Article available at http://www.parasite-journal.org or http://dx.doi.org/10.1051/parasite/1996032183

MADIN DARBY CANINE KIDNEY: A NEW CELL LINE FOR *PNEUMOCYSTIS CARINII IN VITRO* CULTURE

ATZORI C.*, BRUNO A.*, AGOSTONI C.*, NOVATI S.*, GATTI S.* & SCAGLIA M.*

Summary :

Pneumocystis carinii pneumonia (PCP) is a highly frequent cause of morbidity and mortality in immunocompromised subjects, particularly in HIV-infected ones. The biology of *P. carinii* is poorly understood because of the lack of reliable synthetic media or adequate cell lines to grow this opportunistic pathogen in continuous culture. We reported the suitability of the MDCK (Madin Darby Canine Kidney, ATTC CCL 34) cell line to support the temporary microorganism's growth *in vitro* and the experimental pharmacological trials, in comparison with the HEL 299 cell line, used as reference standard.

KEY WORDS : *Pneumocystis carinii, in vitro* culture, MDCK cell line.

Résumé : MADIN DARBY CANINE KIDNEY : UNE NOUVELLE LIGNE CELLULAIRE POUR LA CULTURE *IN VITRO* DE *PNEUMOCYSTIS CARINII*

La pneumonie à Pneumocystis carinii (PCP) est la cause la plus fréquente de morbidité et mortalité chez les sujets immunodéprimés, notamment les patients infectés par le VIH. La biologie de P. carinii *reste encore insuffisamment connue, du fait qu'on ne dispose pas encore de milieux synthétiques ou de lignées cellulaires permettant la culture continue de ce micro-organisme opportuniste. Dans cette étude les auteurs démontrent que la lignée cellulaire MDCK (Madin Darby Canine Kidney, ATCC CCL 34) peut supporter la culture temporaire de* P. carinii *et les tests de pharmacosensibilité tout comme la lignée HEL 299 prise comme référence.*

MOTS CLÉS : Pneumocystis carinii, *culture* in vitro, *ligne cellulaire MDCK.*

INTRODUCTION

Pneumocystis carinii causes severe opportunistic pneumonia (PCP) in immunocompromised hosts. In particular PCP continues to be the most frequent cause of morbidity and mortality in HIV-infected subjects (Glatt and Chirgwin, 1990).

The emergence of AIDS-related PCP has shed new light on the problems related with therapy. Both classic therapeutic regimens, such as cotrimoxazole (TMP-SMZ) and pentamidine and newer ones, such as clindamycin-primaquine, trimetrexate and atovaquone (Sattler and Feinberg, 1992; Hughes *et al.*, 1993), entail a high risk of severe side effects. Research efforts to identify effective drugs have been at least partially hindered by the lack of synthetic media or adequate cell lines to grow *P. carinii* in continuous culture. Since the late 70's, several cell lines have been employed with acceptable results to maintain *P. carinii* vegetative forms in a short- or medium-term culture (Latorre *et al.*, 1977; Pifer *et al.*, 1977, 1978; Bartlett *et al.*, 1979; Cushion *et al.*, 1985).

We report on the satisfactory results we obtained by culturing *P. carinii* on the MDCK (Madin Darby Canine Kidney, ATTC CCL 34) cell line, which has already been employed successfully for the *in vitro* isolation and culture of other microorganisms such as *Cryptosporidium parvum* (Gut *et al.*, 1991; Rosales *et al.*, 1993) and Microsporidia (*Encephalitozoon* spp.) (Canning and Hollister, 1991; Curry and Canning, 1993; Hollister *et al.*, 1993). The HEL 299 line was used as a reference standard because of its known reliability in drug sensitivity tests (Bartlett, 1991; Atzori *et al.*, 1993; Mirovsky and Fishman, 1993; Queener *et al.*, 1993).

MATERIALS AND METHODS

Live *P. carinii* trophozoites were used as inoculum, and were prepared by extraction and purification from the lungs of pharmacologically immunosuppressed rats which had been previously infected by the transtracheal instillation (Bartlett *et al.*,

* Laboratory of Clinical Parasitology, Institute of Infectious Diseases, University-IRCCS S.Matteo, 27100 Pavia, Italy.
Correspondence : Pr. Massimo Scaglia. Tel. : +(382) 502.698.
Fax : + (382) 42.33.20.

1988). The lungs of the infected animals were homogenized in a grinder with MEM medium and gently centrifuged at 500 rpm to remove the larger tissue fragments. The number of microorganisms was obtained by microscopical observation of a sample of supernatant and viability test performed at the same time by using the double staining fluorogenic method described by Kaneshiro (1991).

MDCK and Hel 299 cells were cultured in 12- or 24-well plates (Flow Lab.) at 37 °C in 5 % CO_2 with minimal essential medium (MEM) to which 10 % fetal calf serum (FCS) was added. Once a confluent monolayer had developed, each well was inoculated with 50 µl of lung homogenate to obtain a final concentration of 5-6 × 10^5 trophozoites/ml.

The organisms developed in culture in the extracellular space; some were adherent to the host cells, while others were free in the culture medium, at times forming clusters.

One, 5 and 8 days after the inoculation, organism counts were performed after gentle pipetting the supernatant in order to detach the adherent microorganisms. Each point, plotted in the growth curves, represented the mean trophozoites' number per field (magnification 1,000 ×) obtained by blind count from two observers of 30 fields of a 10 µl drop of supernatant of *P. carinii* cell culture dried onto a 1 cm square slide and Giemsa stained: the mean value has a mathematical correlate, as multiplyied × 400,000 yields to the number of trophozoites/ml. Viability test was performed at the moment of the infection and repeated on day 8.

An experimental trial with TMP-SMZ (9-45 µg/ml) was performed in parallel, by adding the drug to the wells together with the *P. carinii* inoculum. Study control of the direct effect of the drug on monolayer at the same concentration used for *P. carinii* infected cells was also done. Counts were performed by using the same approach described for growth curves.

Each experiment was repeated at least three times before final evaluation, four well replications for each sample.

RESULTS AND DISCUSSION

Repeated experiments showed that the growth curves thus obtained from rat-derived *P. carinii* after an *in vivo* growth stage in the MDCK cell line were comparable to those obtained in the reference HEL 299 cell line (Fig. 1). The increase in the number of organisms was obviously related to the active multiplication of trophozoites, which represented the absolutely prevalent form observed (approximately 99 % of total) as compared to the cysts, with a 95 % viability assessed on day 8. According to our observation, gentle pipetting the culture supernatant is very important in order to obtain detachment of adherent forms, a phenomenon well demonstrated also by different Authors (Aliouat *et al.*, 1993; Bartlett *et al.*, 1994).

The suitability of the *in vitro* culture technique was evaluated by double-blind counts and showed a 10-fold increase in the number of organisms/ml at the end of the culture period (average initial count: 1.4 organisms/field; average final count, after 8 days of culture: over 13 organisms/field).

The results of the pharmacological tests performed with TMP-SMZ were comparable in the two cell lines tested (Fig. 2). Less than 1 % of TMP/SMZ treated microorganisms were viable at day 8.

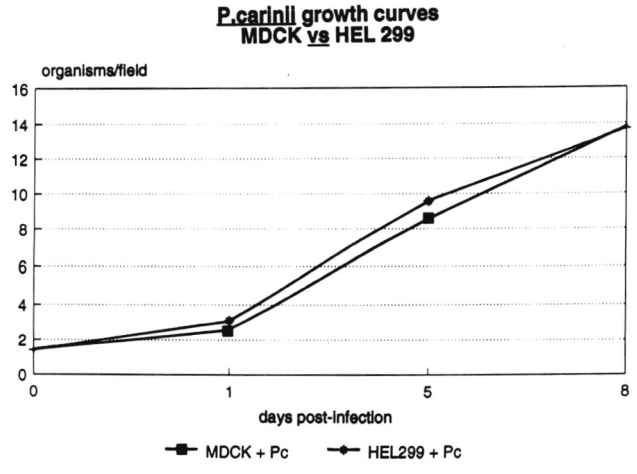

Fig. 1 – Growth curves of rat-derived *P. carinii* (Pc) in MDCK and HEL 299 cell lines.

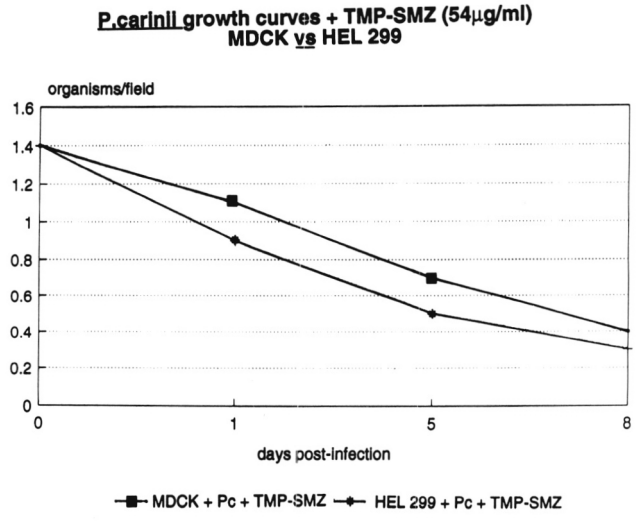

Fig. 2. – *In vitro* culture of *P. carinii* (Pc) in MDCK and HEL 299 cells; response to addition of cotrimoxazole (TMP-SMZ).

Given its already proven efficacy to support the development of human strains of protozoan pathogens (*i.e. C. parvum* and Microsporidia) with good reproducibility of the culture (Gut *et al.*, 1991; Rosales *et al.*, 1993; Canning and Hollister, 1991; Curry and Canning, 1993; Hollister *et al.*, 1993), MDCK cell line may be considered a candidate for the role of « all-purpose » cell line for the isolation, maintenance and pharmacological testing of opportunistic microorganisms with paramount importance in AIDS- and non-AIDS-related human infectious diseases.

ACKNOWLEDGEMENTS

This work was partially supported by ECA; contract # BMH CT94 PL 1118.

REFERENCES

ALIOUAT E.M., DEI CAS E., OUAISSI A., PALLUAULT F., SOULEZ B. & CAMUS D. *In vitro* attachment of *P. carinii* from mouse and rat origin. *Biologie Cellulaire*, 1993, *77*, 209-217.

ATZORI C., BRUNO A., CHICHINO G., BOMBARDELLI E., SCAGLIA M. & GHIONE M. Activity of Bilobalide, a sesquiterpene from *Ginkgo biloba*, on *Pneumocystis carinii*. *Antimicrobial Agents and Chemotherapy*, 1993, *37*, 1492-1496.

BARTLETT M.S. Models for evaluating compounds for activity against *Pneumocystis carinii*. *European Journal of Clinical Microbiology and Infectious Diseases*, 1991, *10*, 199-201.

BARTLETT M.S., VERBANAC P.A. & SMITH J.W. Cultivation of *Pneumocystis carinii* with WI-38 cells. *Journal of Clinical Microbiology*, 1979, *10*, 796-799.

BARTLETT M.S., FISHMAN J.A., QUEENER S.F., DURKIN M.M., JAY M.A. & SMITH J.W. New rat model of *Pneumocystis carinii* infection. *Journal of Clinical Microbiology*, 1988, *26*, 1100-1103.

BARTLETT M.S., GOHEEN M.P., LEE C.H., SHAW M.M., DURKIN M.M. & SMITH J.W. Close association of *P. carinii* from infected rat lung with culture cells as shown by light and electron microscopy. *Parasitology Research*, 1994, *80*, 208-215.

CANNING E.U. & HOLLISTER W.S. *In vitro* and *in vivo* investigations of human microsporidia. *Journal of Protozoology*, 1991, *38*, 631-635.

CURRY A. & CANNING E.U. Human microsporidiosis. *Journal of Infection*, 1993, *27*, 229-236.

CUSHION M.T., RUFFOLO J.J., LINKE M.J. & WALZER P.D. *Pneumocystis carinii*: growth variables and estimates in the A549 and WI-38 human cell lines. *Experimental Parasitology*, 1985, *60*, 43-54.

GLATT A.E. & CHIRGWIN K. *Pneumocystis carinii* pneumonia in human immunodeficiency virus-infected patients. *Archives of Internal Medicine*, 1990, *150*, 271-279.

GUT J., PETERSEN C., NELSON R. & LEECH J. *Cryptosporidium parvum*: *in vitro* cultivation in Madin-Darby Canine Kidney cells. *Journal of Protozoology*, 1991, *38*, 725-735.

HOLLISTER W.S., CANNING E.U., COLBOURN N.I., CURRY A. & LACEY C.J.N. Characterization of *Encephalitozoon hellem* (Microspora) isolated from the nasal mucosa of a patient with AIDS. *Parasitology*, 1993, *107*, 351-358.

HUGHES W., LEOUNG G., KRAMER F., BOZZETTE S.A., SAFRIN S., FRAME P., CLUMECK N., MASUR H., LANCASTER D., CHAN C., LAVELLE J., ROSENSTOCK J., FALLOON J., FEINBERG J., LAFON S., ROGERS M. & SATTLER F. Comparison of atovaquone (566080) with trimethoprim-sulfamethoxazole to treat *Pneumocystis carinii* in patients with AIDS. *New England Journal of Medicine*, 1993, *328*, 1521-1527.

KANESHIRO E.S., WU Y.P. & CUSHION M. Assays for testing *P. carinii* viability. *Journal of Eukaryotic Microbiology*, 1991, *38* (Suppl.), 85-87.

LATORRE C.R., SULZER A.J. & NORMAN L.G. Serial propagation of *Pneumocystis carinii* in cell line cultures. *Applied and Environmental Microbiology*, 1977, *33*, 1204-1206.

MIROVSKY P. & FISHMAN J.A. An improved method for the prolonged maintenance of *Pneumocystis carinii in vitro*. *Journal of Infectious Diseases*, 1993, *167*, 1470-1473.

PIFER L.L., HUGHES W.T. & MURPHY M.J.J.R. Propagation of *Pneumocystis carinii in vitro*. *Pediatric Research*, 1977, *11*, 305-316.

PIFER L.L., WOODS D. & HUGHES W.T. Propagation of *Pneumocystis carinii* in Vero cell culture. *Infection and Immunity*, 1978, *20*, 66-68.

QUEENER S.F., BARTLETT M.S., NASR M. & SMITH J.V. 8-Aminoquinolines effective against *Pneumocystis carinii in vitro* and *in vivo*. *Antimicrobial Agents and Chemotherapy*, 1993, *37*, 2166-2172.

ROSALES M.J., CIFUENTES J. & MASCARO C. *Cryptosporidium parvum*: culture in MDCK cells. *Experimental Parasitology*, 1993, *76*, 209-212.

SATTLER F.R. & FEINBERG J. New developments in the treatment of *Pneumocystis carinii* pneumonia. *Chest*, 1992, *101*, 451-457.

Reçu le 22 septembre 1995
Accepté le 12 février 1996