An Official ATS Workshop Summary: Recent Advances and Future Directions in Pneumocystis Pneumonia (PCP)

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Pneumocystis pneumonia (PCP) is a major cause of morbidity and mortality among immunocompromised persons, and it remains a leading acquired immune deficiency syndrome (AIDS)-defining opportunistic infection in human immunodeficiency virus (HIV)-infected individuals throughout the world. Pneumocystis has proven difficult to study, in part due to the lack of a reliable culture system for the organism. With the development of molecular techniques, significant advances in our understanding of the organism and the disease have been made over the past several years. These advances include an improved understanding of host–organism interactions and host defense, the development of noninvasive polymerase chain reaction (PCR)-based diagnostic assays, and the emerging data regarding the possible development of trimethoprim-sulfamethoxazole–resistant Pneumocystis. In addition, the recognition that patients without PCP may nevertheless be carriers of or colonized with Pneumocystis, and observations that suggest a role for Pneumocystis in the progression of pulmonary disease, combine to signal the need for a comprehensive and accessible review. In May 2005, the American Thoracic Society sponsored a one-day workshop, “Recent Advances and Future Directions in Pneumocystis Pneumonia (PCP),” which brought together 45 Pneumocystis researchers. The workshop included 21 presentations on diverse topics, which are summarized in this report. The workshop participants identified priorities for future research, which are summarized in this document.

Keywords: acquired immune deficiency syndrome; epidemiology, molecular; immunity; immunosuppression; lung diseases, fungal

BASIC BIOLOGY OF Pneumocystis

There are several unique features of Pneumocystis that make its study exciting but quite difficult (1). The organism cannot be cultured reliably outside the lung, complicating the investigation of its life cycle and signaling (Table 1). The organism’s source in nature has not been identified, and so the issue of transmission has been difficult to examine. The organism is strictly host specific, but the antigens or mechanisms that confer specificity have not been elucidated. Finally, Pneumocystis infections are virtually always limited to the lung, but the host characteristics that allow infection are not completely understood.

The Pneumocystis organism was first identified in 1909 by Chagas in guinea pigs, and then by Carini in rat lung (for a comprehensive historical summary, see Reference 2). The Delanoës originated the taxonomic designation Pneumocystis carinii in 1912, when the organism was still considered to be a protist. More recent work demonstrates that Pneumocystis is a member of the fungi. Each mammalian host is infected by a specific Pneumocystis that cannot infect other hosts. Therefore, efforts are being made to reclassify the various Pneumocystis organisms as separate species, and the taxonomy and nomenclature for these organisms is a focus of continuing controversy (3, 4). Some authorities use Pneumocystis jirovecii to refer to the Pneumocystis species that causes human disease. For the purposes of this workshop summary, we refer to Pneumocystis as a general term, and identify the species in which specific experiments were performed in the text. For Pneumocystis carinii, the organism that infects rats, the genome has been identified to be about 7.7 Mb, and consists of 13 to 15 linear chromosomes that range from 300 to 700 kb. Most genes identified to date have numerous short introns (5). An active Pneumocystis genome project exists, and it is likely that homologies between Pneumocystis and other fungi will continue to be explored to identify Pneumocystis gene function.

The life cycle of Pneumocystis consists of a trophic form, a pre cystic form, and a cystic form. Because of the difficulties with in vitro culture, these life forms have been identified by morphologic criteria. Whether trophic forms replicate without an obligate encystment stage remains a matter of controversy. Recently, kinase systems that control life cycle traverse in other fungi have also been shown to control the Pneumocystis life cycle. Pneumocystis has a cdc2 protein kinase, a cdc13 B-type cyclin, and a cdc25 phosphatase that participate in cell cycle regulation (6). The activities of cdc2 and cdc13 appear to be differentially regulated over the life cycle of Pneumocystis, and
TABLE 1. RECOMMENDATIONS FOR FUTURE DIRECTIONS IN Pneumocystis BASIC SCIENCE RESEARCH

<table>
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<th>Recommendation</th>
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<tr>
<td>Improve understanding of interactions of Pneumocystis with epithelial cells, macrophages, dendritic cells, and proteins of the lung, particularly surfactant</td>
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<td>Improve understanding of innate and acquired inflammatory and immune responses to Pneumocystis</td>
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<td>Develop vaccines and novel methods to therapeutically alter lung inflammation during active Pneumocystis infection</td>
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<td>Improve understanding of life cycle, signaling, and culture requirements</td>
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<td>Understand differences in organism growth and host response in developing and adult lungs</td>
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<td>Further investigation of beneficial and detrimental effects of CD4+ and CD8+ T cell responses</td>
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<td>Characterization of antibody responses and immunodominant epitopes in animals and humans to develop protective therapeutics</td>
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the cdcs25 appears to restore a DNA damage checkpoint, but not the DNA replication checkpoint. Therefore, molecular techniques are likely to yield important information about the Pneumocystis life cycle, even in the absence of long-term culture techniques.

Molecular techniques are also being used to characterize the mitogen-activated protein kinases of Pneumocystis. These kinases operate differentially to control the pheromone pathways, cell wall integrity pathways, and invasive growth pathways. For example, it is likely that Pneumocystis engages in sexual replication using pheromone receptors. The putative pheromone receptor PCSTE3 has been cloned, and functional studies, as well as ligand identification, are now underway (7, 76).

Heterologous fungal expression systems, genomics, and proteomics have all provided important insights about the basic biology of Pneumocystis and its life cycle. In addition, the Pneumocystis genome project is likely to continue to provide important information about the basic biology of this organism. However, the lack of a reliable culture system remains a significant problem hampering better understanding of Pneumocystis biology (8).

ANTIGENS RELEVANT TO PROTECTIVE IMMUNITY TO Pneumocystis

Numerous antigens of Pneumocystis have been identified and studied; some of these antigens are species-specific, but others are shared across species. The antigens of Pneumocystis have been the focus of intense clinical and scientific interest, but the critical antigen or antigens that elicit protective immunity remain unresolved. For example, the monoclonal antibody 4F11, which was elicited from Pneumocystis-immunized mice, has been a useful research tool for the investigative community (9). This antibody cross-reacts with organisms obtained from mice, ferrets, rats, macaques, and humans and has been shown to be protective against infection in several animal models. However, its antigenic target and its mechanisms of protection remain unknown. Recent work has examined whether the entire 4F11 molecule is required for protection in vivo. In experiments performed with severe combined immunodeficiency (SCID) mice environmentally exposed to Pneumocystis-infected source mice, it is apparent that the Fc portion of the 4F11 molecule is required for optimal protection. Because F(ab')2 fragments are insufficient for protection, it is apparent that Fc-mediated functions, such as phagocytosis and complement activation, are necessary for optimal host defense.

Despite intensive study, the immunodominant antigen or antigens that elicit protective immunity against Pneumocystis remain unclear. Investigation of the antigens recognized by 4F11 demonstrates that this antibody recognizes at least two Pneumocystis antigens (10). In experiments using mouse-derived Pneumocystis, the first identified antigen is Pneumocystis kexin, which is similar to other fungal proteases. Kexins are typically intracellular molecules, however, and so seem unlikely candidates to elicit host immunity. The second identified antigen is A12, derived from a cDNA clone with little similarity to known proteins. This antigen appears to be the C-terminus of a larger molecule, appears to exist as a single copy gene, and encodes a protein of 278 amino acids. A murine A12:Thioredoxin fusion protein has been generated, and this fusion protein elicits anti-Pneumocystis antibody production in mice. Most importantly, the fusion protein decreases the intensity of Pneumocystis infection in mice depleted of CD4+ T cells. Further cloning and characterization of the A12 sequence is in process. Preliminarily, the sequence is similar to that of glycoprotein A, a long-studied Pneumocystis antigen that is present in abundance on the surface of the organism.

HOST RESPONSE TO Pneumocystis IN NEONATAL MICE

To complement studies examining immunity to Pneumocystis in adult mammals, many recent insights into host response have been obtained by studying responses in neonatal mice (11). Pneumocystis is a ubiquitous organism that infects most children, as shown by the prevalence of specific serum antibodies, and so insights from neonatal mice are both clinically and scientifically relevant. Important mechanisms in which neonatal immunity differs from adult immunity include the requirement of T cells for strong costimulatory signals, the tendency of T cells to favor Th2-like responses, the low expression of major histocompatibility complex type II on professional antigen-presenting cells, and reduced production of tumor necrosis factor (TNF) by alveolar macrophages.

From mouse models, it is apparent that neonatal mice clear Pneumocystis much more slowly than adult mice (12). This delayed clearance of Pneumocystis occurs by several mechanisms. Neonatal mice challenged with organisms demonstrate delayed migration of activated T cells into the alveolar space. Neonatal mice exhibit delayed B cell responses, in both the kinetics of appearance of antibody and in the numbers of specific, antibody-secreting B cells appearing in tracheobronchial lymph nodes (13). Both the upregulation of adhesion molecules (such as intercellular adhesion molecule-1 and vascular cell adhesion molecule-1) and cytokines (TNF, interferon [IFN]-γ, interleukin-6, monocyte chemoattractant protein-1) are delayed in neonatal mice compared with adults. Comparing alveolar macrophages from Pneumocystis-infected and uninfected neonatal mice, both Fe-γ receptor and MHC class II expression increased during infection. Finally, the neonatal lung environment demonstrates enhanced expression of immunosuppressive molecules, such as TGF-β, that further blunt the host response to Pneumocystis (14).

In summary, the delayed clearance of Pneumocystis in neonatal mice occurs by multiple mechanisms. There are delayed cytokine, adhesion molecule, activation marker, and lymphocyte responses, as well as enhanced expression of immunosuppressive molecules. Key unanswered questions in this field include identification of the signals that lead to recognition of Pneumocystis in neonatal lungs, the role of epithelial cells in controlling Pneumocystis growth and clearance in neonatal lungs, and the consequences of inhibiting anti-inflammatory molecules in the developing lung.

CD4+ AND CD8+ T CELLS IN HOST DEFENSE AGAINST Pneumocystis

Multiple investigations in humans and in animal models have examined the roles of CD4+ T cells and CD8+ T cells in defense
against *Pneumocystis*. Depletion of CD4\(^+\) T cells is a hallmark of HIV infection, and was recognized early in the AIDS epidemic to correlate with the development of *Pneumocystis* pneumonia. Immunologic reconstitution with highly active antiretroviral therapy repletes CD4\(^+\) T cells and decreases the risk of *Pneumocystis* pneumonia. Individuals with HIV infections have qualitative, as well as quantitative defects, in CD4\(^+\) T cell responses to *Pneumocystis*. As HIV infection progresses, there is a decrease in the ability of CD4\(^+\) T cells to proliferate when stimulated with *Pneumocystis* antigens in vitro.

Mammalian animal models have provided useful tools to study the roles of T cells in vivo. In animal models, corticosteroids deplete rats of CD4\(^+\) T cells and allow infection, and CD4\(^+\) T cell numbers rebound when corticosteroid treatment stops. Specific depletion of CD4\(^+\) T cells in mice induces susceptibility to *Pneumocystis*, and infection resolves when mice are repopulated with CD4\(^+\) T cells. SCID mice are susceptible to *Pneumocystis*, and reconstitution with donor CD4\(^+\) T cells clears infection. CD4 knockout mice and CD4-depleted rats are similarly susceptible. Overall, then, animal models indicate that lack of CD4\(^+\) T cells is a prime means by which individuals become susceptible to *Pneumocystis*.

Several mechanisms have been investigated to further characterize the importance of CD4\(^+\) T cells, including investigation of immunodominant epitopes, antigen presentation, costimulatory molecules, cytokine production, modulation of lung inflammation, and the roles of regulatory T cells. For example, the costimulatory molecule CD28, expressed on T cells, is essential for host defense against *Pneumocystis* in mouse models (15). However, not all proinflammatory responses to *Pneumocystis* are beneficial to the host. In SCID mice, for example, reconstitution with immune CD4\(^+\) T cells can eliminate organisms, but can also result in hyperinflammatory responses resulting in the death of the host (16). This seeming paradox has recently been illuminated by study of T regulatory cells, which express CD25 (17). While CD25\(^+\) cells reduce *Pneumocystis* burden but cause severe hyperinflammatory responses, reconstitution with CD25\(^+\) cells is not very successful in clearing organisms but does limit lung inflammation. Further investigations are likely to focus on subtypes of CD4\(^+\) T cells in control of infection and inflammation.

In contrast to CD4\(^+\) T cells, evidence demonstrating an important role for CD8\(^+\) T cells in host defense against *Pneumocystis* in humans is not as clearly developed. In animal models, the beneficial and detrimental effects of CD8\(^+\) T cells are more well explored, but also more controversial. SCID mice that are reconstituted with CD8\(^+\) T cells are not protected against infection, and wild-type mice depleted of CD8\(^+\) T cells alone are resistant. However, mice depleted of both CD4\(^+\) and CD8\(^+\) T cells develop more intense infection than mice depleted of CD4\(^+\) T cells alone, implying some beneficial effect during states of chronic CD4\(^+\) depletion (18). It is now apparent, however, that much of the pulmonary inflammation that occurs during *Pneumocystis* infection in the lungs of CD4\(^+\)-depleted mice is driven by CD8\(^+\) T cells.

In some instances, the protective and destructive effects of CD8\(^+\) T cells in animal models may occur by the same mechanisms. For example, T cytotoxic type-1 (Tc1) cells can be induced by treatment of the cells with a vector expressing IFN-γ, and such Tc1 cells exhibit cytotoxicity toward macrophages exposed to *Pneumocystis* (19). In contrast, other investigators have demonstrated that the lung damage and hypoxia in mouse models depend upon the presence of CD8\(^+\) T cells in the lung (20). As with CD4\(^+\) T cells, further work is needed to determine which populations of CD8\(^+\) T cells protect the host against infection, and which precipitate severe lung damage.

### B CELLS IN HOST DEFENSE AGAINST *Pneumocystis*

The alterations in B cell function that occur during HIV infection have been widely documented and include hypergammaglobulinemia, increased expression of B cell activation markers, increased susceptibility to apoptosis, and decreased responses to antigenic stimulation (21). However, the mechanisms by which these defects translate into increased susceptibility to *Pneumocystis* infection remain unclear. The high prevalence of serum antibodies in the general population, and the lack of effective reagents for their study, has hindered this line of investigation. However, some studies have demonstrated a fall in serum antibody titers before clinical *Pneumocystis* pneumonia and/or a rise in antibody titers after infection. In addition, little information is available about the role of local antibody responses in the lungs and in the regional lymph nodes of humans.

In animal models, *Pneumocystis* infection has been investigated in B cell–deficient mice (22). Passive administration of hyperimmune serum or monoclonal antibodies has also been explored. Other investigators have studied active immunization with *Pneumocystis* before depletion of CD4\(^+\) T cells, active immunization with dendritic cells expressing CD40 ligand, and protection by antibodies produced by Th1-like or Th2-like responses.

A pertinent example using humans to study antibody responses has been conducted in Cincinnati (23). In this study, 95 healthy blood donors were compared with 94 individuals with HIV infection (33 of whom had prior *Pneumocystis* pneumonia and 61 of whom did not). Serum was examined for the presence of antibodies directed against three recombinant fragments prepared from full-length major surface glycoprotein (Msg), termed Msg A, B, and C. Analysis of serum demonstrated no significant difference in the numbers of individuals with antibodies directed against Msg A or Msg B, but did show a significant increase in antibody reactivity to Msg C in the group with previous episodes of *Pneumocystis* pneumonia. Furthermore, antibody responses to Msg C have been studied longitudinally in patients with *Pneumocystis* pneumonia in San Francisco. These antibody responses peak at 3 to 4 wk after *Pneumocystis* infection and then decline over ensuing weeks. Antibody directed against Msg C also appears to be promising to follow responses in healthy individuals. For example, these antibodies have been used to examine responses in healthy young children in Chile.

Variants of the Msg C response may provide information about the epidemiology of *Pneumocystis* infection, as well as protective B cell responses. Recently, three variants of Msg C have been generated by PCR from infected human lung. These variants share homology at the nucleotide and amino acid levels, but antibody responses to the variants are divergent in the sera of individuals with prior *Pneumocystis* pneumonia. Further studies will focus on the role of antibodies to Msg C as an independent risk factor in the development of *Pneumocystis* pneumonia, as well as determining the role of local lung antibody concentrations in relation to pneumonia. In addition, development of antibodies to Msg C will be examined in young children and in adults with chronic lung diseases.

### INTERACTIONS OF *Pneumocystis* WITH MACROPHAGES, EPITHELIAL CELLS, AND SURFACTANT

Alveolar macrophages are known to bind, phagocytize, and degrade *Pneumocystis*. Rodent models with reduced numbers of functional alveolar macrophages exhibit poor clearance of the organism. In addition to phagocytosis, macrophages have been shown to be a rich source of TNF-α during *Pneumocystis* infection.
SP-A knockout mice exhibit increased Pneumocystis burden and enhanced lung inflammation at early time points after infection, which become less apparent later during the course of pneumonia (27). In contrast, recent models of SP-D overexpression, which mirror the clinical condition of SP-D accumulation, indicate that excessive SP-D actually promotes exuberant infection and pulmonary inflammation during Pneumocystis pneumonia (28). Thus, the balance of SP-A and SP-D expression are critical to host defense, and additional studies will be required to fully define the relative activity of these surfactant proteins during Pneumocystis pneumonia.

LUNG INFLAMMATION AND INJURY DURING Pneumocystis PNEUMONIA

Multiple observations indicate that pulmonary inflammation more potently contributes to lung injury than direct effects of the organism during Pneumocystis pneumonia. For instance, IL-8 and neutrophil levels correlate more closely with impaired oxygenation than organism numbers in patients with Pneumocystis pneumonia. This contention is further supported by the observed clinical benefit of adjunctive corticosteroid therapy during Pneumocystis pneumonia (1). Recent investigations indicate that both innate and adaptive immune responses contribute to this lung injury associated with Pneumocystis infection. CD8+ T-lymphocytes appear to be of particular importance, in that depletion of these cells reduces lung injury in CD4+-depleted rodent Pneumocystis models (20). In addition, TNF receptor signaling appears to be crucial for both the influx of CD8+ cells as well as neutrophils that accompanies severe Pneumocystis infection. Type I IFN signaling further promotes CD8+ cell recruitment, and MCP-1/CCR2 signaling also appears to be necessary for CD8+-based lung inflammation. In addition, alveolar epithelial cells strongly promote inflammatory cell influx and may function as a link between innate and adaptive immunity during this infection. Adaptive immunity, particularly involving antigen-specific CD8+ cells, appears to represent a significant mechanism of lung injury and provides a potential therapeutic target to ameliorate this lung injury during Pneumocystis pneumonia (20).

Adaptive and innate immunity are both markedly altered in the lung during HIV infection. While the loss of CD4+-based adaptive immunity strongly predisposes patients toward Pneumocystis pneumonia, recent studies support roles for CD4+-independent defense mechanisms. Furthermore, there is emerging evidence that suggests that HIV-induced alterations of innate immune function also contribute to the pathogenesis of lung infections in HIV-infected persons. HIV affects both humoral and cellular components of the innate immune response, at a relatively early stage. For instance, HIV infection is associated with impaired phagocytosis, respiratory burst, and inflammatory activation of alveolar macrophages in response to Pneumocystis (29). In addition, soluble proteins such as SP-A, fibronectin, and vitronectin are expressed in higher concentrations in the lung during HIV infection, further altering the interactions of Pneumocystis with host cells. Specific mechanisms mediating these events are beginning to emerge. Recent studies indicate that HIV infection decreases expression and increases sequestration of macrophage mannose receptors, rendering macrophages less able to phagocytize Pneumocystis (30). The loss of macrophage mannose receptors also promotes accumulation of glycoproteins such as fibronectin and vitronectin in the alveolar spaces. The modulation of pulmonary innate immune responses might provide another approach to prevent and treat Pneumocystis pneumonia during HIV infection.
MODULATION OF HOST DEFENSE AND NOVEL VACCINE STRATEGIES FOR **Pneumocystis**

Additional studies have yielded important insights into alternate strategies through which host defense can be manipulated to enhance clearance of *Pneumocystis*, while avoiding tissue injury. Substantial evidence indicates that depletion of CD4\(^+\) cells both in animals and humans compromises clearance of *Pneumocystis* and predisposes the host to pneumonia. Current antiretroviral strategies to restore functional CD4\(^+\) cells have clearly proven beneficial in reducing the risk of *Pneumocystis* pneumonia (1). In contrast, while CD8\(^+\) cell depletion may also impair clearance of the organisms, recent investigations have shown that recruitment of CD8\(^+\) cells to the lung mediates lung injury over the course of *Pneumocystis* pneumonia (31).

Various cytokines and related soluble components have been considered as potential targets for modulating host defense during *Pneumocystis* pneumonia. While TNF-\(\alpha\) is necessary for organism elimination, the deleterious effect of TNF-\(\alpha\) promoting lung inflammation and injury limits its overall therapeutic potential (32). In contrast, although studies of IFN-\(\gamma\) suggest that it is not essential, in and of itself, for host defense against *Pneumocystis*, enhancing concentrations of this cytokine in the lower respiratory tract enhances clearance of the organism and reduces the intensity of infection, particularly in the setting of decreased CD4\(^+\) cells. Additional studies indicate that administration of GM-CSF may also enhance clearance of this infection. Recent work has further supported a role for the ligands of the CXCR3 chemokine receptors, including IP-10 MIG and I-TAC during infection. *Pneumocystis* inoculation results in CXCR3 ligand lung expression in mice, which is accompanied by recruitment of CD4\(^+\) and CD8\(^+\) lymphocytes into the lung that are CXCR3-positive. IP-10 gene transfer facilitates clearance of *Pneumocystis* in rodents that are CD4\(^+\)-depleted. Thus, immune enhancement strategies may evolve, which will facilitate clearance of infection while limiting tissue injury (32).

Recent research has provided new insights, which will be essential for the development of an effective vaccine against *Pneumocystis*. In addition to the roles already defined above for T cells and macrophages, B cells provide essential activities for host defense against *Pneumocystis*. Immunization with *Pneumocystis* protects mice even after subsequent CD4\(^+\) cell depletion. In addition, B cells may function in a helper capacity in CD4\(^+\) replete hosts (33). Passive immunization of mice with either IgM or IgG1 against Kex1 protein protects mice against subsequent *Pneumocystis* challenge. *Pneumocystis* opsonized with specific antibodies are more rapidly taken up and killed by macrophages. Additional studies have sought to develop strategies to immunize hosts that already are CD4\(^+\) cell deficient. CD4\(^+\)-independent vaccination against *Pneumocystis* has been successfully accomplished in mice using genetically modified dendritic cells that express CD40L (34). These dendritic cells are pulsed with either whole *Pneumocystis* or with Kex1 *Pneumocystis* antigens, prior to reintroducing back into the CD4\(^+\)-deficient mice hosts, rendering the mice protected against subsequent *Pneumocystis* challenge. Additional vaccine strategies, including DNA-based vaccine technologies using prime-boost immunization protocols, are currently under development.

THE EPIDEMIOLOGY OF HIV-ASSOCIATED *Pneumocystis* PNEUMONIA

The epidemiology of *Pneumocystis* pneumonia has changed dramatically over the course of the HIV/AIDS epidemic (Table 2). During the 1980s, *Pneumocystis* pneumonia was the AIDS-defining illness for approximately two-thirds of adults and adolescents with AIDS in the United States, and it was estimated that 75% of HIV-infected persons would develop *Pneumocystis* pneumonia during their lifetime (35). Early in the epidemic, the incidence of *Pneumocystis* pneumonia was almost 20 cases per 100 person-years among HIV-infected persons with CD4\(^+\) cell counts below 200 cells/\(\mu\)L (36).

The introductions of PCP prophylaxis in 1989 and potent combination antiretroviral therapy in 1996 have led to substantial declines in the incidence of *Pneumocystis* pneumonia. In the Centers for Disease Control and Prevention (CDC) Adult and Adolescent Spectrum of Disease (ASD) Project, the incidence of *Pneumocystis* pneumonia decreased 3.4% per year during the period from 1992 to 1995 and then declined 21.5% annually during 1996 to 1998, a time when potent combinations of antiretroviral therapy were beginning to be used (37). In the EuroSIDA study, the incidence of *Pneumocystis* pneumonia declined from 4.9 cases per 100 person-years before March 1995 to 0.3 cases per 100 person-years after March 1998 (38).

Despite its decreased incidence, *Pneumocystis* pneumonia remains the most frequent serious opportunistic infection among HIV-infected persons, and a substantial proportion of persons who develop *Pneumocystis* pneumonia are unaware of their HIV infection or are outside of medical care, therefore limiting opportunities for further reductions in the incidence of the disease (37). Of concern, *Pneumocystis* pneumonia has been increasingly reported in low- or middle-income countries (LMIC). One study from Uganda found that 38.6% of 83 HIV-infected patients who were admitted to the hospital with pneumonia and who had three expectorated sputum smears that were negative for acid-fast bacilli had *Pneumocystis* pneumonia diagnosed on bronchoscopy with bronchoalveolar lavage (BAL) (39).

### TABLE 2. RECOMMENDATIONS FOR FUTURE DIRECTIONS IN *Pneumocystis* CLINICAL RESEARCH

<table>
<thead>
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<th>Recommendation</th>
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<tr>
<td>Improve understanding of the current epidemiology of PCP in the United States and worldwide, including in low- or middle-income countries.</td>
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<tr>
<td>Describe the clinical and radiographic presentation of PCP and the prevalence of tuberculosis co-infection in HIV-infected persons residing in low- or middle-income countries where tuberculosis is endemic.</td>
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<tr>
<td>Examine the impact of PCP prophylaxis and combination antiretroviral therapy on the presentation and diagnosis of HIV-associated PCP.</td>
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<tr>
<td>Continue to develop and validate new methods to diagnose PCP, including the use of noninvasive respiratory specimens such as oral wash combined with molecular tools such as PCR.</td>
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<tr>
<td>In the absence of a culture system for Pc, explore the application of Pc mRNA surrogate viability assays for studies of PCP diagnosis, Pc colonization, transmission, and putative drug resistance.</td>
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<tr>
<td>Continue to study putative drug resistance in human Pc, including the association between mutations in the Pc dldh/pyr Synthase gene and trimethoprim-sulfamethoxazole PCP treatment failure and death.</td>
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<tr>
<td>Further clarify the role of PCP prophylaxis for non-HIV immunocompromised hosts.</td>
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<tr>
<td>Define the incidence of PCP-associated immune reconstitution syndrome (IRS), the risk factors for the development of IRS, the optimal timing of antiretroviral therapy in the setting of newly diagnosed PCP, and improve understanding of the pathogenesis and the management of the syndrome.</td>
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<tr>
<td>Improve understanding of epidemiology of Pc colonization.</td>
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<tr>
<td>Further investigate risk of PCP or transmission of PCP resulting from Pc colonization.</td>
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<tr>
<td>Characterization of response to Pc colonization in the lung.</td>
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<tr>
<td>Further define role of Pc colonization in sudden infant death syndrome and chronic obstructive pulmonary disease.</td>
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Definition of abbreviations: HIV = human immunodeficiency virus; Pc = *Pneumocystis*; PCP = *Pneumocystis* pneumonia; PCR = polymerase chain reaction.
with a subacute course and longer symptom duration than other immunocompromised persons (1). Studies comparing the two groups have found that HIV-infected patients present with a higher arterial oxygen tension and a lower alveolar–arterial oxygen gradient, and their BAL specimens contain significantly greater numbers of \textit{Pneumocystis} organisms and significantly fewer neutrophils compared with patients without HIV infection.

HIV-associated \textit{Pneumocystis} pneumonia develops predominantly in persons whose CD4$^+$ cell count is less than 200 cells/µl (36). Classically, \textit{Pneumocystis} pneumonia presents with fever, cough, and dyspnea on exertion. Physical examination is nonspecific, and the pulmonary examination is often unremarkable, even in the presence of significant disease and hypoxemia. On chest radiograph, \textit{Pneumocystis} pneumonia usually presents with bilateral, diffuse, symmetric reticular (interstitial) or granular opacities (40, 41). Unfortunately, no combination of symptoms, signs, and chest radiographic findings is diagnostic of \textit{Pneumocystis} pneumonia, and its diagnosis currently relies on microscopic visualization of the characteristic cysts and/or trophic forms on stained respiratory specimens. Bronchoscopy with BAL is the preferred diagnostic procedure for \textit{Pneumocystis} pneumonia, with reported sensitivities ranging from 89% to greater than 98% (using any microscopic diagnosis of PCP in the subsequent 30–60 d as the gold standard) (42, 43). At select institutions, sputum induction is performed as the initial diagnostic procedure with reported sensitivities ranging from 74% to 83%, thereby decreasing the need for bronchoscopy (43, 44).

However, most of our current understanding of the clinical presentation and diagnosis of HIV-associated \textit{Pneumocystis} pneumonia is derived from studies conducted in the United States and Europe before the current era of combination antiretroviral therapy. It is also unclear whether the current U.S. and European-based understanding is generalizable to LMIC where the specter of tuberculosis looms significantly.

NEWER METHODS TO DIAGNOSE \textit{Pneumocystis} PNEUMONIA

The molecular diagnoses of infectious diseases are revolutionizing clinical medicine and PCR-based molecular assays currently are used to diagnose a wide range of infectious pathogens. The inability to culture \textit{Pneumocystis} and the requirement for invasive procedures to obtain respiratory specimens for microscopic examination identify \textit{Pneumocystis} pneumonia as an attractive target for molecular diagnosis. Several PCR assays have been developed as possible \textit{Pneumocystis} pneumonia diagnostic assays, including assays to amplify the human \textit{Pneumocystis} MSG, mitochondrial large subunit (mtlsu) rRNA, and the internal transcribed spacer (ITS) region genes. The assays have been tested on BAL, induced sputum, and noninvasive oral wash specimens. In general, PCR assays have been more sensitive but also less specific for \textit{Pneumocystis} pneumonia compared to traditional microscopic methods. In one study, a quantitative touch-down PCR assay targeting the multicopy \textit{Pneumocystis} MSG gene tested on oral wash specimens (obtained by having the subject gargle 10 ml of 0.9% NaCl for 60 seconds) had a sensitivity = 88% and a specificity = 85% for \textit{Pneumocystis} pneumonia (45).

PCR-negative subjects had significantly fewer copies per tube than did PCR-positive subjects, and the \textit{post hoc} application of a cutoff value of 50 copies per tube increased the specificity to 100%. PCR treatment before oral wash collection decreased the sensitivity of the PCR assay, suggesting that specimens should be collected before initiation of treatment if the assay is to be used on noninvasive specimens in clinical settings. Thus, oral wash specimens paired with a sensitive molecular PCR assay offer the potential to diagnose \textit{Pneumocystis} pneumonia in outpatient settings and in inpatient settings where bronchoscopy and the personnel and equipment to perform bronchoscopy are unavailable or limited in availability.

\textbf{Pneumocystis VIABILITY ASSAYS: APPLICATION TO DIAGNOSIS AND DRUG RESISTANCE}

PCR assays for human \textit{Pneumocystis} are being used in studies to improve diagnosis of \textit{Pneumocystis} pneumonia, to examine putative trimethoprim-sulfamethoxazole drug resistance, to study \textit{Pneumocystis} colonization in immunocompromised and immunocompetent hosts, and to address questions regarding the transmission of the disease. Most of these PCR assays amplify human \textit{Pneumocystis} DNA, which is relatively stable and may persist for an indeterminate amount of time after cell death. Therefore, the detection of \textit{Pneumocystis} DNA by one of these assays provides no information concerning the organism’s viability or infectivity. In contrast, RNA, especially mRNA, is much less stable than DNA and is rapidly degraded by endogenous RNAsases after cell death. As a result, the detection of \textit{Pneumocystis} mRNA may be a useful surrogate for organism viability. Previously, a reverse transcriptase (RT)-PCR assay based on detection of the Phsb1 transcript of human \textit{Pneumocystis} was developed (46). The coding and noncoding primers were designed to span the boundaries of the third and fifth introns of the Phsb1 gene, permitting amplification from cDNA but not genomic templates. The assay was shown to distinguish between viable and nonviable, heat-killed \textit{Pneumocystis}. Preliminary studies using this assay on respiratory specimens demonstrated a sensitivity = 100%, specificity = 86% on BAL specimens and a sensitivity = 65%, specificity = 80% on induced sputum specimens. The sensitivity of the assay was even lower on individual oral wash (gargle) specimens. However, several factors were found to influence the sensitivity of the assay. PCR treatment before oral wash collection decreased the sensitivity, while analyzing multiple sequential oral wash samples, obtaining the oral wash after the patient had coughed five times, and collecting the sample after the subject fasted for greater than 3 h increased the sensitivity of oral wash samples. These data, when taken together, suggest that careful attention to clinical sampling parameters will be necessary if the molecular viability assay is to be employed to monitor for viable organisms in a clinically relevant setting, such as testing the hypothesis that clearance of viable organisms is retarded in individuals infected with drug-resistant organisms.

\textbf{PCP TREATMENT AND TRIMETHOPRIM-SULFAMETHOXAZOLE DRUG RESISTANCE}

Trimethoprim-sulfamethoxazole is recommended as the first-line PCP treatment regimen (47). This fixed-dose medication has excellent tissue penetration and is available in intravenous and oral formulations that achieve comparable serum levels. Although intravenous pentamidine has equivalent efficacy compared to trimethoprim-sulfamethoxazole, the incidence of treatment-limiting adverse drug reactions is lower in patients treated with trimethoprim-sulfamethoxazole, thereby leading to its recommendation as first-line therapy.

Trimethoprim-sulfamethoxazole is also recommended as the first-line PCP prophylaxis regimen for HIV-infected persons whose CD4$^+$ cell count is less than 200 cells/µl, persons with oral candidiasis regardless of CD4$^+$ cell count, and patients with \textit{Pneumocystis} pneumonia after completion of PCP treatment (48). The widespread use of trimethoprim-sulfamethoxazole for PCP prophylaxis has been implicated in the increases in
trimethoprim-sulfamethoxazole–resistant bacteria reported in HIV-infected patients. It has also raised concerns about the possible selection of drug-resistant *Pneumocystis*.

The inability to culture human *Pneumocystis* has limited the ability to confirm drug resistance, but several groups have studied putative trimethoprim-sulfamethoxazole drug resistance by sequencing the *Pneumocystis* dihydropteroate synthase (DHPS) gene and correlating the presence/absence of nonsynonymous mutations with clinical outcomes, including PCP treatment failure and death (49). This approach was chosen in part because animal studies demonstrate that the anti-*Pneumocystis* activity of trimethoprim-sulfamethoxazole is almost entirely attributable to sulfamethoxazole, a DHPS inhibitor, and because similar DHPS mutations confer resistance to sulfan drugs in organisms including *Plasmodium falciparum*, *Escherichia coli*, and *Streptococcus pneumoniae*. The DHPS gene is highly conserved in many organisms including *Pneumocystis* isolated from nonhuman mammals.

In humans, DHPS mutations have been associated with the use of trimethoprim-sulfamethoxazole or dapsone, a sulfa DHPS inhibitor used for PCP prophylaxis (49). DHPS mutations have also been associated with the duration of sulfa or sulfone prophylaxis (50). Interestingly, geography is also a strong predictor of the risk of DHPS mutations (51) and settings where use of trimethoprim-sulfamethoxazole or dapsone for PCP prophylaxis is common are more likely to have patients with PCP whose *Pneumocystis* contains DHPS mutations compared with settings in which these medications are uncommon or their use has been restricted. Genetic analysis indicates that DHPS mutations have arisen independently in multiple isolates. Virtually all of the observed DHPS mutations are nonsynonymous point mutations at amino acid positions 55 and/or 57. Homologous mutations in the *E. coli* DHPS gene are located in an active site involved in substrate binding. Taken together, these findings suggest that the use of trimethoprim-sulfamethoxazole or dapsone for PCP prophylaxis selects for DHPS mutations and confers some benefit to those *Pneumocystis* organisms that contain these mutations.

The potential association between the presence of DHPS mutations and clinical outcomes suggestive of trimethoprim-sulfamethoxazole drug resistance, such as PCP treatment failure or death, is less clear (49). One study found that the presence of DHPS mutations was an independent predictor associated with an increased all-cause mortality at 3 mo (hazard ratio [HR], 3.10; 95% confidence interval [95% CI], 1.19–8.06; p = 0.01) (52). Another study failed to confirm an association with an increased mortality, but found that the presence of DHPS mutations was associated with an increased risk of PCP treatment failure with trimethoprim-sulfamethoxazole or dapsone plus trimethoprim (relative risk, or risk ratio, [RR], 2.1; p = 0.01) (50). A third study found no association between the presence of DHPS mutations and PCP treatment failure, mortality at 6 wk, or mortality at 6 wk attributable to *Pneumocystis* (53). A subsequent multivariate analysis from the same group (consisting of 215 subjects, including 70 from the earlier report) found that independent predictors of mortality at 60 d were a low serum albumin level and the need for intensive care within 72 h (54). One consistent finding in all of these studies was the observation that the majority of patients with PCP whose *Pneumocystis* contained DHPS mutations and who were treated with trimethoprim-sulfamethoxazole responded to treatment with this regimen and survived (49). Since mortality in an HIV-infected patient with *Pneumocystis* pneumonia may be related to multiple factors beyond their *Pneumocystis* infection, the definitive answer to the question of drug resistance will likely require substantial numbers of subjects and a multivariable analysis to control for all potential factors.

**PCP PROPHYLAXIS AND IMMUNE RECONSTITUTION**

HIV-infected patients appear to have the highest risk for the development of *Pneumocystis* pneumonia, followed by patients immunosuppressed after solid organ transplantation or bone marrow transplantation, those with malignancies undergoing chemotherapy, and those receiving chronic immunosuppressive medications. Each of these groups is susceptible to *Pneumocystis* pneumonia at certain points in their disease course, and treatment and PCP prophylaxis is recommended during these periods in an attempt to prevent disease. In general, trimethoprim-sulfamethoxazole is the recommended first-line PCP prophylaxis regimen (48). Dapsone, with or without pyrimethamine, represents the main second-line alternative, followed by aerosolized (or occasionally intravenous) pentamidine, atovaquone, and clindamycin plus primaquine. Once started, PCP prophylaxis should be continued until or unless the specific risk factor for the disease improves or resolves.

One of the most exciting advances in the past decade of the HIV epidemic has been the dramatic decline in HIV-associated opportunistic infections and AIDS-associated mortality as a result of potent combinations of antiretroviral therapy (55). Antiretroviral therapy has also allowed HIV-infected persons to discontinue prophylaxis or maintenance therapy for opportunistic infections including *Pneumocystis* (48). Previously, persons who began PCP prophylaxis remained on prophylaxis for life. Multiple studies have since demonstrated that primary and secondary PCP prophylaxis can be safely discontinued in patients whose CD4+ cell counts increase above 200 cells/μl for at least three months as a result of potent combination antiretroviral therapy (48). Current guidelines recommend that persons who meet these criteria can discontinue PCP prophylaxis and remain off prophylaxis as long as the CD4+ cell count remains above 200 cells/μl.

Another important consequence of the recovery of immune function resulting from antiretroviral therapy is the immune reconstitution syndrome (IRS), also called the immune reconstitution inflammatory syndrome (IRIS). The incidence of *Pneumocystis*-associated IRS appears to be less than that with several other opportunistic infections, including mycobacterial and fungal infections (56, 57). In persons with *Pneumocystis* pneumonia, the initiation of antiretroviral therapy during the course of PCP treatment has been associated with a paradoxical worsening of *Pneumocystis* pneumonia with a relapse in their symptoms and a deterioration in their respiratory status, occasionally causing acute respiratory failure (58). Persons with clinically silent *Pneumocystis* pneumonia may have their pneumonia unmasked as a result of the initiation of antiretroviral therapy. The syndrome occurs as a result of an improved immune response directed against active infection or residual antigen (56). Most patients recover, occasionally requiring the temporary discontinuation of antiretroviral therapy and/or a short course of corticosteroids.

**EPIDEMIOLOGY AND SIGNIFICANCE OF PNEUMOCYSTIS COLONIZATION**

Colonization with *Pneumocystis* has recently gained attention as a potentially important phenomenon. In general, colonization is defined as isolation of a microbe that does not result in sufficient damage to cause clinical disease, but may alter the host homeostasis. For *Pneumocystis* in particular, colonization has been defined as detection of the organism in respiratory samples from a host who does not progress to acute *Pneumocystis*
pneumonia (59). The detection of Pneumocystis colonization has been greatly facilitated by the development of sensitive PCR techniques. Although colonization can occasionally be found using traditional staining methods or single round PCR, nested PCR is generally employed because of its greater sensitivity. Nested PCR of the mtLSU rRNA locus is the technique most commonly used to determine colonization because the mtLSU is a multicycle gene and its use enhances detection.

**COLONIZATION IN ANIMALS**

Colonization occurs in both animals and humans. In a recently developed nonhuman primate model, simian immunodeficiency (SIV)-infected rhesus macaques are intrabronchially inoculated with Pneumocystis (60). Although some animals progress to acute Pneumocystis pneumonia, a number of them develop a prolonged colonization phase. The colonization period is marked by significant pulmonary inflammation as determined by bronchoscopy with BAL. An influx of CD8+ T lymphocytes and neutrophils occurs in the lungs, and inflammatory cytokines such as IL-8 and TNF-α are elevated, suggesting that the Pneumocystis is altering the host immune response.

**COLONIZATION IN CHILDREN**

Children, particularly infants, may play an important role in the epidemiology of Pneumocystis. Pneumocystis is common in normal infants with and without respiratory symptoms, and these infants may represent a human reservoir for Pneumocystis. Primary exposure to Pneumocystis is widespread and likely occurs early in life as Pneumocystis antibodies are detectable in 85% of children by 20 mo of age (61). Rates of detection of Pneumocystis colonization in nasopharyngeal aspirates (NPAs) are approximately 10.5% in healthy children, and approximately 15% of infants with respiratory symptoms and/or bronchiolitis are colonized.

Intriguing studies have focused on Pneumocystis colonization in infants who die in the community and/or die from sudden infant death syndrome (SIDS). In one study, all 58 infants less than 1 yr of age and without underlying immunodeficiency who died from various causes had detectable Pneumocystis in their lungs (62). In another study, 52% of infants dying at home, mostly from SIDS, had Pneumocystis detected in their lungs, compared with only 20% of those who died in the hospital (63). Although these findings are based on Pneumocystis DNA detection by nested PCR, some infants have Pneumocystis organisms apparent by microscopy of autopsy lung samples. Studies comparing the incidence of Pneumocystis in infants dying from different causes including accidents are in progress. The highest prevalence of colonization is found in infants dying around 4 mo of age, the same age at which peak incidence of Pneumocystis pneumonia occurs in children with HIV.

**COLONIZATION IN ADULTS**

In adults, Pneumocystis colonization occurs in both HIV-infected and non–HIV-infected subjects, and certain populations appear to have a higher risk of colonization. Recently, there have been increasing reports of Pneumocystis colonization. This increase could represent an improvement in detection techniques or a growing prevalence of colonization in the community.

In the HIV-infected population, the number of colonized subjects varies from 10% to 69% (64–67). This large variation likely results from the use of different detection techniques, different respiratory samples, and different populations. Several studies have examined the contribution of various risk factors to development of colonization. Although it might be assumed that lower CD4+ cell counts increase the risk of colonization, the relationship between CD4+ count and colonization is debated, and there does not seem to be a clear-cut association. Use of PCP prophylaxis and history of Pneumocystis pneumonia are also not associated with risk of colonization. Cigarette smokers have an increased risk of colonization, and HIV-infected subjects living in different cities may have different colonization risks (64).

Non–HIV-infected persons can also become colonized. In general, the prevalence in non–HIV-infected populations is somewhat less. Most healthy people are not Pneumocystis-colonized (68), but 7% to 19% of patients with an underlying chronic illness or various respiratory disorder have detectable colonization (69–71). Pregnant women also seem to have a higher risk, with one study reporting that 16% of pregnant women were colonized (72). Increased risk of colonization has been reported in patients with chronic obstructive pulmonary disease (COPD) (73–75). This risk is higher than that in other lung diseases, and cigarette smokers who are colonized have worse airway obstruction than smokers who are not colonized, suggesting that colonization might play a role in progression of COPD.

Many important questions remain unanswered about Pneumocystis colonization. The answers to these questions have potential to impact patient care. For example, are those persons who are colonized with Pneumocystis at risk for developing Pneumocystis pneumonia and can they transmit the infection to others? If a colonized subject takes Pneumocystis prophylaxis for a long period of time, is there a risk of developing drug-resistant organism? Animal data indicate that Pneumocystis colonization provokes an intense inflammatory response in the lungs. Does a similar response occur in humans and is it detrimental to the lung? Might such inflammation play a role in the progression of COPD in cigarette smokers? Is there a role of Pneumocystis in SIDS? These questions should be the subject of future study to define better the epidemiology and the importance of Pneumocystis colonization.

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