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Colonization by *Pneumocystis jirovecii* and Its Role in Disease

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INTRODUCTION

Despite the introduction of combination antiretroviral therapy for the human immunodeficiency virus (HIV), *Pneumocystis* pneumonia (PCP) remains one of the leading causes of morbidity and mortality (7). Although PCP has been recognized as a cause of pneumonia in immunocompromised hosts for many years, the first cases described in the 1980s that were associated with the AIDS epidemic heralded the onset of PCP as a more common clinical disease. PCP has long been the most common AIDS-defining opportunistic infection in the United States (7). With the development of combination antiretroviral therapy (ART) for treatment of HIV infection, there has been a decrease in incidence of PCP in those who have access to ART (238); however, PCP remains a leading cause of disease in HIV-infected persons who do not have access to ART, who are unable to tolerate ART or in whom ART is not effective, or who do not know that they are HIV infected (141). Since the increased clinical importance of PCP at the start of the AIDS epidemic, much has been discovered about the biology of *Pneumocystis*, including changes in the classification of the organism, insight into its transmission, and the discovery of a state of colonization that may be important in the development of PCP as well as other lung diseases. This review discusses the biology of *Pneumocystis*, the host response to the organism, current understanding of PCP epidemiology, diagnostic modalities, treatment, and colonization and its role in disease.

CLASSIFICATION AND NAMING

Carlos Chagas originally identified *Pneumocystis* in the lungs of rats and guinea pigs in 1909. Chagas believed the organism to be a form of *Trypanosoma cruzi*, and he subsequently identified *Pneumocystis* in the lungs of patients who died of *Trypanosoma* infection (34, 160). Antonio Carini also noted these cysts in rats with trypansomiasis but thought that they might be an unrelated organism. In 1912, Pierre and Marie Delanoë classified *Pneumocystis* as a new species but believed it to be a parasite (60, 123). It was reported as a cause of pneumonitis in three infants in the Netherlands in 1942 (291) and was then identified by Vanek and Jirovec as a cause of interstitial plasma cell pneumonia in malnourished infants during World War II (123, 134, 292). It remained relatively uncommon as a cause of disease until the onset of the AIDS epidemic in the 1980s, with 80 cases reported by 1973, including a series of 15 children with PCP and congenital immunodeficiencies (27).

Pneumocystis research has been hampered by the lack of a system for sustained propagation of the organism. It is an obligate extracellular pathogen and exists in trophic and cystic forms. Initially classified as a protozoan, it was reclassified as a fungus based on greater DNA sequence homology with fungal organisms (74, 282). *Pneumocystis* infects only mammals and is species specific. Historically, all forms of *Pneumocystis* were referred to as *Pneumocystis carinii* with a special form designating the host. In recognition of the specificity of different *Pneumocystis* organisms, the human form was renamed *Pneumocystis jirovecii* in honor of Otto Jirovec. *Pneumocystis carinii* is now reserved for the rat form of *Pneumocystis*.

TRANSMISSION OF *PNEUMOCYSTIS*

Pneumocystis is thought to be a ubiquitous organism given the universal serologic response seen in humans (206, 240, 243, 295, 310). Historically, it was thought that *Pneumocystis* infection was acquired during childhood and that PCP occurred via reactivation of latent infection when the host's immune system was compromised. It has recently been recognized that *de novo* exposure either from the environment or from individuals with PCP or colonized with *Pneumocystis* may result in transmission (32, 56, 57, 94, 139, 215, 236, 246, 306, 307, 322). The mechanism of transmission of *Pneumocystis* has important clinical and public health implications. For example, if reactivation of latent infection is the primary cause of PCP, there is little reason to isolate patients with PCP when they are in the hospital as the risk of person-to-person transmission would be low. Alternatively, if person-to-person transmission is a key component of the disease process, avoidance of exposure to infected persons, particularly by immunocompromised patients, would be important. Similarly, if particular environmental reservoirs of *Pneumocystis* are detected, at-risk patients should be counseled to avoid those exposures.

Reactivation of Latent Infection

According to the reactivation theory, *Pneumocystis* is commonly encountered in the environment during childhood. The organism does not cause clinical disease but is harbored within the host and can subsequently reactivate to cause PCP if the host's immune function declines. Several lines of evidence, including characteristics of *Pneumocystis*, a high seroprevalence of anti-*Pneumocystis* antibodies in the population, a high rate of PCP in immunocompromised infants, and detection of *Pneumocystis* in normal hosts support, the idea that reactivation contributes to the development of PCP.

Pneumocystis has several characteristics that suggest that it is carried long-term in a host. *Pneumocystis* can vary its major surface glycoproteins, indicating that it can evade the host immune system over a prolonged period of time (95, 283). The specificity of *Pneumocystis* for a particular mammalian host also argues that the organism has evolved along with its host (98). In addition, the inability to culture *Pneumocystis* suggests that it has evolved to require a very specific environment that is not easy to reproduce outside its host.

The majority of persons appear to be exposed to *Pneumocystis* and develop antibody responses to the organism early in life (206, 240, 243, 295, 310). One study of healthy children found that anti-*Pneumocystis* antibodies were detectable by 7 months of age, with 83% of children having titers of at least 1:16 by the age of 4 years (243). Other series from various geographic regions have

reported a prevalence of antibodies ranging from 70% to 100% in healthy children, with a prevalence of about 70% in both HIV-infected and non-HIV-infected adults (240, 311).

HIV-infected infants also have a high rate of PCP, particularly during the first year of life. In a cohort of 3,665 infants with perinatally acquired HIV, 37% developed either definitive or empirically diagnosed PCP during 10 years of follow-up, with more than half of the cases presenting at between 3 and 6 months of age (269). These findings suggest that early exposure and infection are common, but they cannot distinguish between the possibility that early acquisition of infection leads to reactivation or that PCP results from *de novo* exposure.

De Novo Infection

Although reactivation of latent infection may be one mechanism of PCP infection, human and animal studies imply that *de novo* infection also occurs. For example, studies of immunosuppressed rodents with PCP demonstrate that the majority of animals have no detectable *Pneumocystis* by PCR after immune reconstitution and do not develop PCP after repeated immunosuppression (36, 296). Other evidence that supports *de novo* infection are the observations that *Pneumocystis* is cleared after infection and the presence of genotype switching in repeat episodes of PCP (146, 147, 149, 234, 265).

Environmental reservoirs. Data suggest that there may be environmental reservoirs of *Pneumocystis* and that geographic and climatic factors affect PCP risk. *Pneumocystis* DNA has been detected in pond water and in air from both outdoor and indoor settings (32, 139, 236, 306, 307). One study also found that patients with PCP were significantly more likely to have a history of recent gardening or hiking and camping than age- and $CD4^+$ cell count-matched controls (odds ratio of 5.38 and 95% confidence interval of 1.39 to 20.8 for gardening; odds ratio of 7.68 and 95% confidence interval of 1.34 to 44.1 for hiking/camping), suggesting that soil exposure may be important for disease transmission (226). PCP risk also varies in different parts of cities (69, 223), and several studies have shown that frequencies of *Pneumocystis* genotypes vary in different cities and countries (13, 172). In addition, the strains seen in each city reflect the patient's current residence rather than place of birth, suggesting a recently acquired infection (13). This infection could have resulted either from an environmental reservoir or from an infected host but would be unlikely to result from reactivation. A series of studies have also examined seasonal variation in PCP risk in an effort to distinguish the impact of various environmental factors on disease, but these studies have reached various conclusions, with peaks of PCP in both summer and winter months or no relation to season at all (210, 271, 293).

Person-to-person transmission. (i) Evidence from animal models. Transmission of *Pneumocystis* has been documented in both immunocompromised and immunocompetent animals, strongly supporting the hypothesis that transmission can also occur between people. Immunosuppressed rodents housed in a room with other rodents with acute PCP will develop PCP (246, 322). In a series of experiments, investigators found that immunocompetent mice exposed to mice with PCP could transmit *Pneumocystis* to other immunocompetent mice (4, 99). When these immunocompetent, PCP-exposed mice were cohoused with immunosuppressed mice, the immunosuppressed animals developed PCP, thus directly implicating intraspecies transmission as an important mechanism. Simian immunodeficiency virus-infected macaques also can develop spontaneous PCP when cohoused with infected animals (304).

(ii) Evidence from human studies. The possibility of person-to-person transmission of PCP was first raised after reports of PCP outbreaks in oncology and transplant units (35, 38, 260, 272). Many other clusters of PCP cases have since been reported, including both immunosuppressed and HIV patients (56, 57, 94, 215). A recent study of hospital inpatients with PCP tested air samples in patient rooms and outdoors (39). *Pneumocystis* DNA was detected in 80% of air samples collected within 1 meter of the patient. Both the organism burden and percentage of positive samples decreased with increasing distance from the patient, but *Pneumocystis* remained detectable in about a third of air samples taken from the adjacent corridor. Other studies that support person-to-person transmission of *Pneumocystis* have examined the presence of mutations in the dihydropteroate synthase (DHPS) locus. These mutations develop in the setting of exposure to sulfa-containing medications such as trimethoprim-sulfamethoxazole (TMP-SMX) and have not been found to occur spontaneously in free-living mammals (62). Studies have found these mutations in patients with PCP without previous exposure to sulfa, suggesting that they acquired their disease from a patient who had a history of sulfa use, although unreported sulfa exposure is possible as well (112, 120).

Health care workers may be an intermediate host in *Pneumocystis* transmission, in a manner similar to that found from the studies of immunosuppressed and immunocompetent animals described above. Studies of health care workers have examined either anti-*Pneumocystis* antibody responses or colonization with *Pneumocystis* as determined by detection of *Pneumocystis* DNA by PCR of oral or nasal samples. Although some studies have found no measurable difference in antibody titers in health care workers exposed to PCP, others have detected increases in those with PCP exposure (176, 186). A recent study of antibodies to the major surface glycoprotein (Msg) of *Pneumocystis* found that those individuals with a clinical occupation (and presumed exposure to PCP) had higher antibody levels to Msg fragments that nonclinical workers without PCP exposure (288). Studies of colonization after PCP exposure have found that staff and family members can become *Pneumocystis* colonized after contact with PCP patients, but not all studies have replicated these results (51, 176, 187, 209, 299). If person-to-person transmission is an important mechanism of disease spread, there are direct implications for patient care. In most hospitals, patients with PCP are not placed in respiratory isolation and may be at risk of transmitting disease directly to other immunosuppressed patients or infecting health care workers, who then serve as a reservoir. Current guidelines do not recommend respiratory isolation of patients suspected to have PCP, but some centers do use isolation for these patients.

SEROLOGY

Humoral Response

Studies of seroprevalence to *P. jirovecii* have provided important information regarding the epidemiology and protective immune responses. Development of sensitive and reliable serologic assays for *Pneumocystis* infection and colonization would be useful as a noninvasive diagnostic test and as an epidemiologic tool. Reagents that could distinguish past from current *Pneumocystis* infection or

colonization from active disease would be particularly useful. Although serologic studies have been conducted for many years, there are several impediments to successful translation of these studies to clinical applications (46, 51–53, 55, 67, 68, 233, 288, 312). Much of the difficulty is due to the lack of an *in vitro* culture system for human *Pneumocystis*, which has limited the development and evaluation of specific diagnostic reagents. Serologic studies using crude antigenic extracts derived from infected human or rodent lung tissue have been used extensively (93, 240, 252, 295). Although these studies have demonstrated that the prevalence of seroreactivity is high and varies by geographic location, assays using crude extracts have produced conflicting results regarding seroprevalence in surveys of healthy and immunocompromised hosts and generally fail to distinguish active infection from previous exposure, and thus these reagents have been of limited clinical use. With the development of recombinant *Pneumocystis* antigens, more recent studies investigating the utility of specific antigens for serologic studies are producing promising results in this area (22, 52, 173, 203).

Pneumocystis Antigens

Major surface glycoprotein. Early studies of *Pneumocystis* surface moieties revealed a predominant surface glycoprotein, the major surface glycoprotein (also referred to as glycoprotein A) (188, 281). Msg is encoded by a large gene family consisting of over 100 copies. Molecular examination of Msg genomic localization and expression revealed that the *msg* gene undergoes extensive genomic rearrangement, resulting in variations in its antigenic properties (148, 161, 203, 280, 281). The expressed *msg* gene copy is positioned at a specific expression site in the genome, with a single protein isoform expressed at one time (73, 283, 305). Variation of the Msg protein appears to occur through transcriptional regulation and involves complex genomic rearrangement resulting in variant expression of *msg* genes. Whether variation of this abundant surface protein results in immune evasion, as has been shown with other pathogens that undergo antigenic variation, has not been clearly determined *in vivo*. Biochemical characterization of the Msg protein revealed that it is a highly glycosylated protein complex of ~90 to 120 kDa. Several studies have established a role for Msg in interactions with host molecules, including fibronectin, vitronectin, and surfactants, as well as a role in attachment to alveolar epithelial cells (177, 180, 201, 302). In a series of studies utilizing recombinant Msg fragments that cover the entire protein-coding region, Walzer and colleagues developed an enzyme-linked immunosorbent assay (ELISA) that has shown promise in diagnostic testing and epidemiologic studies (53, 54, 312). Of particular interest is a fragment that encodes the relatively conserved carboxy-terminal region of the protein (MsgC) (46, 53–55, 67). HIV-infected patients with active PCP have significantly higher antibody titers to MsgC than patients with pneumonia due to other causes (68). Recent studies have supported the utility of MsgC titers as indicators of acute or recent PCP (102).

Serologic assays, particularly those using well-defined, recombinant antigens such as the Msg-based ELISAs, may also be of use, in conjunction with DNA-based methods, for detecting the organism, in dissecting patterns of *P. jirovecii* transmission, and in epidemiologic studies. While host-to-host transmission has been demonstrated in various animal models, it remains to be determined whether there is significant person-to-person transmission of *Pneumocystis* from healthy hosts who may be transiently colonized with *Pneumocystis* and whether colonized individuals can be a significant source of infection for immunocompromised individuals. A clearer picture of the mechanism of transmission may emerge with more extensive use of molecularly defined, recombinant reagents as the immunologic targets, rather than antigenically complex targets. For example, Tipirneni and colleagues found that individuals in clinical care occupations had higher levels of antibody to a relatively conserved region of Msg (MsgC) than did nonclinical workers (288). In contrast, occupation (clinical or nonclinical) was not significantly associated with antibody responses to more variable regions of the protein. Thus, it appears that health care workers may have occupational exposure to *P. jirovecii*, and these studies provide insight into the utility of serologic assays for the tracking person-to-person transmission. Longitudinal studies using defined antigens such as MsgC as epidemiologic tools to assess *P. jirovecii* exposure in health care workers may be useful in determining the nature and extent of transmission in the clinical setting and may guide the adaption of precautions and respiratory isolation in the care of patients with PCP.

Kexin. Recently, a second *Pneumocystis* protein has been investigated for potential use in serologic studies (102, 154, 219). The *Pneumocystis* protease, kexin (Kex1, Prt1), possesses similarity to the fungal subtilisin-like serine proteases Kex1 from *Kluyveromyces lactis* and Kex2 from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (165, 173, 181, 182). The *Pneumocystis*-derived *kex1* gene isolated from *P. murina* (173), as well as that from *P. jirovecii* (165), is a single-copy gene, whereas a multigene kex1 family has been characterized in rat-derived *P. carinii* (279). Kex is hypothesized to be involved in the proteolytic processing of *Pneumocystis* surface antigens, in particular, Msg, and in this respect may represent a new therapeutic target. Several recent studies with human subjects and with experimental animal models suggest that recombinant derivatives of *Pneumocystis* Kex may be a useful target for serologic studies and a potential target for immunologic control of *Pneumocystis* infection, as discussed below (154, 316, 328).

In experimental studies to evaluate the dynamics of the humoral response to *Pneumocystis* colonization and infection in macaques, our group has shown that an ELISA using recombinant Kex1 protein covering a 110-amino-acid internal conserved region was effective in tracking natural transmission of *Pneumocystis* among immunocompetent macaques cohoused with *Pneumocystis*-colonized primates (153). In a nonhuman primate model of HIV and *Pneumocystis* coinfection using a humanized simian immunodeficiency virus (SHIV) and natural *Pneumocystis* transmission by cohousing (154), anti-Kex antibody titers were shown to be effective in evaluating changes in *Pneumocystis* colonization status, and higher baseline plasma anti-Kex IgG titers prior to SHIV-induced immunosuppression correlated with prevention or delay of *Pneumocystis* colonization after SHIV immunosuppression and *Pneumocystis* exposure. In contrast, macaques with low or undetectable anti-Kex titers at baseline were more likely to become colonized with *Pneumocystis* following SHIV infection.

These experimental studies are supported by a recent human study in which plasma anti-Kex and anti-Msg IgG levels were evaluated in HIV-infected patients with PCP and those with another AIDS-defining illness, both before and after their AIDS-defining illness (102). Antibody titers to *Pneumocystis* Msg and Kex increased after acute PCP, but low baseline Kex levels were associated with subsequent development of PCP, even in persons

not yet at risk for PCP by CD4 cell count criteria. Together with the nonhuman primate studies described above, these findings suggest that low plasma anti-Kex IgG levels may be a novel, early marker of future PCP risk in HIV-positive individuals.

PNEUMOCYSTIS PNEUMONIA

Recent Epidemiology of PCP in HIV-Infected Patients

The first cases of PCP that heralded the onset of the HIV epidemic were reported in men who had sex with men and in intravenous drug users in 1981 (6, 197). For many years, PCP was the leading cause of morbidity and mortality in people with HIV. With the widespread use of ART and anti-*Pneumocystis* prophylaxis, the incidence of PCP has declined, particularly in high-resource countries where individuals have access to these medications. In the EuroSIDA study of over 8,500 HIV-infected persons, the PCP incidence fell from 4.9 cases per 100 person-years to 0.3 case per 100 person-years after ART became widely available (317). In the United States, the HIV Outpatient Study (HOPS) recently reported that the incidence of a first episode of PCP was 3.9 cases per 1,000 person-years for the period from 2003 to 2007 (26). A large study of hospital discharges in the United States found that the percentage of HIV-infected patients discharged from the hospital with PCP decreased from 31% at the beginning of the HIV epidemic to 9% in the years after the introduction of combination ART (150). In the United States and Europe, PCP still occurs primarily among persons who do not know that they are HIV infected, who do not seek medical care, or who do not comply with or respond to antiretroviral therapy or *Pneumocystis* prophylaxis (47, 141, 185, 247, 287).

The genetic susceptibility of HIV-infected subjects to PCP has not been widely examined; however, polymorphism in the gene encoding the Fc segment of IgG (FcγRIIa) has been shown to influence the risk of PCP (86). Forthal et al. found that functional polymorphisms of FcγRIIa, which affect immune complex binding and clearance, influence susceptibility to PCP in HIV-infected subjects (86). The role of FcγRIIa genotype in susceptibility to the development of PCP is unclear, but it is possible that opsonization of *Pneumocystis* organisms at attachment to alveolar macrophages (AMs) (which bear FcγRIIa) may influence infection.

The incidence of PCP in low- and middle-income countries may differ from that in the developed world. PCP appears to be infrequent in Africa (1, 11, 84, 218). A recent study of 353 patients admitted to Mulago Hospital in Uganda with greater than 2 weeks of cough found that only one percent had a diagnosis of PCP (166). The lower incidence of PCP in Africa may be from a truly low rate of infection or could be due to a lack of diagnostic facilities or to a high mortality from diseases such as tuberculosis and bacterial pneumonia that cause mortality before individuals become susceptible to PCP. Recent reports of low *Pneumocystis* colonization in Africa support the hypothesis that the organism is not prevalent in the area, perhaps due to geographic or climatic factors (133). In contrast, PCP appears to be more frequent in other resource-limited countries such as Ukraine and India (92, 192).

PCP mortality has also decreased in recent years, with current estimates of in-hospital PCP mortality ranging from 7 to 11% (150, 250, 313). A study from London examining PCP from 1985 to 2006 found that mortality decreased over time from a high of 17% in the early years to 10% in the later years of the study (313). Mortality in critically ill PCP patients remains high, with recent

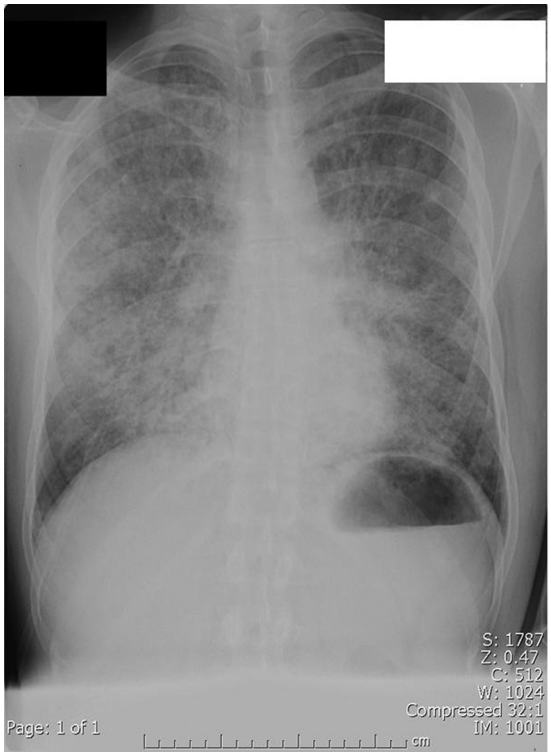

FIG 1 Chest radiograph from an HIV-infected patient with *Pneumocystis* pneumonia, demonstrating diffuse bilateral infiltrates.

estimates of 29 to 62% for PCP patients admitted to intensive care (48, 65, 152, 221, 250). Factors associated with increased mortality risk in critically ill patients with PCP include higher age, lower serum albumin, need for mechanical ventilation, development of a pneumothorax, greater alveolar-arterial oxygen gradient, and lower hemoglobin (2, 79, 92, 150, 208, 221, 250, 313). Some studies have reported that a lower $CD4^+$ cell count is associated with a greater mortality, but not all cohorts have replicated this result (79, 92, 150, 250).

The strongest risk factor for PCP is a $CD4^+$ cell count below 200 cells/μl, with the risk increasing the lower the $CD4^+$ count declines below this level (242, 276). Previous PCP, oral candidiasis, and persistent fevers are also risk factors for PCP. A recent study found that an increasing number of PCP cases in the United States were seen in black persons, women, and individuals who lived in the South (150).

HIV-infected patients with PCP typically present with the subacute onset of cough, dyspnea, and fevers (137, 140, 159). One study found an average of 1 month of symptoms prior to presentation in HIV-infected patients with PCP (159). Purulent sputum and pleuritic chest pain are uncommon in PCP and are more predictive of bacterial pneumonia. Serum lactate dehydrogenase (LDH) is often elevated, but a normal LDH level does not rule out the diagnosis. The chest radiograph usually demonstrates bilateral reticular or granular infiltrates (Fig. 1) (61). The infiltrates are typically bilateral and extend out from the hilum. A normal chest radiograph can also be seen, often in early disease (61, 151, 237). Other atypical presentations include focal consolidation, unilateral infiltrates, or nodules (151). PCP rarely causes intrathoracic adenopathy or pleural effusion.

TABLE 1 Summary of characteristics of novel diagnostic tests for *Pneumocystis* pneumonia

Test	Sample(s)	Advantages	Disadvantages
PCR	Oral wash, induced sputum, BAL fluid	Can be used on noninvasive samples, does not require experienced laboratory, high sensitivity	Not widely available; may detect colonization as well as pneumonia, so low specificity
(1→3)-β-D-Glucan	Serum	Noninvasive, does not require experienced laboratory, good sensitivity and specificity depending on population	May detect other fungal diseases, most useful as ancillary test
S-Adenosylmethionine	Serum	Noninvasive, does not require experienced laboratory, increases in levels may correspond with clinical improvement	Specificity and sensitivity debated

Recent Epidemiology of PCP in Non-HIV-Infected Immunosuppressed Populations

In contrast to the case for the HIV-infected population, the number of non-HIV-infected immunosuppressed patients at risk for PCP has been increasing with the increasing use of organ transplantation and the introduction of anti-tumor necrosis factor alpha (anti-TNF-α) agents. However, the absolute number of cases of PCP is much lower in these patients, as prophylaxis appears to be more effective than in HIV infection, with an estimated risk reduction of 91% (105, 254). The PCP incidence in cancer patients without PCP prophylaxis varies by diagnosis (e.g., 22 to 45% for lymphomas and 25% for rhabdomyosarcomas) (125, 264). Hematopoietic stem cell transplantation and solid organ transplantation also increases the risk of PCP, and these patients are generally offered PCP prophylaxis (72, 75, 106, 325). Patients with collagen vascular disease have an elevated risk for PCP, particularly those with Wegener's granulomatosis (103, 235). Chronic corticosteroid use also increases PCP risk. Studies indicate that most patients who present with PCP had received a daily dose of corticosteroids equivalent to greater than 16 to 20 mg of prednisone for greater than 1 month (324). The recent development of TNF-α inhibitors has been associated with a risk of PCP of 0.4% to 0.23% in Japan and approximately 0.01% in the United States (143, 157, 286). The use of PCP prophylaxis in these patients is currently debated.

Mortality in the non-HIV-infected immunosuppressed population is generally higher than that in HIV infection. A study of 17 non-HIV-infected patients with PCP reported mortality of 53% (77). Mortality appears to be higher in non-HIV-infected PCP patients, possibly secondary to a greater pulmonary inflammatory response to the organism (179, 213). In one study of intensive care unit patients with PCP, mortality was 48% in non-HIV-infected patients, compared to 17% in those who were HIV infected (213). Another study of 30 non-HIV-infected PCP patients found an in-hospital mortality of 67% (80). Predictors of mortality in this population include development of a pneumothorax, delay in intubation, longer duration of mechanical ventilation, and higher initial illness severity based on APACHE scores (80, 85).

The $CD4^+$ cell count is less helpful in determining PCP risk in this population, although many patients do have low counts at the time of illness (77, 80). Chronic corticosteroid use is associated with increased risk, particularly in association with another immunosuppressive medication or condition (109, 157). In a case series of patients with rheumatoid arthritis receiving TNF-α inhibitors, patients who developed PCP were taking a second immunosuppressive agent and were more likely to be older and to have a comorbid condition (157). Many non-HIV-infected immunosuppressed patients are not receiving PCP prophylaxis at the time of their admission (80).

Non-HIV-infected patients with PCP typically present more acutely than those with HIV and PCP (159, 213, 324). They often are more hypoxemic and acutely ill (77, 159, 179). They also tend to be older than HIV-infected PCP patients (77, 213). Although non-HIV-infected individuals typically have lower organism burdens than seen in those with HIV, they have higher bronchoalveolar lavage (BAL) fluid neutrophilia and a greater inflammatory response (179, 213). Lactate dehydrogenase (LDH) is often elevated, but this is nondiagnostic (80). Chest radiographic appearance is similar to that in persons with HIV, and diffuse, bilateral infiltrates are common.

New Diagnostic Methods

Traditional methods. Because *Pneumocystis* cannot be cultured, diagnosis relies on visualization of the organism in respiratory samples, including induced sputum, BAL fluid, or lung tissue. Sputum induction and BAL are the most commonly used, although non-HIV-infected patients with PCP may require lung biopsy for diagnosis. Standard staining methods include methenamine silver, toluidine blue-O, Giemsa stain, or Diff-Quik. Monoclonal antibodies can be used to detect *Pneumocystis* with a rapid, sensitive, and easy-to-perform immunofluorescence assay (101, 158, 232).

These methods have several limitations. Many patients with PCP cannot tolerate sputum induction. BAL and lung biopsies are invasive. Also, histological detection of *Pneumocystis*, particularly in induced sputum, relies heavily on the expertise of the laboratory. In addition, techniques that do not require specialized equipment or expertise would aid in diagnosing PCP in low- and middle-income countries. For these reasons, there is growing interest in finding alternative, less invasive methods of detection (Table 1).

PCR. Assays using PCR have been applied to improve the diagnostic sensitivity of BAL fluid and induced sputum and to allow the use of noninvasive oral washes for diagnosis. These assays are based on detection of *Pneumocystis* DNA using amplification of various genetic loci. Sensitivity is increased by selecting either a multicopy gene target (such as the Msg or the mitochondrial large-subunit rRNA [mtLSU] gene) or by using a nested PCR that increases detection by employing two rounds of PCR (309, 310). The most commonly used PCR detects the multicopy mtLSU gene (222, 309, 310). In studies of BAL fluid, it has excellent sensitivity but less specificity, as the increased sensitivity of using a multicopy

TABLE 2 Treatment regimens for PCP[a]

Disease severity	First choice	Alternatives	Notes	Adjunctive corticosteroids
Mild-moderate	TMP-SMX (15–20 mg/kg TMP and 75–100 mg/kg p.o. per day, divided q8h, or 2 DS tabs p.o. q8h)	Dapsone (100 mg p.o. q.d.)-trimethoprim (15–20 mg/kg/day p.o. divided q6h), clindamycin (300–350 mg p.o. q6h–8h)-primaquine (15–30 mg p.o. q.d.), atovaquone (750 mg p.o. b.i.d.)	Renal dosing for TMP-SMX: with creatinine clearance of 15–30 ml/min, full daily dose divided every 12 h for 24–48 h, then decrease daily dose by 50% and give every 24 h; with creatinine clearance of <15 ml/min, full daily dose every 48 h; on hemodialysis, full daily dose before dialysis and 50% dose after dialysis. Dapsone: check glucose-6-phosphate dehydrogenase levels prior to starting dapsone.	Not indicated
Moderate-severe[b]	TMP-SMX (15–20 mg/kg TMP and 75–100 mg/kg i.v. per day, divided q6h or q8h)	Clindamycin-primaquine, pentamidine (3–4 mg/kg i.v. per day)	Renal dosing for pentamidine: with creatinine clearance of 10–50 ml/min, 3–4 mg/kg i.v. q24h–q36h; with creatinine clearance of <10 ml/min, 3–4 mg/kg i.v. q48h; on hemodialysis, 3–4 mg/kg i.v. q48h.	Prednisone (40 mg p.o. b.i.d. for 5 days, then 40 mg p.o. q.d. for 5 days, then 20 mg p.o. q.d. for 11 days), methylprednisolone i.v. at 75% of prednisone dose, start at time of antibiotic initiation or at least within 72 h

[a] Abbreviations: b.i.d., twice daily; i.v., intravenous; p.o., orally; q.d., every day; q8h, every 8 h; TMP-SMX, trimethoprim-sulfamethoxazole.
[b] Moderate to severe is defined as a pO_2 of <70 mm Hg or an alveolar-arterial oxygen gradient of >35 mm Hg while breathing room air.

gene detects patients who are colonized with *Pneumocystis* but do not have clinical pneumonia (10, 132). A recent study of real-time PCR at the heat shock protein 70 gene in BAL fluid from HIV-infected patients found that a cutoff of 10 copies/reaction had a sensitivity of 98% and specificity of 96% compared to routine staining (124). These performance characteristics were better than those for mtLSU PCR. Other loci such as the cdc2 gene are also under investigation in an attempt to maximize the sensitivity and specificity of these molecular assays (88, 319). PCR techniques have also been applied to analyses of oral washes (gargles) in an attempt to establish a noninvasive test for PCP (9, 83, 198). Quantitative touchdown PCR of the Msg locus in oral washes from HIV-infected patients has a sensitivity of 88% and a specificity of 85% (169). Certain characteristics of oral wash collection can improve the sensitivity, such as collecting the specimen prior to treatment initiation (169). PCR assays for *Pneumocystis* are not yet generally available clinically but are often used in research settings.

BG. (1→3)-β-D-Glucan (BG) is a polysaccharide that is present in the *Pneumocystis* cyst wall as well as in the walls of most fungi (81, 199). It triggers an innate immune response and can be detected in BAL and serum specimens from patients with PCP (81, 104). There has been recent interest in using serum BG levels as a noninvasive diagnostic test for PCP. Several studies have examined the performance characteristics of commercial BG assays and found a sensitivity ranging from 90 to 100% and a specificity of 88 to 96% in non-HIV-infected immunosuppressed patients with pneumonia, with various cutoffs used depending on the BG kit and the population (58, 64, 315). In a study of 111 HIV-infected PCP patients, median serum BG levels were significantly higher in PCP patients than in controls, and a cutoff of 23.2 pg/ml had a 78 to 98% sensitivity and a specificity of 77 to 94% (113, 128, 315). Although one study has found that BG levels correlate with organism burden, most others do not find a relationship of BG levels to organism burden, PCP severity, or response to therapy (58, 113, 315). Because BG is also increased in other fungal infections such as *Aspergillus*, it is most useful as an ancillary test in patients with a high suspicion for PCP.

AdoMet. *S*-Adenosylmethionine (AdoMet) is important for methylation reactions and polyamine synthesis in *Pneumocystis* (205). Because *Pneumocystis* does not synthesize AdoMet, it must scavenge the compound from the host (205); therefore, it has been postulated that low plasma AdoMet levels might be a marker for PCP (273). Two studies have found that low AdoMet levels are able to predict a diagnosis of PCP and that increases in AdoMet correspond with clinical improvement (273, 274). Another study of 21 non-HIV-infected patients with PCP and 10 patients with other causes of pneumonia did not find a relationship of AdoMet levels to PCP (58), and the test cannot currently be recommended for clinical use.

Treatment

Antimicrobials. Selection of an initial anti-*Pneumocystis* regimen depends on the severity of the patient's illness, other comorbid conditions, and the patient's ability to tolerate a specific agent (Table 2). Mild disease is defined as partial O_2 pressure (pO_2) of greater than 70 mm Hg while breathing room air and an alveolar-arterial gradient of less than 35 mm Hg (8, 140, 178). Trimethoprim-sulfamethoxazole (TMP-SMX) is the agent of choice for treating PCP of any severity, even if the disease has occurred while the agent for prophylaxis was being received (8, 178). It is

more effective than other regimens and equally effective as intravenous pentamidine, with fewer serious adverse reactions (140). For mild disease, oral TMP-SMX can be given at a dose of 2 double-strength tablets (160 mg TMP and 800 mg SMX) every 8 h. For more severe disease, intravenous therapy is preferred, with a dose of 15 to 20 mg/kg TMP and 75 to 100 mg/kg SMX divided every 6 to 8 h (140). Treatment can be switched to oral therapy once clinical stability has been attained and the patient can tolerate oral intake. Therapy is continued for 21 days, and there is no utility to repeated diagnostic testing since detection of the organism in respiratory samples does not indicate treatment failure. Many patients suffer treatment-limiting toxicities with TMP-SMX, including fever, rash, headache, pancytopenia, hyperkalemia, and renal dysfunction (140). Other complications include Stevens-Johnson syndrome and distributive shock. In general, if the reaction is mild, supportive care should be given with an attempt to continue treatment (140). More serious toxicity may necessitate switching to another agent. Adverse effects seem to be more common in persons with HIV, with studies suggesting that about 25% are unable to tolerate a full course of TMP-SMX (140).

If a patient with moderate to severe PCP is unable to tolerate TMP-SMX or if intravenous TMP-SMX is not available, alternative choices would be intravenous pentamidine or intravenous clindamycin plus oral primaquine (8, 140, 262, 289, 318). In less severe cases in patients with good oral absorbance, oral bactrim may be considered if intravenous bactrim is not available. Intravenous pentamidine is given at a dose of 4 mg/kg daily, although some studies suggest that a dose reduction to 3 mg/kg/day is effective and has fewer side effects (8, 42, 43, 140). Pentamidine also is associated with adverse side effects, many of which are serious. Renal dysfunction, hypo- and hyperglycemia, pancreatitis, or cardiac arrhythmias can occur. Clindamycin (600 to 900 mg intravenously every 6 to 8 h) with primaquine (15 to 30 mg base orally daily) is another alternative for moderate to severe disease. One study found that the combination was the most effective for PCP patients failing therapy, but no prospective trials have been conducted (20, 114). Atovaquone (750 mg orally given twice daily), dapsone (100 mg orally daily) plus TMP (15 mg/kg/day orally in three divided doses), and primaquine (15 to 30 mg base orally daily) plus clindamycin 300 to 450 mg orally every 6 to 8 h) are alternatives for mild disease (140).

There has been recent interest in the potential to use newer antifungal agents such as echinocandins for treatment or prophylaxis of *Pneumocystis*. These agents target BG synthesis. Case reports of PCP patients treated with echinocandins in addition to standard PCP therapy have reported mixed outcomes (5, 138). Animal studies have shown that echinocandins are active against the cystic form but not the trophic form (50, 131, 245, 263). In one study, infected mice treated with anidulafungin did not transmit PCP to other mice but were not cured of infection (50). Current data do not support use of these agents for PCP treatment or prophylaxis.

Corticosteroids. The widespread institution of adjunctive corticosteroids for moderate to severe PCP greatly improved outcomes from the disease. Randomized studies and a Cochrane database review demonstrated that glucocorticoids given to HIV-infected patients with a pO_2 of less than 70 mm Hg or an alveolar-arterial oxygen gradient of greater than 35 mm Hg while breathing room air improved survival (24, 105, 140, 178). The anti-inflammatory properties of the corticosteroids decrease the pulmonary inflammation that occurs in response to dead or dying organisms and prevent the clinical deterioration that is often seen after several days of treatment. Prednisone can be administered orally at a dose of 40 mg twice daily for 5 days, then 40 mg daily for 5 days, and then 20 mg daily for 11 days (8, 140, 178). Although few data exist to guide clinicians in the use of corticosteroids in patients without HIV, most physicians will add or increase corticosteroids in non-HIV-infected immunosuppressed patients with PCP.

Drug resistance. Although patients may worsen early during PCP treatment due to a transient inflammatory response to the organism, true treatment failure may also develop. Given the long-term use of single-agent prophylaxis for PCP, it seems possible that drug resistance might develop and decrease antibiotic effectiveness. *Pneumocystis* develops mutations in the DHPS gene with exposure to sulfamethoxazole or dapsone (144, 145, 190). Although drug resistance cannot be directly tested in *Pneumocystis*, similar mutations develop in bacteria after trimethoprim-sulfamethoxazole exposure and lead to antibiotic resistance (122). Several clinical studies have attempted to link DHPS mutations to treatment failure and poor outcomes in PCP, but the relationship is not entirely clear. Studies have found that DHPS mutations predict a higher 3-month mortality or increased risk of treatment failure (115, 144), but others have not replicated these results (45, 190, 225). Despite the theoretical concern for drug resistance, trimethoprim-sulfamethoxazole remains the treatment of choice even in those with previous sulfa exposure. If a patient does not improve clinically after 8 to 10 days of treatment, consideration should be given to switching antibiotics, but other causes of worsening, such as a new or additional infection, pulmonary edema, or pulmonary embolism, should be sought.

COLONIZATION

Definition

Pneumocystis colonization, in contrast to *Pneumocystis* infection, occurs in persons without signs or symptoms of acute pneumonia (29, 49, 222). Colonization can occasionally be detected using routing immunohistochemical staining of respiratory specimens, but because of the low organism burden associated with colonization, PCR-based techniques are most often necessary to determine the presence of the organism's DNA. Most commonly, nested PCR of the mtLSU locus is used, but other loci or real-time PCR may be employed. Colonization has been found in lung tissue samples, BAL fluid, expectorated and induced sputum, oral washes, and nasal aspirates. Interest in colonization as an important step in the life cycle of *Pneumocystis* and as an agent in other lung disease has increased, and current understanding of the epidemiology and clinical significance of colonization is discussed below.

Epidemiology

Colonization in animals. Colonization appears to occur naturally in both free-living and captive mammals. *Pneumocystis* has been detected in the lungs of many species of rodents, horses, and primates (167, 168, 218). In laboratory rodents, nested PCR of oral swabs has demonstrated that close to 100% of adult rats are colonized with *Pneumocystis* shortly after receipt (127). Similarly, spontaneous colonization occurs in both free-living and laboratory nonhuman primates (23, 44, 63). In a free-living macaque colony, all animals had detectable colonization at some point over

TABLE 3 Risk factors for *Pneumocystis* colonization

Risk factor
Associated medical conditions
Chronic lung disease, especially COPD
Pregnancy
HIV infection
Autoimmune disease
Young children, especially during upper respiratory infections
Malignancy
Organ transplantation
Medications
Corticosteroids
TNF-α inhibitors
Other immunosuppressives
Clinical risk factors
Low $CD4^+$ cell count
Cigarette smoking
Geographic location
History of recent PCP exposure
Lack of PCP prophylaxis

a 2-year observation period, and the duration of colonization averaged 2 months (63). In a study of laboratory macaques, over 95% had antibodies to *Pneumocystis*, and healthy animals became transiently colonized with *Pneumocystis* (153). When these animals were immunosuppressed after inoculation with simian-immunodeficiency virus (SIV), 60% developed spontaneous colonization when cohoused with *Pneumocystis*-infected animals. Colonization occurred despite prophylaxis with TMP-SMX. In another study of simian-human immunodeficiency (SHIV)-infected macaques, 68% of animals became colonized after immunosuppression (154).

Colonization in humans. The risk factors for colonization in humans are summarized in Table 3.

(i) Children. Children appear to have a higher prevalence of colonization than adults. Primary exposure to *Pneumocystis* likely occurs early and is widespread given the increasing anti-*Pneumocystis* antibody titers seen in the first few years of life (252, 295, 311). Colonization occurs in healthy children and is detectable both by PCR and, in some cases, by direct staining of organisms. It also appears to be common in children during upper respiratory infection. One study found that 32% of infants with mild respiratory symptoms were colonized with *Pneumocystis* (295). Another study of infants with bronchiolitis found that 24% were colonized (231). In another study of 60 children of HIV-infected mothers, 3.5% were *Pneumocystis* colonized, and those with colonization had upper respiratory symptoms (275). Other investigators have documented colonization in immunocompetent pediatric populations with either acute respiratory syndromes or chronic lung diseases (142, 170, 290). Although one study found a high proportion of colonization in infants dying from sudden infant death syndrome, this association has not been supported by subsequent studies (14, 298, 300).

(ii) Nonimmunosuppressed adults. Many studies have failed to find evidence of colonization in nonimmunosuppressed nonsmoking adults without chronic diseases. For example, one autopsy study of 15 nonimmunosuppressed adults did not find evidence of colonization, but that study did not use the more sensitive nested PCR (241). Other studies of BAL fluid, oral washes, nasal swabs, or induced sputa from healthy volunteers using nested PCR have also failed to find colonization (175, 198, 229, 301). More recent studies, however, have discovered colonization in about 20% of healthy subjects (202, 228). The amount and type of sample may affect the ability to detect colonization. One recent study of older adults found a colonization prevalence of 13% when examining only oral washes but of 23% when also including nasal swabs (297). Ponce et al. analyzed DNA from 3% of the weight of the right upper lobe of the lung in individuals undergoing autopsy in Santiago, Chile (244). They found that 65% of individuals were determined to be *Pneumocystis* colonized when this amount of lung tissue was used. It is difficult to compare these findings to other studies of normal subjects as this amount of lung tissue is not generally available, but the findings suggest that very low levels of colonization may be more common than previously thought.

(iii) Pregnant women. Pregnancy is associated with changes in the immune system that might predispose to colonization. One study examined *Pneumocystis* colonization in 33 third-trimester pregnant women and found that 16% were colonized based on nested PCR of deep nasal swabs. None of the 28 nonpregnant control women were colonized (301). Whether colonization is increased further in HIV-infected pregnant women or whether it plays a role in early acquisition of colonization and infection in infants is not currently known.

(iv) Immunosuppressed populations. Patients receiving immunosuppressive therapy for various medical disorders have an increased risk of colonization. In many cases, the underlying disease can also confer increased colonization susceptibility. For example, patients with lung transplantation are at risk of colonization. One study found that 9% of subjects were colonized after lung transplantation, and another series reported that 18% of symptomatic transplant patients were colonized despite use of prophylaxis (111; S. Saleh, personal communication). A recent study of patients with various autoimmune diseases examined induced sputum and found that 16% of patients were *Pneumocystis* colonized (204). Use of corticosteroids and a decreased lymphocyte count were independent predictors of colonization risk. Corticosteroid use has been linked to colonization in other populations. A study of subjects undergoing diagnostic bronchoscopy found that 44% of those using more than 20 mg per day of prednisolone were *Pneumocystis* colonized, compared to 12% who were not receiving corticosteroids (195). Another study confirmed this association in patients with bacterial pneumonia (116). Seventy-five percent of colonized subjects in this study had received corticosteroids. A lower $CD4^+$ lymphocyte count also seems to confer an increased risk. In one study, approximately 30% of subjects were colonized when the $CD4^+$ cell count was lower than 400 cells/μl or the $CD4^+/CD8^+$ T lymphocyte ratio was less than 1. With the recent increases in use of TNF-α inhibitors, it has been recognized that these patients have an increased risk of colonization. A recent study by Wissmann et al. of 78 patients with rheumatologic disease found that 25% were *Pneumocystis* colonized (320, 321). Use of infliximab for greater than 3 years and use of corticosteroids were risk factors for colonization (320, 321). Whether colonization indicates a need for prophylaxis in this patient group is currently unknown. Colonization has also been noted in patients with various chronic disease such as diabetes mellitus, sarcoidosis, and multiple myeloma (230).

(v) HIV-infected populations. *Pneumocystis* colonization is easily detectable in individuals with HIV infection. The range of colonization prevalence varies from 20 to 69% (107, 121, 174, 217, 230, 249, 285, 308, 310). This variability likely results from differences in the patient populations studied, the samples collected, and the use of different detection methods. A higher prevalence of colonization is more common in inpatients with pneumonia or in HIV-infected individuals dying from causes other than PCP (121, 217). The most recent study of colonization in HIV examined oropharyngeal washes from 20 HIV-infected teenagers without respiratory symptoms and found colonization in 20% (107). The relationship of colonization to $CD4^+$ cell count in HIV is debated. One study found that colonization increased from 10% in persons with a $CD4^+$ cell count of greater than 400 cells/μl to 40% in those with counts of less than 60 cells/μl (175). Other studies have not replicated this relationship, and it is clear that colonization does occur even in individuals with higher CD4 cell counts as well as those on antiretroviral therapy or anti-*Pneumocystis* prophylaxis (121, 217). Other risk factors for colonization in HIV include cigarette smoking and geographic location (217).

(vi) Patients with chronic lung diseases. *Pneumocystis* colonization can be found in individuals with various chronic lung diseases. The role of colonization in disease progression, particularly in chronic obstructive pulmonary disease (COPD), has been investigated and is discussed in detail below. The prevalence of colonization varies with the population and the underlying disease. For example, *Pneumocystis* colonization is detected in individuals with cystic fibrosis, and the prevalence ranges from 1% to 22% (90, 253, 270). Patients with interstitial lung diseases appear to have a higher prevalence of colonization, especially if they are receiving corticosteroids. In one series of patients with suspected interstitial lung disease, 34% were colonized, and similar to the case for the HIV-infected population, smokers were more likely to be colonized (303). One small study found that 100% of patients with small-cell lung cancer were *Pneumocystis* colonized, compared to 20% with non-small-cell lung cancer (55). A recent study found that 29% of patients with lung cancer, primarily non-small-cell lung cancer, were *Pneumocystis* colonized, so a specific relationship of colonization to small-cell lung cancer is unclear (59, 214). It has been noted that patients with COPD and chronic bronchitis have an increased prevalence of colonization as well. Calderon et al. have found that 41% to 100% of subjects with chronic bronchial disease have detectable colonization (28, 30). Other groups have found that *Pneumocystis* is detectable in 37% of patients with severe COPD and only 5% of those with mild disease. Others have not reported increases in colonization in COPD, but they included only those with mild disease (195).

Role in Disease

As described above, *Pneumocystis* colonization occurs in many populations. Colonization is likely more than just an interesting phenomenon, as it has several important clinical implications. It may be involved in the development or transmission of disease, suggesting possible utility of treatment of colonization or of respiratory isolation of patients. It may also play a role in development or progression of various lung diseases, particularly COPD. The importance of colonization has been an increasing focus of research in the last several years.

Development and transmission of PCP. A period of colonization most likely precedes the development of acute PCP, but the duration of this colonization and the efficacy of treating colonization to prevent disease are unknown. Small studies have documented development of PCP after detection of colonization by PCR (107). For example, a study of BAL fluid found that 9 of 96 lung transplant recipients were *Pneumocystis* colonized and that the one case of PCP that developed during the study was in a previously colonized individual. Other studies have found disease following colonization, usually with *Pneumocystis* with the same genotype. *Pneumocystis*-colonized individuals might also serve as a reservoir for disease transmission. As discussed above, colonized animals can transmit colonization to other animals and can also transmit disease to immunocompromised hosts (4, 99). Evidence of person-to-person transmission of PCP as well as detection of colonization in health care workers suggests that the organism might pass through a colonized host before causing disease (56, 57, 94, 176, 186, 215, 288).

COPD. The increased prevalence of *Pneumocystis* colonization in those with COPD as described above has led to an interest in understanding its role in the disease. It is possible that *Pneumocystis* colonization in COPD represents an epiphenomenon secondary to structural lung damage, immunosuppression from corticosteroids, or increased risk from cigarette smoking, but a series of animal and human studies suggest that the organism might actually be linked to disease in some individuals. Studies in a non-HIV-infected population have demonstrated that *Pneumocystis* colonization is a risk factor for more severe COPD, independent of smoking history or corticosteroid use (220). In an HIV-infected population, *Pneumocystis* colonization was associated with an increased risk of airway obstruction (odds ratio of 8.8) (216). This effect was seen even with adjustment for smoking history. In addition, *Pneumocystis* colonization was associated with increased lung levels of matrix metalloprotease 12, a protease that has been implicated in COPD pathogenesis (41, 82, 212, 224). Others have found that *Pneumocystis* colonization in COPD is associated with an increased systemic inflammatory response, including higher peripheral lymphocyte counts and increases in interleukin-6 (IL-6), IL-8, and TNF-α (294), suggesting that the *Pneumocystis* is not just an innocent bystander in the lung but is provoking an immune reaction (31).

Although human studies can suggest a role of *Pneumocystis* in COPD, it is difficult to prove causation in these types of investigations. Animal studies can prospectively define the role of *Pneumocystis* in COPD, and two studies have demonstrated directly that *Pneumocystis* colonization is linked to subsequent COPD development. A rat model of *Pneumocystis* colonization in nonimmunosuppressed rodents demonstrated that animals exposed to *Pneumocystis* and cigarette smoke had increased physiologic and anatomic changes of emphysema compared to animals exposed to either alone (40). Another study in nonhuman primates infected with humanized SHIV found that animals spontaneously developed *Pneumocystis* colonization (268). Colonized animals demonstrated airflow obstruction, increases in radiographic emphysema, and lung airspace enlargement.

IMMUNE RESPONSE TO *PNEUMOCYSTIS*

T Cells

$CD4^+$ cells. The adaptive host response to *Pneumocystis* infection involves humoral and cellular immune responses, as well as alveolar macrophages, dendritic cells, neutrophils, and cytokines

working in concert to promote clearance of infection. The central role of $CD4^+$ T cells in controlling *Pneumocystis* infection has been well established and is most clearly evident in the strong correlation between diminished peripheral blood $CD4^+$ T cell numbers in HIV-infected individuals and an increased risk of development of PCP (242). In studies of HIV-infected individuals and in experimental murine models of $CD4^+$ T cell depletion, peripheral blood $CD4^+$ T cell counts of below 200 cells/μl are associated with development of PCP (242). The introduction of ART and successful reconstitution of $CD4^+$ T cell levels have dramatically decreased the frequency of PCP in HIV-infected individuals, further demonstrating the importance of $CD4^+$ T cell levels in prevention and control of *Pneumocystis* infection (141). Others have shown a similar correlation between CD4+T cell counts and susceptibility to PCP in non-HIV-infected patients receiving immunosuppressive therapy after transplantation or following chemotherapy for malignancy (193).

While the level of $CD4^+$ T cells clearly correlates with control of *Pneumocystis* infection, the mechanism by which these cells contribute to clearance of the organism is less clear. Several studies have sought to examine the relative contributions of T helper subsets in the control of *Pneumocystis* infection. Shellito et al. examined recruitment of T helper 1 and 2 lymphocytes to the lungs following experimental *Pneumocystis* infection in mice and found that both subsets were increased in the lungs following infection; however, there was an increased frequency of Th2 cells (267). Similarly, a mixed Th1 and Th2 response was observed early after *Pneumocystis* infection in a SHIV-infected macaque model (268). In these studies, SHIV-infected macaques that became colonized with *Pneumocystis* had increased levels of Th2 cytokines (IL-4, IL-5, and IL-13) in BAL fluid compared to monkeys infected with SHIV alone. Gamma interferon (IFN-γ) and TNF-α were only transiently increased in the *Pneumocystis*-colonized monkeys, and this increase occurred at later time points during the infection.

Studies focused on T cell costimulatory signaling, which is critical for optimal T cell function, have demonstrated that mice deficient in costimulatory molecules CD2 and CD28 develop PCP despite normal T cell numbers (15). These mice showed decreased antibody titers and dysregulated cytokine responses, particularly in IL-10 and IFN-γ expression. Interestingly, mice deficient in CD28 alone were susceptible to acute *Pneumocystis* infection, with an influx of naïve $CD8^+$ T cells into the lungs, but eventually cleared the infection. Depletion of $CD8^+$ T cells did not result in an increased organism burden, suggesting that these cells did not contribute to control of the infection (255). Together, these studies emphasize the requirement for optimal $CD4^+$ T cell signaling for early control of *Pneumocystis* infection.

In addition to studies of the relative contributions of Th1 and Th2 cells, recent studies have begun to explore the role of T helper 17 $CD4^+$ T cells in *Pneumocystis* infection. Th17 T cells are distinguished from other T helper cells by expression of IL-17, IL-21, and IL-22, while IL-23 is necessary to maintain this subset (71). These cytokines have been shown to play an important role in the effector response to extracellular pathogens, particularly at mucosal sites (71). In an examination of the role of the IL-17/IL-23 axis in experimental models of *Pneumocystis* infection, Rudner and colleagues determined that *Pneumocystis* exposure induced IL-23 expression by alveolar macrophages and that the IL-23 response was required for optimal IL-17 production during the infection (259). Furthermore, the importance of the IL-17/IL-23 cytokine response was demonstrated in studies showing that protection from infection was impaired in IL-23 knockout mice (259).

$CD8^+$ cells. While the central role of $CD4^+$ T cells in control of *Pneumocystis* infection is clear, the contribution of $CD8^+$ T lymphocytes in protection is more controversial, particularly in the context of $CD4^+$ T cell depletion. In experimental *Pneumocystis* infection of immunocompetent mice, both $CD4^+$ and $CD8^+$ T cells accumulate in the lung and the infection is controlled (16). Unlike the exquisite sensitivity of $CD4^+$ T cell knockout mice to *Pneumocystis* infection (19), β_2-microglobulin knockout mice (lacking $CD8^+$ T cells) are not susceptible to *Pneumocystis* (108). Additionally, in mice selectively depleted of $CD4^+$ T cells, infection with *Pneumocystis* leads to an influx of $CD8^+$ T cells in the lung, though the mice fail to control the infection (18). Further studies of T cell reconstitution in severe combined immunodeficiency (SCID) mice using splenocytes depleted of either $CD4^+$ T cells or $CD8^+$ T cells also failed to show a positive effect of $CD8^+$ T cells on protection from infection (257). In a model of HIV and *Pneumocystis* coinfection, we have shown that in simian immunodeficiency virus (SIV)-infected primates, *Pneumocystis* infection resulted in significant accumulation of $CD8^+$ T cells in BAL fluid compared to that in animals infected with SIV alone; however, the dramatic influx of $CD8^+$ T cells to the lungs in this model did not appear to be sufficient for clearance of *Pneumocystis* (44).

In contrast to the above studies, lines of evidence exist suggesting that $CD8^+$ T cells may contribute to a protective response against *Pneumocystis* infection, particularly in the context of immunosuppression (17). Evidence supporting a protective role of $CD8^+$ T cells is provided by studies in a murine model of *Pneumocystis* infection with selective depletion of either $CD4^+$ T cells, $CD8^+$ T cells, or both. While mice depleted of $CD8^+$ T cells alone were not susceptible to infection, mice depleted of both cell types had a higher organism burden than those depleted of $CD4^+$ T cells alone, suggesting at least partial protection in the absence of $CD4^+$ T cells (17).

In more detailed studies that also examined T cell function, Kolls et al. demonstrated that delivery of IFN-γ to the lungs resulted in control of *Pneumocystis* infection in a $CD4^+$ T cell-depleted murine model and that the protective effect was associated with increased $CD8^+$ T cell recruitment (156). Further studies demonstrated that the protective effect was conferred by T cytotoxic type 1 $CD8^+$ T cells and that these cells promoted enhanced macrophage-mediated killing of *Pneumocystis in vitro*, whereas T cytotoxic type 2 cells promoted lung pathology (200).

Taken together, these studies suggest that the differences in experimental models, i.e., reconstitution, depletion, or genetic alterations, may contribute to the disparate results summarized above. In addition, the studies of Kolls and colleagues that delineated the role of $CD8^+$ T cell subsets in protection versus pathology further explain some of the discrepancies of earlier studies (200).

$\gamma\delta$ cells. The role of gamma-delta ($\gamma\delta$) T cells in *Pneumocystis* infection has not been as extensively examined; however, studies suggest that they may play a role in controlling inflammation and pulmonary injury associated with infection and possibly a protective role in the context of diminished alpha-beta T cells. In clinical studies, $\gamma\delta$ T cells were increased in blood and BAL fluid specimens from HIV-infected individuals with PCP (3, 136). In murine models of *Pneumocystis* infection, early studies suggested that $\gamma\delta$ T cell-deficient mice were not susceptible to progressive *Pneumocys-*

tis infection, while other studies indicated that these cells may play a role in control of infection in alpha-beta T cell-deficient models (3). Steele and colleagues demonstrated that γδ T cells accumulate in the lungs of immunocompetent mice in response to *Pneumocystis* infection. Interestingly, in γδ T cell-deficient mice, resolution of infection was improved and correlated with increased $CD8^+$ T cell recruitment and IFN-γ production (278). These results suggest that one role of γδ T cells may be to influence recruitment of $CD8^+$ T cells to the lungs and possibly modulate their inflammatory effect.

B Cells

In addition to the profound loss of $CD4^+$ T cells, HIV infection results in significant loss and derangement of the B cell compartment (211). As such, alterations in both T and B cell numbers and effector capabilities may play a critical role in the increased susceptibility of HIV-infected individuals to the development of PCP. The importance of B cells in protection from *Pneumocystis* infection is evident from experimental animal models of B cell deficiencies (183, 184, 191, 194), as well as clinical studies where PCP has been reported following B cell-targeted chemotherapy (126, 164). The B cell effector mechanisms that contribute to control of *Pneumocystis* infection have been extensively investigated, and these studies indicate that both antibody production and activation of $CD4^+$ T cells are important components of a protective response.

Role of antibodies in protection. Reports of PCP in patients with genetic mutations affecting immunoglobulin production (207), along with the observations that the majority of healthy adults are seropositive and rarely present with *Pneumocystis* infection, provide strong evidence that anti-*Pneumocystis* humoral immunity is a critical component of protective immunity to *Pneumocystis*. Several studies of antibody responses following exposure of normal animals to *Pneumocystis* also support the correlation between a rise in anti-*Pneumocystis* antibody titers and prevention of overt infection (89, 153). Direct evidence for the role of antibodies in protection from *Pneumocystis* infection has come from experimental infections in animal models, which can help to distinguish the role of antibodies from other B cell effector functions. In early studies of corticosteroid-treated rats, Walzer and Rutledge demonstrated that tapering of steroid treatment in *Pneumocystis*-infected rats resulted in increased *Pneumocystis* antibody titers and a reduction in organism burden (314). More direct evidence of antibody-mediated protection was reported by Gigliotti and Hughes, who showed that passive transfer of a *Pneumocystis*-specific monoclonal antibody promoted clearance of *Pneumocystis* in corticosteroid-immunosuppressed rats and ferrets (100). These results were further supported by the studies in which passive transfer of hyperimmune *Pneumocystis* antisera or monoclonal antibodies conferred protection in murine models of PCP (12, 97, 258).

In addition to the role of class-switched anti-*Pneumocystis* antibodies in protection, recent studies have explored the role of natural immunoglobulin in control of *Pneumocystis* infection (251). Natural antibodies, predominantly IgM, are produce by the B-1 subset of B cells and are generated without exogenous antigenic stimulation; thus, they are thought to contribute to an early defense system against a wide range of common pathogens. The specific role of natural IgM antibodies that recognize common fungal cell wall carbohydrate antigens was recently examined in murine models of *Pneumocystis* infection (251). Rapaka and colleagues showed that these antibodies are conserved across many species and contribute to early host defense against *Pneumocystis* infection (251). Natural IgM antibodies influence recognition of fungal antigens by dendritic cells (DC), enhancing DC maturation and migration to the draining lymph nodes. Additionally, these studies showed that natural IgM appear to be required for the adaptive Th2 response, Th17 cell differentiation, and immunoglobulin class switching. Given that Th2 responses appear to be an important component of the protective response to *Pneumocystis* infection (91, 267) and the emerging evidence of a role for IL-17 in control of *Pneumocystis* infection (259), it may be that early IgM responses influence the T helper environment following exposure of *Pneumocystis* in the local draining lymph nodes and promote the development of a protective, adaptive response.

Active immunization and humoral immunity. Antibody-mediated protection against *Pneumocystis* infection has been demonstrated by both passive and active immunization in experimental models. While studies of passive immunization have clearly demonstrated the importance of antibodies in mediating protection (12, 97, 258), the potential for active immunization for the purpose of vaccine development has also been pursued. In studies that supported the role of antibody-mediated protection as a result of active immunization, Harmsen et al. showed that immunization of normal mice with whole *Pneumocystis* organisms induced high-titer antibodies and that following $CD4^+$ T cell depletion, mice were protected against *Pneumocystis* challenge infection (110). Likewise, following intranasal immunization with a *Pneumocystis* crude antigen preparation, Pascale et al. demonstrated protection from *Pneumocystis* challenge in $CD4^+$ T cell-depleted mice (239).

While these studies suggest the feasibility of immunization, there remains a need to define specific protective antigens in order for *Pneumocystis* vaccine development to progress. Msg, which is highly immunogenic, is not a suitable candidate for protective immunization, due to extensive antigenic variability (280). In an effort to identify other potentially protective antigens, Gigliotti and colleagues examined the reactivity of murine hybridomas derived from draining lymph nodes of mice following resolution of infection (96). In contrast to the findings from studies of systemic antibody specificity, the majority of these hybridomas reacted to antigens other than Msg, suggesting a clear difference between local and systemic humoral responses. Studies of a specific monoclonal antibody that was protective upon passive administration led to the identification of the *Pneumocystis* Kex and a related antigen as potential vaccine candidates (96, 173). Wells et al. further demonstrated that immunization with the recombinant Kex-related protein A12 reduced the organism burden in mice following $CD4^+$ T cell depletion and *Pneumocystis* challenge (316). Additionally, CD4-deficient mice immunized with DNA vaccine constructs encoding *Pneumocystis* Kex and CD40L have a reduced *Pneumocystis* burden in a *Pneumocystis* challenge model compared to mice immunized with Kex alone. Immunized mice produced high anti-*Pneumocystis* IgG titers and opsonic antibodies that promoted *Pneumocystis* killing in an *in vitro* assay (328).

We have reported evidence that *Pneumocystis* Kex or Kex-related antigens may play a role in protective immunity against *Pneumocystis* infection in a nonhuman primate model of HIV and *Pneumocystis* coinfection (153, 154). In these studies, we found that a baseline anti-Kex plasma IgG titer of >1:12,500 in healthy macaques correlated with protection from *Pneumocystis* infection

following SHIV immunosuppression and *Pneumocystis* exposure challenge (154). In contrast, monkeys with baseline titers of <1:3200 were susceptible to *Pneumocystis* colonization following SHIV infection. A further association between BAL fluid Kex-specific IgA and prevention of colonization was also noted (154). Although $CD4^+$ T cells and viremia levels were comparable in the *Pneumocystis*-positive and *Pneumocystis*-negative groups throughout the study, plasma anti-Kex antibodies remained elevated in the high-baseline anti-Kex antibody group, along with earlier detection of class-switched anti-Kex antibodies in BAL fluid, compared to the low-baseline-titer group. These results suggest that B cell responses in the SHIV-infected macaques were adequate despite substantial losses in the $CD4^+$ T cell compartment and that T cell help for antibody production may be derived from alternative sources under these conditions. Taken together, these studies support the concept that protection from *Pneumocystis* infection may be induced by active immunization and that strategies to develop this response in the context of low $CD4^+$ T cell help, such as in HIV-infected or other immunosuppressed individuals, may be feasible.

B cells and $CD4^+$ T cell activation. While *Pneumocystis*-specific antibodies promote protection against *Pneumocystis* infection, B cells also have a critical role in T cell priming. In a series of studies to examine the B cell-T cell interaction, Lund et al. investigated $CD4^+$ T cell responses to *Pneumocystis* using bone marrow chimeric mice that express major histocompatibility complex (MHC) class II on all antigen-presenting cells (wild-type chimeras) and bone marrow chimeric mice that express MHC class II on all antigen-presenting cells except B cells. They found that although *Pneumocystis* was rapidly cleared by wild-type chimeric mice, organism levels remained high in chimeric mice that lacked MHC class II on B cells. In addition, the number of activated $CD4^+$ T cells in the lungs of these mice was reduced relative to the number in the lungs of wild-type chimeric mice. In adoptive transfer studies, they further demonstrated that SCID mice that received $CD4^+$ T cells from normal mice were able to clear *Pneumocystis* infection more efficiently than mice that received CD4 cells from B cell-deficient mice. Together, these studies indicate that B cells play a critical role in priming and activation of $CD4^+$ T cells in response to *Pneumocystis* infection and that this interaction is required for clearance of *Pneumocystis* in the lungs (183).

Macrophages

The interaction of *Pneumocystis* with alveolar macrophages (AMs) has been the focus of intense research (70, 76, 130, 135, 227, 277). AMs are the principal phagocytic cells of the lower respiratory tract, and when stimulated by inflammatory cytokines such as IFN-γ, they are the primary cell type responsible for uptake and killing of respiratory pathogens, such as *Pneumocystis*. AMs promote direct killing of *Pneumocystis* trophozoites and cysts and in addition play a central role in directing the inflammatory and adaptive immune responses to this organism.

Mechanisms and consequences of attachment and phagocytosis. Several host proteins and receptors have been implicated in the uptake and clearance of this organism, and the specific interactions between *Pneumocystis* and host proteins and downstream effects on the inflammatory response have been under investigation. As is the case with other fungal organisms, the *Pneumocystis* cell wall contains highly mannosylated glycoproteins, principally the major surface glycoprotein described above. The interaction of *Pneumocystis* with the host mannose receptor (MR) on AMs was first reported by Ezekowitz et al., and the importance of this mechanism of uptake was revealed in studies of the interaction of *Pneumocystis* with AMs from HIV-infected patients, where phagocytosis of *Pneumocystis* was significantly diminished compared to that with cells from healthy controls (78, 162). Subsequent studies revealed that *Pneumocystis* induced shedding of MR from AMs, and coating of *Pneumocystis* with soluble MR inhibited phagocytosis in experimental murine models (87). Interestingly, *in vivo* studies in normal mice did not reveal a downregulation of surface MR in response to *Pneumocystis* infection, and MR knockout mice depleted of $CD4^+$ T cells did not have a fungal burden significantly different from that of wild-type mice (284). These results suggest that innate effector mechanisms involving phagocytosis may be redundant and that the opsonic effect of other molecules may compensate for lack of MR.

In addition to mannose, β-glucan moieties are important components of fungal cell walls that are involved in induction of host inflammatory mediators and interactions with host receptors such as complement receptor 3 and dectin 1 (25, 256). *In vitro* studies have shown that dectin 1 mediates uptake and killing of *Pneumocystis* associated with the generation of reactive oxygen species (277). Dectin 1 knockout mice are more susceptible to *Pneumocystis* infection than wild-type mice, and macrophages from knockout mice also showed impaired production of reactive oxygen species (261).

Additional pattern recognition molecules such as scavenger receptor A and Toll-like receptors 2 and 4 on AMs influence the inflammatory response to *Pneumocystis*, where the principal effect is regulation of cytokine production (66, 118, 326, 327) (discussed below). Other studies have shown that osteopontin, which participates in innate immune responses to other infectious agents, is highly upregulated in AMs of *Pneumocystis*-infected immunosuppressed rats (37). Recently, Inoue et al. have shown that the intracellular isoform of osteopontin is involved as an adaptor molecule in the collaboration of Toll-like receptor 2, dectin 1, and mannose receptor following interaction of *Pneumocystis* with AMs (129). Additional studies demonstrated a role for intracellular osteopontin in enhancement of phagocytosis and clearance of *Pneumocystis* (129).

Inflammatory responses. The interaction between *Pneumocystis* and AMs leads to the induction of a variety of inflammatory mediators that influence eradication of this organism by the host (21, 33, 117, 155, 189, 196). While upregulation of inflammatory mediators is critical to control of the infection, several studies have examined the role that these response elements play in the progression of lung injury during the course of *Pneumocystis* infection. Understanding the nature of the inflammatory response to *Pneumocystis*, particularly the interaction of *Pneumocystis* with AMs, is important because there is strong evidence that pulmonary impairment and mortality are more closely associated with lung inflammation than organism burden (179, 323).

Several studies have examined the effects of interaction of *Pneumocystis* with AMs in culture and animal models (33, 37, 78, 117, 302). As the epithelial lining fluid of the alveolar spaces is a complex milieu of proteins and other molecules, the association of *Pneumocystis* with molecules such as vitronectin, fibronectin, surfactants, immunoglobulin, and complement components and the interaction of the organism with alveolar macrophages are likely to be more complex than can be represented with *in vitro* culture

systems. Downstream events such as cytokine and chemokine regulation and other cellular responses are likely to be interconnected and overlapping and to have a significant influence on control of infection. These studies are further complicated by alterations in the lung environment associated with changes in immune status (immunocompetent versus immunosuppressed) and the presence of coinfection (e.g., HIV). Comprehensive evaluations of the effects of *Pneumocystis* on gene expression of AMs and lung tissue are beginning to address the complexity of the host-pathogen interaction (37, 248). In this regard, in order to assess the influence of *Pneumocystis* in the lung in the association with SIV infection, Qin et al. evaluated cytokine and chemokine gene expression changes in lung tissue in SIV-infected macaques with and without PCP (248). The most upregulated immune genes were found to be those for IFN-γ, IFN-γ-inducible CXCR3, CCR5 ligands, and their cognate chemokine receptors. Changes were greatest in monkeys with SIV and *Pneumocystis* coinfection. These studies are important because they identify specific inflammatory pathways in models of both HIV and PCP and have the potential to identify and test the effects of therapeutic agents that target these pathways and which may be useful in the specific control or interruption of potentially damaging inflammatory responses in the lung.

Mechanisms of *Pneumocystis* killing. Several studies have demonstrated that *Pneumocystis* is readily bound and internalized by normal AMs and that following internalization and phagolysosomal formation, it is degraded by induction of the oxidative burst and hydrogen peroxide generation (119, 196, 277). Studies using rodent models of *Pneumocystis* infection have shown a correlation of AM numbers and control of *Pneumocystis* in the lungs (119, 171). The importance of reactive oxygen species produced by AMs is supported by studies showing that AMs from HIV-infected individuals with $CD4^+$ T cells counts of less than 200 cells/μl had reduced hydrogen peroxide production compared to those from HIV-uninfected individuals, and the effect was most pronounced in patients with PCP (163). Generation of nitric oxide and reactive nitrogen intermediates (RNI) by IFN-γ- and TNF-α-activated macrophages has also been shown to promote killing of *Pneumocystis* (70, 266). As these cytokines are produced by lymphocytes and lymphocyte-activated macrophages, the potential for reduced RNI production in HIV-infected persons with low $CD4^+$ T cell levels may also contribute to increased susceptibility to *Pneumocystis* infection.

CONCLUSIONS

Pneumocystis continues to be an important pathogen in both HIV-infected and non-HIV-infected immunosuppressed populations. The epidemiology of PCP has shifted over time with advances in HIV care, changes in critical care medicine, and development of new immunosuppressive medications. Future work on improving therapy and prevention is needed for this disease. Understanding of the immune response to *Pneumocystis* is also increasing and may offer potential areas for vaccine development or immunomodulatory therapy. The understanding and recognition of the importance of *Pneumocystis* colonization have increased substantially over the past several years, with studies suggesting that transmission of *Pneumocystis* occurs and may be important for development of disease, that certain risk factors increase colonization risk, and that *Pneumocystis* colonization might play a role in the development and progression of chronic lung disease such as COPD.

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