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Highlights and Summaries of the 11th International Workshops on Opportunistic Protists

EDNA S. KANESHIRO^a, MELANIE T. CUSHION^b, FRANCINE MARCIANO-CABRAL^c, LOUIS M. WEISS^d, LIHUA XIAO^e

^aDepartment of Biological Sciences, University of Cincinnati, Cincinnati, Ohio,

^bVeterans Administration Medical Center, University of Cincinnati College of Medicine, Cincinnati, Ohio,

^cDepartment of Microbiology and Immunology, Medical College of Virginia, Richmond, Virginia,

^dDepartment of Medicine, Albert Einstein College of Medicine, Bronx, New York,

^eDivision of Foodborne, Waterborne and Environmental Diseases, National Center of Emerging and Zoonotic Infectious Diseases, Center for Disease Control and Prevention, Atlanta, Georgia

Abstract

The 11th in the series of International Workshops on Opportunistic Protists (IWOP-11) was held in August 2010 on the Big Island of Hawaii. These meetings are devoted to agents of infections that cause serious problems in AIDS patients and other individuals with defective immune systems. International Workshops on Opportunistic Protists serves as a forum for exchange of current research information on *Pneumocystis*, *Cryptosporidium* and the Microsporidia, *Toxoplasma*, free-living amoebae, kinetoplastid flagellates and other pathogens that are particularly pathogenic in immunodeficient hosts. Studies on interactions between host and pathogen, especially host responses, were highlighted in this year's symposium. The lack of in vitro cultivation methods for luxuriant growth of *Pneumocystis*, *Cryptosporidium* and the *Enterocytozoon bieneusi* remains a major hindrance to understanding the basic biology of these organisms and precludes genetic manipulations. However, slow but steady progress is being achieved by hard work including data mining of some completed or partially completed genome sequencing of several IWOP organisms. Of great concern is evidence for dramatic decline in research funding for these pathogens and the lack of appreciation by the larger scientific community concerning the state of art and challenges faced by researchers working on these organisms that can provide critical insight into emerging and reemerging pathogens.

Keywords

Acanthamoeba; Blastocystis; Cryptosporidium; Giardia; Microsporidia; Pneumocystis; Toxoplasma

THIS article presents updates on significant advances in, and major challenges of, research efforts in understanding the biology of immunodeficiency-associated opportunistic protists and their interactions with animal hosts. Abstracts of all presentations at 11th International Workshops on Opportunistic Protists (IWOP-11) are available at http://www.uga.edu/protozoa/meetings/iwop/iwop_2010_abstracts.pdf. Numbers corresponding to these abstracts are cited below.

SYMPOSIUM AND ROUND TABLE DISCUSSIONS

Symposium: host responses to immunodeficiency-associated disease-causing protists.

The symposium was chaired by Chao-Hung Lee who presented an introduction and background on host-defense mechanisms (S1) and also reviewed work done by his group on interactions of *Pneumocystis carinii* and alveolar macrophages (AM). They showed that *Pneumocystis* pneumonia (PcP) causes (1) over-expression of the antizyme inhibitor which enhances uptake of exogenous polyamines and hence intracellular polyamine levels, which in turn leads to increased apoptosis of AM cells; (2) decreased AM phagocytosis can be explained by down-regulation of the transcription factor PU.1, which regulates the expression of several macrophage receptors, including the mannose receptor, dectin-1, CD11b/CD18, FcγR and the scavenger receptor; (3) down-regulation of calmodulin in AM results in decreased phagocytosis of latex and zymosan beads by treatment of normal mouse AM with the calmodulin inhibitor W-7. Because calmodulin is required for iNOS dimerization, its down-regulation may explain the defect in nitric oxide production by AM during PcP.

In an impressive gesture, Guan Zhu stood in for Honorine Ward and competently presented the talk she had prepared on the role of glycans in *Cryptosporidium* infection (S2). This parasite has surface mucins or mucin-like glycoproteins that are involved in attachment to host cells. Her group had identified over 30 mucins or mucin-like glycoproteins with terminal GalNAc α1–3-Ser/Thr or Gal(β1–3)GalNAc. As lectins and antibodies specific for them blocked attachment and infection in vitro and/or in vivo, these mucin-like glycoproteins probably mediate infection of host cells. The genome data mining also led to the identification of a Gal/GalNAc-specific glycan-binding protein (lectin) p30, which binds specifically to mucins on host cells as well as Gal/GalNAc glycans on mucin-like glycoprotein (gp900 and gp40) on *Cryptosporidium*, serving as a bridge between parasite and host glycans. Furthermore, clinical isolates were found to have highly polymorphic genes for these glycoconjugates, suggesting they might be under selective pressure in response to host immune responses.

Beth Garvy's investigations into pulmonary immune responses to *Pneumocystis murina* by normal infant mice indicate significant differences between neonatal and adult immune defenses against the pathogen (S3). Not surprisingly, neonatal responses are delayed compared with those in adults. However, she presented evidence that there are clear differences between neonatal and adult immune cells such as AM responses to cystic forms with respect to signaling of NF-κB activation. Interestingly, she also reported that there are differences in responses to different life cycle stages of the organism (trophic vs. cystic forms) in both adults and neonates.

Craig Roberts reviewed and updated what is known about host neuropsychiatric responses such as schizophrenia and depression to toxoplasmosis (S5). While most reports in the literature remain anecdotal and questionable, a controlled laboratory study on mice that showed a change in reaction to cat urine resulting from the infection remains the most convincing evidence for behavioral changes in response to toxoplasmosis. Assuming there are neuropsychological alterations, his group is investigating the possible role in toxoplasmosis of two aromatic amino acid hydroxylases for which the genes have been identified in the *Toxoplasma gondii* genome.

Roundtable discussion: future of funding for opportunistic protist research.

Spearheaded by Anthony Sinai and Melanie Cushion, grave concern over future funding for opportunistic protist research was brought into an open discussion. This session will result in a white paper after input from the broader community to include comments from those investigators who were not in attendance.

Roundtable discussion: nomenclature: How should we abbreviate genes, cDNA, gene products, etc. in opportunistic protist publications? What are being used and can we achieve uniformity?

Co-chaired by Andrew Limper and Louis Weiss (Discussant, Lihua Xiao), this roundtable reviewed the various ways in which the literature is becoming complicated by a lack of uniformity in use or guidelines for abbreviations for genes and gene products from different organisms. While acknowledging that authors are required to conform to specific journal instructions, a white paper is being prepared describing the problems and possible suggestions that might help standardize the literature.

CONTRIBUTED REPORTS

FREE-LIVING AMOEBAE

Free-living amoebae belonging to the genus *Acanthamoeba* cause amoebic keratitis (AK), a painful disease of the cornea. Treatment is difficult partly due to the organism's resistant double-walled cysts. Amoebic keratitis has been associated with certain contact lens solutions that induce the formation of pseudocysts (incomplete encystment). Pseudocysts with a single-layer wall are capable of giving rise to viable trophozoites. It was recently demonstrated that *Acanthamoeba* formed pseudocysts after exposure to propylene glycol, which is found in some contact lens cleaning solutions (PL20). These observations show that the use of such formulations might lead to increased numbers of trophozoites that could help establish AK infections.

Acanthamoeba also causes granulomatous amoebic encephalitis (GAE), a chronic fatal infection of the central nervous system (CNS) in which granulomas form around the amoebae. These granulomas consist of microglia, macrophages and lymphocytes that produce proinflammatory cytokines. Recent studies nicely demonstrated that *Acanthamoeba* secretes serine proteases that degrade chemokines and cytokines as an immune evasion mechanism (PL29). A particularly interesting finding was that *Acanthamoeba culbertsoni*, but not *Acanthamoeba astronyxis*, induces apoptosis apparently through protease-activated

receptors. This observation implicates the pathway that may be responsible for infection-induced neuropathogenesis of *A. culbertsoni* infection and thus a potential drug target for treating GAE. They showed that *Acanthamoeba* can induce apoptosis in BV-2 mouse microglial-like cells in vitro and concluded that cell contact is not required for pathogenesis if free-living amoebae gain entry into the CNS.

BLASTOCYSTIS—Although *Blastocystis hominis* is commonly present in human stool, the taxonomy and pathogenicity of this organism remains controversial. Over the years a few reports on this group of organisms have been presented at IWOP. This year evidence was presented for pathogenicity by experiments in vitro using the HT-29 human polarized intestinal epithelial cell line and two strains of the parasite (PL34). While evidence for parasite entry into the cells was not observed, decreased transmembrane electrical resistance and ultrastructural changes of cytoskeletal elements were observed. Also evidence was obtained of monolayer cells apoptosis and increased TNF- α production.

CRYPTOSPORIDIUM

Post-whole genome sequencing.: In the era of whole genome sequencing, these studies benefited from the successful sequencing of *Cryptosporidium parvum* and *Cryptosporidium hominis* genomes 6 yr ago. Thus, through comparative genomics, similarities and differences in metabolic pathways between *Cryptosporidium* and the related *Ascogregarine tawainensis* or other apicomplexans were identified, especially those involved in energy and fatty acid synthesis (PL10). This had also led to the identification of a *Cryptosporidium*-unique type II thioesterase that hydrolyzes C₆- to C₁₂-length acyl-CoAs (PO13). On the host side, integrin A2 (ITGA2) was identified as a potential receptor on host cells for host-parasite interaction, as reduced parasite invasion of host cells was noted in ITGA2 knockdown cells (PL2). The availability of the whole genome sequence data had also facilitated the identification by two-dimensional PAGE and MALDI-TOF MS peptide fingerprinting of several proteins that might be involved in protection of *Cryptosporidium* from gamma irradiation, such as proteasome subunit alpha type 4, Ntn hydrolase fold, and a thioredoxin peroxidase-like protein (PO22). The identification of *Cryptosporidium*-unique metabolic pathways and key components involved in host-parasite interactions and environmental survival may lead to the identification of potential targets for the development of interventions against cryptosporidiosis.

Epidemiology.: Likewise, the availability of the whole genome sequence data of *Cryptosporidium* spp. has also greatly facilitated the development of molecular epidemiologic tools. A recent screening of *Cryptosporidium muris* whole genome sequence data for microsatellite and minisatellite sequences led to the identification of 13 potential loci. Sequence analyses of various *C. muris* and *Cryptosporidium andersoni* isolates at four of the loci formed the basis for the development of a multilocus sequence typing tool for the two common *Cryptosporidium* spp. and the identification of a potential new gastric *Cryptosporidium* species (PO17). Using sequence and population genetic analyses of 32 microsatellite markers identified in chromosome 6, a valley of reduced genetic diversity and increased linkage disequilibrium was identified in four markers surrounding the gp60 gene in association with parasite virulence. As population differentiation between the virulent and

avirulent parasites occurred only in this area, genes near gp60 are potential *Cryptosporidium* virulence factors (PL26).

Several studies examined the epidemiology of *Cryptosporidium* spp. in humans and animals and public health importance of parasites of animal origins. A study in South Korea assessed the prevalence of *Cryptosporidium* spp. in over 100,000 patients with diarrhea. An overall prevalence of 0.5% was observed and was highest among children 2–9 yr of age (PO14). A slightly higher infection rate (2.0%) was seen in over 5,000 children hospitalized in Shanghai, China for non-gastrointestinal illnesses, with infants younger than 6 mo having the highest infection rate (7.3%). Most of the children were infected with two subtypes (IaA14R4 and IdA19) of *C. hominis*, indicating that anthroponotic transmission plays a major role in cryptosporidiosis in urban areas in China (PL18). In contrast, the transmission of *Cryptosporidium* in HIV+ patients in a rural area of China could be through a zoonotic pathway, as *Cryptosporidium meleagridis*, *C. parvum* and *Cryptosporidium suis* were found in five of the seven positive specimens, and sheep/goat farming was significantly associated with *Cryptosporidium* infection (PO36). Surveys in China also identified *Cryptosporidium* spp. in 8.1% of birds in pet shops (PO11) and 34% of rabbits on farms (PO31). DNA sequence analyses identified several host-adapted *Cryptosporidium* spp. species/genotypes in these animals. However, two of these species, *C. meleagridis* in birds and *Cryptosporidium cuniculus* in rabbits, are important zoonotic pathogens. Most of these studies in molecular epidemiologic studies used DNA sequence analysis of the gp60 gene for subtyping *Cryptosporidium* spp. In a study presented, the sensitivity and specificity of 43 published primers were compared by sequence alignment and polymerase chain reaction (PCR) analyses. Primers S60.ATGF/gp15extR in primary PCR and AL3532/AL3535 in secondary PCR were recommended based on results of primer evaluations (PO12).

An assay that uses propidium monoazide in conjunction with PCR enables monitoring drinking water for viable oocysts as well as obtaining *Cryptosporidium* genotypes (PL9). This type of analyses would improve on the current US EPA Methods 1622 and 1623 that regulate the drinking water industry but do not distinguish live from dead parasites nor identify *Cryptosporidium* species/strains. As food safety is of public health concern laser-scanning confocal microscopy together with fluorescein-labelled monoclonal antibodies demonstrated that *Cryptosporidium* oocysts can adhere to stomatal openings and become interiorized into the mesophyll of spinach leaves as well as in crevices of apple surfaces (PL33). It is known that cancer can result from some parasite infections and a group in France is studying the progression of cryptosporidiosis-induced neoplasia into invasive adenocarcinomas in dexamethasone-treated SCID mice by standard histological analyses (PO32).

GIARDIA—Cysts of several distinct assemblages of *Giardia duodenalis* isolated from feces in cats and dogs were compared by morphometric differences. The identity of the parasites was established by DNA sequencing of the triosephosphate isomerase and β -giardin gene (PO28). The observed morphometric differences supported the previous suggestion on the existence of multiple assemblages or strains of this parasite based on genetic characterizations.

MICROSPORIDIA

Microsporidia are recognized as pathogenic protists in both invertebrate and vertebrate hosts. They are obligate intracellular organisms defined by the presence of a unique invasion organelle consisting of a single polar tube, polaroplast and anchoring disc. Phylogenetic analysis has placed the Microsporidia with the fungi. While the majority of reported cases of human microsporidiosis involve diarrhea, the spectrum of diseases caused by these organisms has expanded to include: keratoconjunctivitis, disseminated disease, hepatitis, myositis, sinusitis, kidney and urogenital infection, ascites, cholangitis and asymptomatic carriage. The phylum Microsporidia contains over 150 genera representing about 1,200 species of which the following have been demonstrated in human disease: *Nosema* (*Nosema corneum* renamed *Vittaforma corneae* and *Nosema algerae* reclassified initially as *Brachiola algerae* and now as *Anncaliia algerae*), *Pleistophora*, *Encephalitozoon*, *Enterocytozoon*, *Septata* (reclassified as *Encephalitozoon*), *TrachiPleistophora*, *Brachiola*, *Anncaliia*, and *Microsporidium*.

Epidemiology, diagnostic and clinical findings.—Our knowledge of these infections continues to expand with new pathogens being found in model systems as well as new infections being described for previously identified pathogens. A novel case was reported of microsporidiosis due to *A. algerae* presenting as hoarseness and cough and involving the false vocal cords (PL23). This illustrates the ability of this insect pathogen to infect humans and extends the tissue types in which infection has been described. A study of *A. algerae* sensitivity to albendazole showed exposure resulted in abnormal morphologies and inhibition of spore production (PO10). Also, its β -tubulin gene showed that five of the six amino acids known to be associated with benzimidazole sensitivity were conserved in *A. algerae* suggesting albendazole as a possible affordable and widely available long-term treatment for this infection.

A major issue in the study of microsporidiosis has been the environmental source of infections. *Enterocytozoon bieneusi* has been identified in many animals since its initial description in humans. However, the relationship of these zoonotic isolates to those seen in humans remains to be determined. A multilocus typing tool for *E. bieneusi* has been developed (PL7) using data from the *E. bieneusi* genome project (available on <http://www.EuPathDB.org>). After validation, four loci (MS1, MS3, MS4 and MS7) were identified as useful in typing this organism. Phylogenetic analysis with these loci was similar to that with the ITS (rRNA) locus. These new tools should facilitate studies on the epidemiology of this organism. Using PCR and genotype analysis, microsporidiosis (*E. bieneusi* and a *Vittaforma*-like Microsporidia) was found more frequently in feces from children compared with adults in Portugal (PO7). Although with lower prevalence, *Encephalitozoon intestinalis* was detected in urine and *Encephalitozoon cuniculi* and *Vittaforma*-like Microsporidia were detected in pulmonary specimens.

The zebrafish, *Danio rerio*, has become an important model organism for the study of disease. Two microsporidial pathogens, *Pleistophora hypohessobriconis*, which causes myositis and *Pseudoloma neurophilia* which causes CNS infections and wasting have been identified and characterized (PL13, PO29). The genome of *P. neurophilia* is in progress and,

with the completed zebrafish genome, will provide a new pathogen model system for studies of the pathogenesis of neural infections.

Cell biology, metabolic studies, molecular biology and immunology.—A new web based database, MicrosporidiaDB, <http://microsporidiadb.org/micro/>, has been developed for studies on Microsporidia (PL32). MicrosporidiaDB is part of EuPathDB and is being expanded to include all available genome data on these organisms, which should greatly facilitate research on these pathogenic protists.

An exciting discovery reported at this meeting was that of a previously unrecognized filamentous structure in the parasitophorous vacuole (PL8). This discovery occurred by applying proteomic methods to the analysis of the invasion apparatus of *E. cuniculi*. In addition to finding novel components of mitosomes and the developing spore wall, the previously unrecognized filamentous network was discovered, external to the parasite but within the lumen of the parasitophorous vacuole. This illustrates the usefulness of a proteomic approach for characterizing these enigmatic pathogens. In another study using both in vitro and in vivo (mouse intestinal loop models) systems, the mechanism of spore activation is being investigated to understand microsporidial invasion of intestinal epithelial cells (PL35). It was demonstrated that spore wall proteins bind to glycans of the host cell glycocalyx (i.e. EnPI has heparin binding motifs). It is likely, that such binding is important in infection as it brings spores into close proximity to intestinal cells and provides a specific microenvironment that may promote germination.

There continues to be growth in research on these ubiquitous intracellular protists and a broadening of our informational databases. The small genome of these organisms and reduction in the size of their genes has proven to be of interest to many biological disciplines. Techniques for genetic manipulation of these protists are still needed. Such techniques will facilitate studies on important cell biology questions, such as differentiation. There still remains a need for an in vitro cultivation system for *E. bieneusi*. Nonetheless as this conference demonstrated, the advances in basic research and epidemiology that have occurred have significant implications for the relationship between host species and the Microsporidia.

PNEUMOCYSTIS

Glucan, cell wall and anti-glycan therapy.—In addition to the role of glycans in *Cryptosporidium* interactions with host cell surface (see above for symposium) a topic that garnered high interest and attention during the workshops was that of the role of glucan, specifically 1,3- β -D-glucan, in the host immune response, diagnosis and its biological implications during infection with *Pneumocystis*. The cyst form of *Pneumocystis* contains β -1, 3-D-glucan while little to none is detectable in the trophic forms. Determining levels of β -1,3-D-glucan in the sera of PcP patients has value as a useful diagnostic procedure (PL27). Although infections with other fungi produce circulating glucans, patients with PcP produce the highest levels, usually ranging from several hundred to >38,000 pg/ml. The presence of *Pneumocystis* cysts in colonized patients, such as those with Chronic Obstructive Pulmonary Disorder (COPD), albeit with lower circulating levels of glucan, is being implicated as a

potential co-morbidity factor. That the β -glucans induced IL-1 β that favored a Th1 phenotype with subsequent IFN- γ production has been reported and further studies presented at the workshop showed that glucan from *Pneumocystis* could activate the IL-23/IL-17 axis through the classical and alternative pathways of NF- κ B in dendritic cells using glycosphingolipids as co-stimulatory molecules (PL3). Because there are inhibitors available to block this pathway, this may provide a new tool to modulate the inflammatory response associated with the presence of glucan. Another intriguing finding was the apparent presence of chitin in the *P. carinii* cell wall, which could also contribute to the detrimental inflammatory response observed in PcP. The presence of chitin was earlier demonstrated by cytochemical techniques but remained controversial due to scant biochemical or morphological evidence. However, the identification of a chitin synthase gene homolog (PO20), and homologs of transcription factors that might control expression of chitinases and glucanases (PL15) support the presence of chitin biosynthetic and repair pathways in *P. carinii*. Further study is essential to better understand the host response to these pro-inflammatory factors in different patient populations. During the meeting, questions were raised about the potential for pathology by these pro-inflammatory factors, including the very striking bone marrow failure shown in lymphocyte competent mice lacking type I IFN signaling which was shown to be a specific response to *P. murina* (PL6). Because *Pneumocystis* cysts clearly make glucan, a likely treatment would be the glucan synthase inhibitors, called pneumocandins and echinocandins. However, it was recently reported that treatment with clinically available glucan synthase inhibitors (caspofungin, anidulafungin, micafungin) significantly reduced cyst forms during PcP in rodent models of infection, while sparing the trophic forms. The ability of the trophic forms to replicate DNA during pneumocandin treatment was verified by quantitative cytochemical analysis of trophic nuclei using fluorescent stains, suggesting that these drug-treated trophic forms might be capable of undergoing mitosis (PO19). Thus, pneumocandins and echinocandins can be used as a means to investigate the life cycle of *Pneumocystis*, but might not be effective as monotherapies.

Insight into possible implications on *P. carinii* physiology was the finding that the glucan synthase had an optimal temperature range between 4 °C and 25 °C, lower than 37 °C of the host lung (PO1). Interestingly, lower than 37 °C was earlier found to favor the establishment of *P. carinii* axenic cultures, and a broad plateau between 20 °C and 30 °C was observed in metabolic radiolabeling studies of mixed life cycle stages. These independent observations suggest that some life cycle forms efficiently proliferate at 37 °C in the host lung while others are better suited for surviving conditions outside the mammalian lung.

Colonization.—There is increasing evidence and increasing interest in the role of colonization (asymptomatic infections in immunocompetent hosts) by *Pneumocystis* in the context of other chronic diseases in humans and experimental animals. In humans, colonization and poor outcome has been mainly associated with COPD and the use of TNF- α therapy in Crohn's and rheumatoid arthritis patients. In a study of patients receiving the TNF- α agonist infliximab conducted in Spain, colonization was detected in 32 of the 125 patients (~ 26%), which was significantly and independently associated with infliximab therapy (PO3). Another group of susceptible patients, those with idiopathic pulmonary

fibrosis, were shown by PCR to be colonized (reaching close to 50%), and the role of *Pneumocystis jirovecii* was raised as a contributing factor in exacerbating the associated fibrosis (PO25). Patients with emphysema were also shown to be significantly colonized (42%) and the distribution of organism foci in the lung was variable, which might confound proper diagnosis (PO33). Increased colonization (45%) was also noted in a study of elderly patients with community-acquired pneumonia in Chile (PO35). On the other hand, it appears that primary infection is highest in infants (2–4 mo old) with highest occurrence in the upper lung lobe (PO25). These studies all suggest a role in co-morbidity for *P. jirovecii* in these and other underlying disease or immunodeficiency states. Among healthy people, analysis of antibody levels to recombinant *P. jirovecii* major surface glycoprotein (Msg) fragments over time confirmed higher antibody responses among health care clinical personnel compared with people with non-clinical occupations (LB1). In a related serology study, HIV+ patients with and without *P. jirovecii* infection showed that patients exhibit responses to recombinant Msg and kexin peptide fragments after PcP and that HIV+ patients appear to have low KEX1 IgG before PcP independent of CD4 cell numbers, which becomes elevated after the infection (LB3).

Several commercial rodent colonies have been reported to be cryptically infected with *P. carinii* and *P. murina*. Because these infections can interfere with immunological and other studies, as well as cause death in mouse strains with immunological defects, the factors influencing the degree and length of colonization must be better understood. Using the rat model, investigators reported that gender was not a factor in colonization, but did show that juvenile rats vs. weanlings or adults were the first to contract the infection after exposure to infected rats (PO9). Also, while *P. carinii* could be harbored by immune competent rats for up to 6 mo, the organism was mostly infective to the hosts during the first 2 mo of carriage. These studies show that *Pneumocystis* can be harbored for long periods by immune competent hosts and investigators should be aware of the status of their animals before use.

Treatment and drug resistance.—Early in the previous decade, an association of mutations in the dihydropteroate synthase (DHPS) gene and prophylaxis failure with the most efficacious anti-PcP therapy, trimethoprim-sulfamethoxazole was reported by several laboratories. Dihydropteroate synthase, an enzyme in *Pneumocystis* folic acid synthesis is blocked by sulfa drugs. Dihydropteroate synthase double mutation was shown to rapidly expand in the human populations residing in the United States and the United Kingdom. In Brazil, these mutations were in very low numbers as previously reported (PO4), while in Santiago, Chile, the prevalence was much higher (46%) (PO5). Although the prevalence of PcP was low (7/133) in a large study in Uganda, the double DHPS mutation was observed in 4/6 PCR-amplifiable samples (PO15). It is likely that the mutations in DHPS will reach most countries with susceptible patients. However, in some cases, therapeutic doses of sulfa drugs can clear *P. jirovecii* harboring these mutations necessitating evaluation of other DHPS gene loci or in another gene relevant in folic acid synthesis. New high throughput methods for identifying *P. jirovecii* haplotypes are being applied to correlate PcP clinical data (e.g. drug resistance and virulence) with single nucleotide polymorphisms (PL12, PL19) that could be applied to evaluate larger human populations providing greater accuracy in epidemiological studies.

Recent reports on the numbers of AIDS cases in resource limited settings (2–3 million) that far outpace the capacity (0.5–1 million cases) for adequate treatment and the late presentation of about 50% of cases in Europe and the United States should signal deep concern for the impact on increased transmission, higher numbers of opportunistic infections and the inability to eradicate HIV (PL14). Over 5 million HIV+ persons do not have access to anti-retroviral therapy and for those that do, approximately £400,000 (US\$625,760) is estimated to cost a patient over a lifetime in developed countries. This would create an enormous financial burden on health care systems. Without an adequate vaccine strategy for HIV, it is necessary to continue identifying new drug targets and therapies for PcP and other opportunistic infections. A new approach using an in silico high throughput screening strategy for selection of anti-PcP therapy in combination with in vitro verification used the *P. carinii* oxidosqualene cyclase as target and identified two to three potential compounds with low host cell toxicity (PL5, PO8). Another laboratory focused on decreasing available S-adenosylmethionine (SAM, AdoMet), which the organism takes up from its environment (culture medium or lung alveolus). Decreased AdoMet is achieved by increasing spermine/spermidine acetyltransferase in lung cells that lead to increased activity of the polyamine pathway in these host cells and decreased available AdoMet for *P. carinii*. Possible PcP therapy strategies include limiting host levels of AdoMet available for the pathogen while maintaining polyamine levels (PL11). Also, discovery of a *P. carinii* mitochondrial AdoMet transporter gene and its expression in a yeast strain lacking this gene holds promise as a potential system for evaluating drugs that block AdoMet transport (PL37). In vitro treatment of biofilm and suspension cultures with sodium nitrite was shown to dramatically reduce viability of the suspension cultures, but had less effect on established biofilms (PO26). These new approaches offer novel strategies for approaching discovery of new candidates for PcP therapy.

Host responses to PcP.—The host immune responses to colonization and frank infection with *Pneumocystis* are complex and our appreciation for all the facets is evolving. As the role of specific T-cell subsets in mediating host resistance against *P. murina* infection remains unclear a series of cytokines and different STAT transcription factor knockout (KO) mice were used to evaluate the role of Th1, Th2 and Th17 cells (LB3). The results from this extensive study showed that Stat4 KO, Stat6 KO and Stat4/Stat6 KO mice were resistant to *P. murina*. The Th17 response was intact and slightly elevated in Stat4/Stat6 double KO mice. Stat3 expression was required in CD4+ T cells for *P. murina* Th17 responses. IL-22 KO mice showed increased *P. murina* burdens in the lung. Although IL-17 RA mice cleared the infection, IL-17RA mice crossed to IL-22 KO mice exhibited a higher *P. murina* burden as compared with IL-22 KO mice. Collectively, the results indicated that IL-23 is required for Th17 responses to *P. murina* infection but that Th1 immunity, while independent of IL-12 and IL-23, is dependent on IFN γ .

It is known that treatment of infected rodents with the pneumocandins and echinocandins can provide a population of trophic forms devoid of cysts. Use of these populations with those that were cyst-replete showed that cysts were more likely to bind the AM than trophic forms (PO6), perhaps due to the presence of glucan via dectin-1 and other surface-associated molecules (PL31). Most animal studies have used juvenile or adult mice or rats, but the

response to the organism in neonates is quite different, being mostly suppressed in this model. Strikingly, neonatal mice were shown to exhibit delayed and reduced responses to *P. murina* cysts (see above B. Garvy in the symposium), but were able to translocate NF- κ B to the AM nucleus in response to the yeast cell wall preparation, zymosan (PO21). Evidence was presented that B cells play a role in the expansion and survival of CD₄ T cells that aid in the defense against *P. murina* by comparing CD₄ T cells from B-cell deficient mice and from wild type mice (PL24). Although it has long been known that AM in adult rodents have a defect in clearance of the infection, the mechanisms or defects are now being identified (C.-H. Lee above in the symposium).

Genetic studies.—Genetic tools which would allow for KO and knock-in of genes to determine function are not available for any species of *Pneumocystis* due to a lack of an in vitro culture system that allows good growth and indefinite sub-cultivation. This has significantly hindered progress in understanding many of the intrinsic processes of these organisms. However, expression in other systems, especially yeast mutants, has helped better understand *Pneumocystis* gene function and also show conservation among the very different fungal species. It was previously shown that the *Erg11* gene that codes for 14 α -demethylase activity (14DM) of *P. carinii* could complement function in a yeast deletion strain and showed evidence that the *P. carinii* 14DM is inherently resistant to azole drugs. Work from another laboratory reported at these meetings demonstrated complementation by biochemically analyzing the sterols found in wild type and transformed yeast strains (PL30). Yeast cells expressing either the *Saccharomyces cerevisiae* or *P. carinii* 14DM produce the same >20 sterols with ergosterol as the major component. They also predicted that both the yeast and the *P. carinii* 14DM had a preference for 4 α ,14 α -methyl sterol substrates over lanosterol and that the *P. carinii* and yeast 14DM had broad substrate specificity giving rise to branched pathways in sterol biosynthesis. The *P. jirovecii* *Erg11* gene is also being analyzed in an attempt to evaluate the possibility that *P. jirovecii* *Erg11* mutants might correlate with previously reported populations of organisms that have and others that lack 14DM activity. Initial analysis of a 1,000-bp segment of the 1,800-bp gene in nine *P. jirovecii* specimens detected two alleles, but these were located in an intron (PO16). Unlike the *P. carinii* *Erg11* in the yeast *Erg11* deletion mutant, the *P. carinii* *Erg6* gene (optimized for yeast codon usage) that codes for SAM:C-24 sterol methyltransferase appears less compatible with the yeast *Erg6* deletion mutant's metabolic processes (PL16). These observations are consistent with earlier studies indicating some unique properties of this *P. carinii* enzyme. The function and localization of the *P. carinii* oxidosqualene cyclase (coded by *Erg7*) in yeast was reported by another group who showed that localization of the enzyme to lipid particles in yeast cells relied on the presence of 3-ketoreductase (coded by *Erg27*) (PL4). Because another group previously reported that this recombinant protein also expressed in yeast failed to localize to lipid particles, this suggests the possibility that different yeast strains transformed with the same plasmid might differ significantly in their cellular functions but differences in detection techniques has not been ruled out.

Phylogenetics.—The *Erg11* gene sequence from various mammalian host species is being used to evaluate whether phylogenetic analysis of this gene supports long-term co-evolution with mammalian host species (PL17). In a much larger study phylogenetic relationships

were reported for *Pneumocystis* in wild rodents from Cambodia, Laos, and Thailand (PL38). This study included 16 different rodent species within the Murinae. Polymerase chain reaction analysis of *Pneumocystis*-specific DNA sequence detected high prevalence (41%) comparable to that in humans, high genetic diversity and verified host species specificity of these associations with *Pneumocystis* (pneumocystosis was not observed).

Proteomics.—Several groups are now using state of art proteomic methods to identify *Pneumocystis* proteins and their differential expressions under various clinical and experimental conditions (PO23, PO24). While only a few of the large number of proteins in the organism have been identified, this approach is providing valuable insights into the organism's responses to changes in its environment as well as potential differential expression of mRNA (PL36) and proteins in cystic compared with trophic stages. One approach being tested to gain information on the *P. carinii* life cycle uses a *S. cerevisiae* proteome microarray (PL39). Several candidate protein substrates for the described previously *P. carinii* regulatory serine/threonine kinase genes (*Cbk1*, *Ste20*) were identified including substrates involved in growth and development. This avenue of investigation holds promise in providing information on *P. carinii* signaling cascade in the control of changes in life cycle stages. An exciting highlight of this conference was the use of proteomics evaluating lung cell endoplasmic reticulum (ER) unfolded protein response (UPR) and cell death pathway (apoptosis). This response reflects the reduced capability of the ER to fold, modify and transport proteins when the cell is under stress leading to accumulation of unfolded proteins. Protein folding involves chaperones and a foldase/isomerase and downstream signaling pathways for expression of proteins in anti-oxidant defense. Comparison of proteins in normal rat lungs and in those infected with *P. carinii* identified several up-regulated and down-regulated proteins; several up-regulated proteins in infected lungs were identified as proteins implicated in UPR leading to apoptosis (LB4).

The role of the environment.—Exploration of the effects of oxygen on *P. carinii* exposed to anaerobic, microaerophilic and 5% carbon dioxide atmospheres revealed that genes involved in sterol and lipid metabolism, protein folding and stress response were down-regulated during hypoxia and genes involved in autophagy were up-regulated (PO27). Unlike yeast, the change in oxygen was not necessary to initiate sterol scavenging, which was operational under the various conditions. Another study which looked outside the pulmonary milieu, and evaluated the effects of climate and air pollutants on hospital admissions for PcP and antibody responses found that higher admission rates were associated with higher temperature and ozone levels, but lower humidity, rainfall, CO, NO₂ and SO₂ were not (PO2). Different seasons were significantly associated with human antibody responses to various regions of the Msg. These studies suggest that both intrapulmonary and extrapulmonary environmental conditions influence organism functions.

TOXOPLASMA

Infection by *T. gondii* in healthy individuals usually results in minor symptoms, but can cause death or defects in brain development in a fetus carried by pregnant mothers lacking immunity to this parasite. Immunodeficient adults are also at risk for toxoplasmosis, and can develop life-threatening encephalitis. Sporulated oocysts can survive for years in soil and

water and are resistant to drinking water disinfectants. Because pigeons are ground feeders, investigators evaluated these birds in the urban environments in Portugal and found that 37% of flocks were seropositive for *T. gondii* (PO30). Infections were verified by growth of organisms isolated from brain tissues in cell cultures and by PCR of rRNA.

Oocysts are recalcitrant to drinking water disinfectants and present a public health threat. Thus, various chemical and physical treatment methods are being evaluated for efficacy in inactivating oocysts by quantitative reverse transcriptase real-time PCR of ACT1 and SporSAG mRNA, an oocyst plaque TOP assay and a SCID mouse model of infection (PL22). Oocysts were found resistant to bleach, ethanol and iodine but susceptible to formalin. Importantly, as some drinking water companies are incorporating UV to eliminate viable and infective spores and oocysts of various pathogens, they found 1- to 3-log inactivation of *T. gondii* oocysts with 4–10 mJ/cm² exposure.

Studies on resistance of some mouse strains to *T. gondii* infection provide insights into the virulence of different *T. gondii* strains. Evidence was presented at this meeting that virulent strains can inactivate IRG proteins (GTPases related to dynamin) that cause rupture of the parasitophorous vacuole membrane leading to death of the parasite (PL28). There is control of the GTPases and inhibition of its dissociation keeps the IRG proteins inactive. Furthermore, it was suggested that the IRG system in some mouse strains is more resistant to *T. gondii* strains and the interactions of parasite and host IRG determines virulence and resistance.

Autophagy is an important cellular process in which intracellular organelles undergo self-destruction. To better understand this process in *T. gondii*, genes involved in the autophagy pathway were identified by homology to 31 known ATG genes in the *S. cerevisiae* autophagy pathway (PL21). The *T. gondii* ATG1 product is associated with mitochondria and appears essential for parasite viability. Also, unlike other systems studied, the ATG8 gene product is apparently a constitutive component of *T. gondii* membranes and very likely plays a distinctive and important role in this parasite.

IWOP-12

The 12th IWOP meetings are tentatively planned for 2012. Contact information: Louis Weiss (Louis.Weiss@einstein.yu.edu) and Melanie Cushion (melanie.cushion@uc.edu).

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