DETECTION OF RAT *PNEUMOCYSTIS CARINII* PROTEINASES AND ELASTASE AND ANTIPNEUMOCYSTIS ACTIVITY OF PROTEINASE INHIBITORS *IN VITRO*

ATZORI C.*, MAININI A.*, AGOSTONI F.*, ANGELI E.*, BARTLETT M.**, BRUNO A.***, SCAGLIA M.*** & CARGNEL A.*

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 dexamethazone immunosuppressed transtracheally infected rats were cultured in spinner flask suspension cultures to minimize host cell contamination. At pH 8.3, seven *Pc*-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.

Résumé : DÉMONSTRATION DES PROTÉASES ET ÉLASTASE DE *P. CARINII* DE RAT ET DE L'ACTIVITÉ ANTIPNEUMOCYSTIS *IN VITRO* DES INHIBITEURS DE PROTÉASES

Les protéases jouent un rôle important pour la survie des micro-organismes et pour la pathogénie des maladies. Par un système de gel en SDS-gélatine-polyacrylamide modifié, on a mis en évidence des bandes de digestion protéolytique qui sont dues à des protéases de P. carinii. *Les organismes de* P. carinii, *obtenus à partir de rats immunodéprimés par la dexaméthasone et infectés par voie trans-trachéale, ont été cultivés en suspension dans des flacons "spinner flasks" pour réduir au maximum (< 1 %) la contamination par les cellules-hôtes. A pH 8,3, on a compté sept bandes protéolytiques spécifiques de* P. carinii *qui peuvent être rassemblées en trois groupes de poids moléculaires clairement différents de ceux des cellules-hôtes. De plus, en employant des pH variés, on a partiellement caractérisé les activités protéolytiques de différentes préparations d'organismes et de milieux de culture aussi bien infectés que non infectés. La sécrétion d'élastase a été démontrée avec un modèle de digestion de l'élastine. Des inhibiteurs des protéases ont été expérimentés pour leur aptitude à inhiber le développement de* P. carinii *dans une monocouche de cellules HEL299 en cultures à court terme. Les résultats démontrent que les activités protéolytiques sont impliquées dans la prolifération des micro-organismes, puisque la leupeptine exerçait une activité antipneumocystis* in vitro, *alors que l'aprotinine stimulait le développement de* P.carinii.

MOTS CLÉS : *protéases, élastase, inhibiteurs des protéases de* P. carinii.

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

* II Div. Infect. Dis., L.Sacco Hospital, Milan, Italy.
** Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, USA.
*** Parasitology Lab., IRCSS/Policlinico S. Matteo, Pavia, Italy.
Correspondence: Chiara Atzori, II Division Infectious Diseases, L. Sacco Hospital, Via G.B. Grassi 74, CAP 20148, Milan, Italy.
Tel: 39-2-35799572 – Fax: 39-2-38200909.
E-mail: aidsaiuto@pn.itnet.it

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 *Pc*-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 homogeneized in MEM, centrifuged slowly to settle large lung pieces and the supernate used for inoculum at a final concentration of 5×10^5 trophic forms per ml of culture medium (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 $15 \times 15 \times 0.75$ 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 Comassie 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 % bromophenol blue), were used (20 µl/well.) for electrophoresis: 1) Feeder Cells (FC): a pellet of uninfected HEL 299 feeder cells (about 5×10^6), 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: 2*a*) Whole *P. carinii* (WPc): freshly harvested, cold phosphate buffered saline (PBS) washed *P. carinii* trophozoites (about 5×10^9) were osmotically disrupted in distilled water then resuspended 1:1 in LB. 2*b*) *P. carinii* Pellet (PPc): cold PBS-washed pellet of WPc after centrifugation at 10.000 rpm for 15 min at 4 °C. 2*c*) Disrupted *P. carinii* supernatant (DPcS): supernatant of centrifuged WPc after osmotic disruption. 2*d*) 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 5×10^6 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-Npropionyl-Lleucyl-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 dimethilsulfoxide (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 % CO_2. 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 cm^2 square etched on a glass slide, fixed with methanol, and stained with Giemsa (Bio-Optica). Each slide was examined microscopically with a 100 × 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 4×10^5 (determined by estimating the number of fields per cm^2 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

Fluorescein 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 $6\text{-}8 \times 10^9$ *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 preliminarly shown (Atzori *et al.*, 1991). By incubating the gelatin-PAGE in 1 M glycine buffers at different pH's, 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

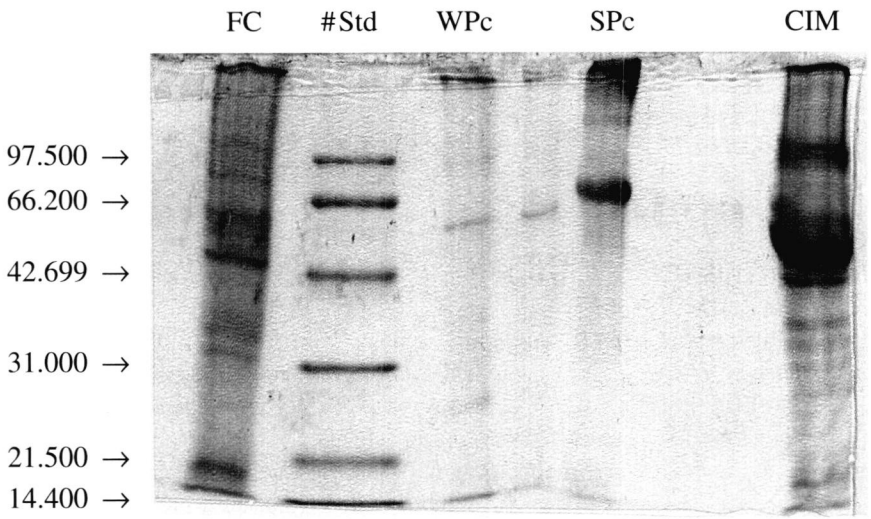

Fig. 1. – Picture of Comassie Blue stained gelatin-PAGE gel showing the characteristic patterns of protein separation in the following reference samples: HEL 299 feeder cells (FC), whole and sonicated *P. carinii* (lanes are labeled as WPc and SPc, respectively) and culture infected medium (CIM): clear 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.

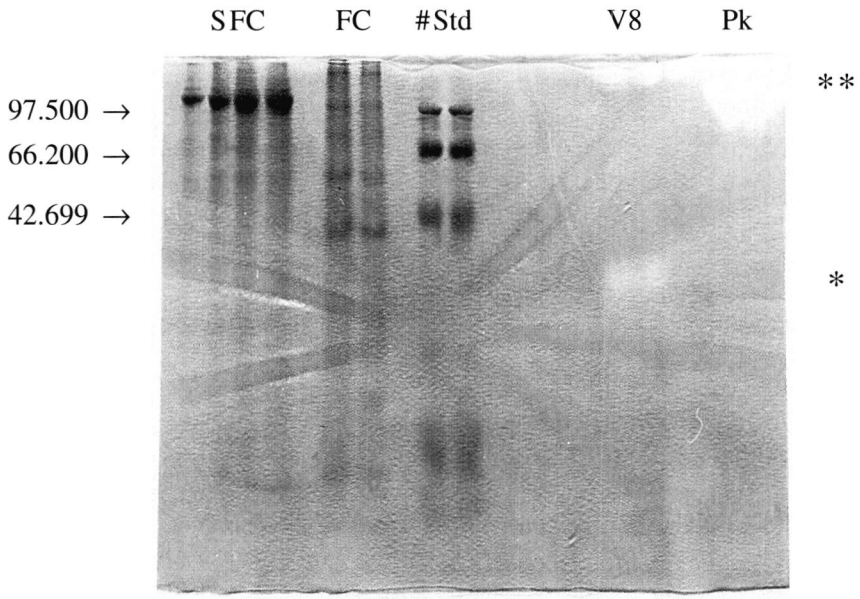

Fig. 2. – The picture shows gelatin-SDS-Page analysis of sonicated *P. carinii* (SPc) and HEL 299 feeder cells (FC) proteinases: no proteolytic activity is detectable in the first lanes containing samples (SPc and FC); areas of proteolytic digestion with staphylococcal proteinase (V8*) and Proteinase 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.

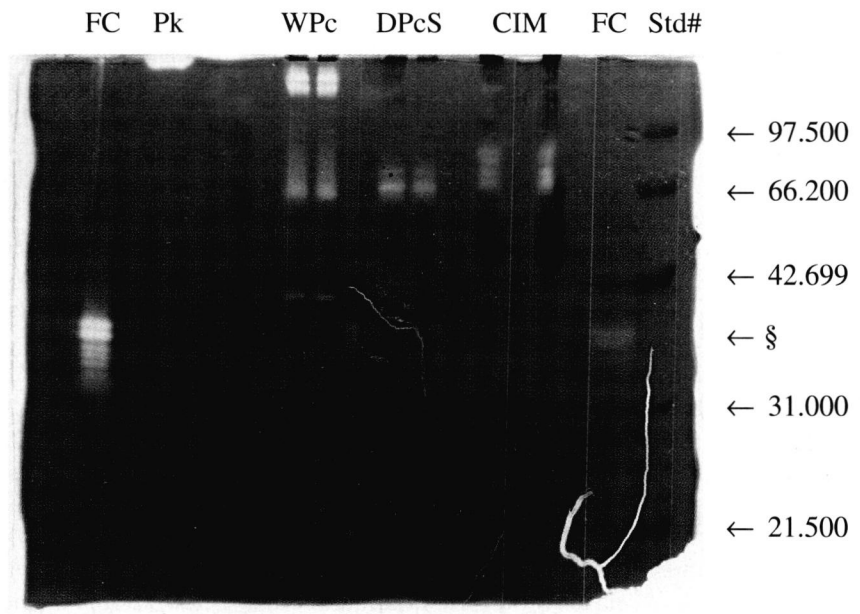

Fig. 3. – Gelatin-SDS-PAGE analysis of *P. carinii* proteinases. The gel is 11 % acrylamide containing 0.1 % gelatin. Background is stained with Comassie blue. PK and V8 are control proteinases. Mol wt (Std) is Biorad low molecular weight marker lane. WPc indicates osmotically disrupted *P. carinii* 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.

Proteinases mol wt	FC	SPc	WPc	PPc	DPcS	CUM	CIM
> 98 kDa (a)	–	–	+	+	–	–	–
> 98 kDa (b)	–	–	+	+	–	–	–
70 kDa	–	–	+	–	+	–	+
65 kDa	–	–	+	–	+	–	+
60 kDa	–	–	+	–	+	–	+
55 kDa	–	–	+	–	+	–	+
40 kDa	–	–	+	–	–	–	–

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 $5\text{-}8 \times 10^8$ *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

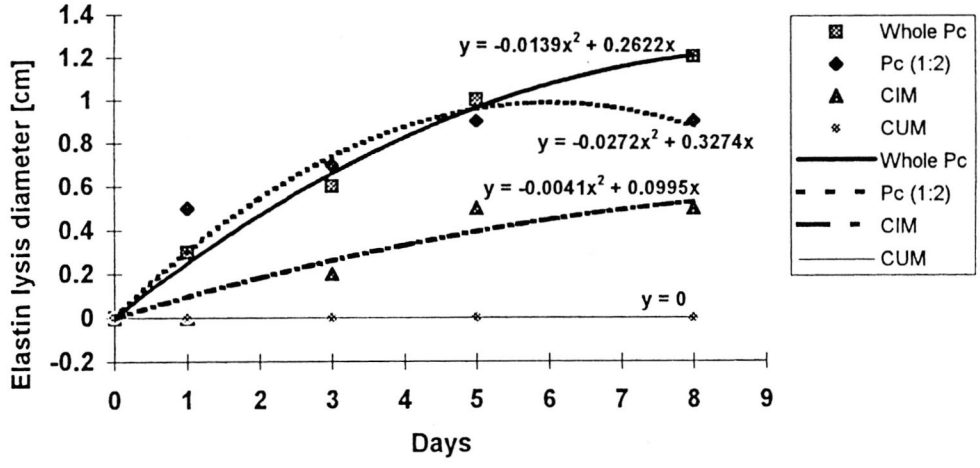

Fig. 4. – Elastase production by cultured *P. carinii* trophozoites detected as diameter of elastin clearing on solid medium (Agar 1.5 %, containing 0.5 % elastine, 0.01 % Rose Bengal) at the time shown.

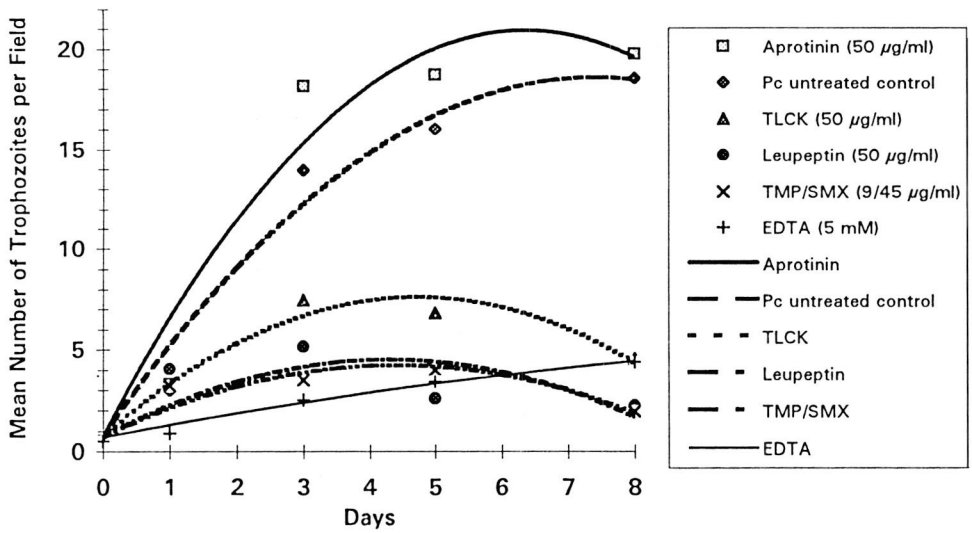

Fig. 5. – Effect of proteinase inhibitors on growth of cultured *P. carinii* assesed by counting the numbers of organisms in culture supernatant at the times shown. Each set of experimental data was interpolated by binomial parabolic plot, after verifying the good fitting of the regression curve ($0.82 < R^2 < 0.98$).

clearly different electrophorectic 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 immunoelelectrophoresis (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

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 antimalarial mode of action of quinoline 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. falciparum* 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 (Kothary *et al.*, 1984; Frosco *et al.*, 1992).

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