 5 mM EDTA, suggesting a requirement for divalent cations. The fungal-derived enzyme

**Fig. 5.** Effect of proteinase inhibitors on growth of cultured P. carinii assessed by counting the numbers of organisms in culture supernatant at the times shown. Each set of experimental data was interpolated by binomial parabolic plot, after verifying the good fitting of the regression curve (0.82 < R^2 < 0.98).
acts optimally at pH 7.4 at 45 °C in 50 mM Sodium Borate buffer, but in Tris HCl the pH optimum shifted to 8.8. Further study is warranted in order to characterize P. carinii elastase. TLCK and TPCK, both potent serine proteinase inhibitors, are highly reactive molecules with sulfhydryl groups and can also modify other amino acid lacking SH residues such as histidine. Other authors (Harth et al., 1993) using fluromethylketones at various concentrations did not observe toxicity to macrophages, fibroblasts or epithelial cells in culture experiments with T. cruzi. However, in HEL cell cultures, TPCK at 50 μg/ml damaged the monolayer, thus results on inhibition of P. carinii growth could not be interpreted. Many drugs active against malaria, like primaquine and other 8-aminoquinolines, also demonstrate antipneumocystis activity (Bartlett et al., 1991; Queener et al., 1993). According to Krugliak (Krugliak & Ginsburg, 1991), who studied the aminolar mode of action of quinolone containing drugs (also active against P. carinii), leupeptin inhibited digestion of ingested host cell cytosol, and thus inhibited parasite growth, though reversibly. Leupeptin is a naturally occurring proteinase inhibitor isolated from the culture filtrates of various species of Actinomycetes. The inhibitory effect of leupeptin at 50 μg/ml towards P. carinii growth in vitro deserves further investigation, after the demonstration of low (43 %) viability of residual organisms from in vitro culture, in accordance with the cytostatic but not cytocidal effect which has been observed with W2 clones of P. falciplanum whose proliferation was arrested when treated with leupeptin at a MIC of 50 μM, but treated organisms were still at least 80 % viable as judged by a cytocidal assay (Young et al., 1993). Aprotinin, a serine proteinase inhibitor obtained from bovine pancreas, did not inhibit P. carinii growth in vitro, on the contrary at 50 μg/ml exerted a slight enhancement in speed of active multiplication. Each time, in the presence of aprotinin the number of cultured microorganisms reached their mean maximum on day three from infection of the monolayer, the cessation of further proliferation may be due to the rapid consumption of culture media nutrients. The untreated controls approached a similar maximum number only on day eight. P. carinii trophozoite growth was strongly inhibited in vitro by 5 mM EDTA, indicating that the removal of endogenous or contaminant divalent cations by chelation is critical for the microorganism growth. This fact can be due to the inhibition of metallo proteinases, and could explain the known susceptibility of P. carinii to ironchelator agents, like desferoxamine (Weinberg, 1994; Merali et al., 1995) and the inhibition of P. carinii attachment to rat alveolar macrophage described by Pottratz (Pottratz et al., 1990). In conclusion, P. carinii trophozoites contain an array of proteinases, distinct from those of monolayer HEL 299 host cells as shown by the active digestion of gelatin containing PAGE samples. The preparation from washed, osmotically disrupted P. carinii exhibited a maximum of seven proteolytic bands at pH 8.3. While studying the possible effect of several proteinase inhibitors on P. carinii growth in vitro, leupeptin was found to be an effective inhibitor of P. carinii proliferation (p < 0.001), whereas aprotinin at the same concentration acted as a growth factor. Most of the proteolytic activity of P. carinii seems due to serine-cysteine proteinases, with the possibility that metallo proteinases are also represented raising several questions with regard to function, localization and regulation of the individual enzymes. According to North (North et al., 1990), there are some similarities between the proteinase contents of many protozoa, notably most of them contain multiple proteinases of the cysteine type. The role of P. carinii proteinases in P. carinii pneumonia is indeed unknown. Further studies are now in progress in order to ascertain the role of P. carinii proteinases in the pathogenesis of lung damage as a virulence factor as has been observed for Aspergillus fumigatus elastase (Kothy et al., 1984; Fosco et al., 1992).

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