Quantitation of Anti-\textit{Pneumocystis jiroveci} Antibodies in Healthy Persons and Immunocompromised Patients

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To facilitate studies of the epidemiology of \textit{Pneumocystis jiroveci} infection in both healthy persons and immunocompromised patients, we developed a quantitative ELISA with recombinant major surface glycoprotein (MSG) fragment MSG-14, a \textit{P. jiroveci}-specific protein that includes a highly conserved region of the MSG protein family. By immunoblot, all samples reacted with the carboxyl portion of MSG-14; by ELISA, immunocompromised patients with \textit{Pneumocystis} pneumonia (PCP) who were immunocompromised for reasons other than AIDS had higher antibody levels than did either patients with AIDS with PCP ($P = .01$) or healthy persons ($P = .005$). Longitudinal observations of 8 patients with AIDS showed no correlation between time of diagnosis of \textit{Pneumocystis} infection and change in antibody levels. Eleven percent (4/35) of healthy persons demonstrated a $>4$-fold change in antibody titers during 1 year of observation. This ELISA assay allows quantitation of anti-\textit{P. jiroveci} antibodies in human serum samples and should be useful in better understanding the epidemiology of \textit{P. jiroveci} infection in humans.

\textit{Pneumocystis jiroveci} causes \textit{Pneumocystis} pneumonia (PCP) in immunocompromised persons, including patients with AIDS, transplant recipients, and malnourished infants. Serological studies suggest that a high proportion of healthy, nonimmunocompromised persons have been infected with \textit{P. jiroveci} at an early age [1]. A better understanding of the reservoir and the mode of transmission of human infection, as well as the course of infection in healthy and immunocompromised persons, should allow development of strategies to prevent or better manage disease in immunocompromised patients. However, the epidemiology and natural history of \textit{P. jiroveci} infection in healthy persons, as well as in immunocompromised patients, remain poorly understood, in large part because of the lack of broadly available \textit{P. jiroveci}-specific reagents.

In the past, because of the difficulties in obtaining \textit{P. jiroveci}-specific antigens, antigens derived from \textit{Pneumocystis}-infected rats (\textit{Pneumocystis carinii}) and mice (\textit{P. carinii f. sp. muris}) were frequently used for studies of human immune responses to \textit{P. jiroveci} [2–5]. However, such an approach can lead to potentially erroneous results, because each species of \textit{Pneumocystis} is antigenically and molecularly distinct. \textit{P. jiroveci} antigens have also been used in serological studies [1, 6–8] but are available in limited quantities and may be contaminated by host proteins.

Recombinant technologies have the potential to reproducibly provide defined proteins in quantities sufficient to overcome the above limitations. To date, the major surface glycoprotein (MSG or gpA) is the only antigen of \textit{P. jiroveci} that has been well characterized [9–11]. MSG is encoded by a multicopy family of related but unique genes that presumably allow \textit{Pneumocystis} to evade host immune responses by use of antigenic vari-
ability. Although multiple MSG variant genes of P. jiroveci have been cloned, expression of full-length MSG has been very difficult [12, 13]. In a recent study, immunoblotting was used to detect serum antibodies against a chimeric full-length recombinant P. jiroveci MSG that was expressed in 3 overlapping fragments [12]. However, immunoblotting does not provide quantification, making it difficult to compare antibody responses and to determine changes in such responses over time. In the present study, we have expressed a recombinant P. jiroveci MSG in 2 fragments and have developed an ELISA to quantitate antibodies in the serum samples of both healthy and immunocompromised persons.

**PATIENTS, MATERIALS, AND METHODS**

**Patients.** Serum samples were primarily obtained before 1990 and were stored at −70°C. Samples were obtained from 45 patients with AIDS, 39 of whom had a history of PCP. Serial samples were available from 8 of the latter patients. Of the 45 patients with AIDS, CD4 cell counts were available for 22 (range, 0–789 cells/μL; median, 25 cells/μL; mean, 78 cells/μL). Plasma human immunodeficiency virus (HIV) loads were unavailable for these patients. We also obtained serum samples from 20 patients immunocompromised for reasons other than AIDS, of whom 16 had a history of PCP, and from 53 healthy persons. Serial samples were available from 35 of the latter subjects.

**Protein expression and purification.** HuMSG14 (GenBank accession no. AF033209 [13]) was used as a polymerase chain reaction template, to generate 2 fragments, HuMSG-14amino (MSG-14Am; bases 1–1800) and HuMSG-14carboxy (MSG-14Ca; bases 1801–3030), which were cloned into pET32a(+) expression vector (Novagen). PET 32a(+) encodes a 6X His tag at the amino terminus that permits rapid purification of the recombinant protein by use of a nickel column. Recombinant p11 [14] cloned into pET 32 was used as a negative control. The sequences of all plasmids were confirmed by automated sequencing.

Plasmids were transformed into Escherichia coli BL21-Codon-Plus (DE3)-RIL cells (Stratagene) and were induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 30°C. Recombinant HuMSG-14 fragments were partially purified by solubilizing organisms in 0.01 M Tris-HCl, 0.1 M NaH₂PO₄, 20 mM 2-mercaptoethanol, 10 mM imidazole, and 10% glycerol; the partially purified fragments were then pelleted, and the supernatant was discarded. The pellets, containing HuMSG-14 fragments, were solubilized by sonication in the same buffer plus 6 M guanidine. Recombinant p11 protein (p11) and the peptide encoded by pET32a with no insert (pET32a) were both soluble under nondenaturing conditions. Recombinant proteins were purified by nickel–nitrilotriacetic acid agarose, according to the manufacturer’s instructions (Qiagen), by use of 50 mM NaH₂PO₄, 300 mM NaCl, 20 mM 2-mercaptoethanol, 40 or 100 mM imidazole, and 10% glycerol for elution.

**Western blotting.** Western blotting was performed, as described elsewhere [15], with human serum samples (1:100) blocked with puriﬁed pET32 protein (to minimize nonspeciﬁc reactivity) or penta-His antibody (Qiagen) (as primary antibodies).

**Pneumocystis ELISA.** ELISAs were performed, as described elsewhere, with either ~10 μg of MSG-14Ca/well or 7 μg of p11/well of a 96-well Nunc Immunomodule plate (Nalge Nunc) [15]. Penta-His antibody was used to ensure that approximately equimolar amounts of antigen had bound to the plate. Human serum samples (1:100) were blocked with a 1:20 dilution of pET32 crude solubilized peptide. Each sample was run in duplicate with both MSG-14Ca and p11, and the difference in mean optical densities was calculated. To quantitate results, a standard curve was generated by use of serial dilutions of a mixture of 5 serum samples with high levels of anti–MSG-14Ca antibodies and was plotted by a 4-parameter graph model with Softmax Pro Software (Molecular Devices). We assigned 100 U to a 1:100 dilution of the standard. Samples with high quantities (>50 U) of anti–MSG-14Ca antibodies were diluted to obtain a value on the linear portion of the standard curve, a value that was then multiplied by the dilution factor, to obtain the final result. Serial samples from a single patient were run on the same day, on a single plate. Penta-His antibody (Qiagen) and a sample with known high-level reactivity were included in each run, to ensure that adequate antigen had bound to the plate. Intraday and interday variability were ~15% and 20%–25%, respectively.

**Cytomegalovirus (CMV) ELISA.** CMV IgG ELISAs were run according to manufacturer’s instructions (Zeus Scientific).

**Statistical analysis.** Antibody levels determined by ELISA were analyzed by a 2-tailed unpaired t test, to determine significant differences among the different populations.

**RESULTS**

MSG-14 was successfully expressed in 2 fragments, in a bacterial expression system. To examine the speciﬁcity of the recombinant products, rabbit serum from a rabbit immunized with a synthetic peptide, EVFKKNIKASYIIEF, corresponding to aa 634–648 in HuMSG-14, was used in immunoblot studies. As expected, this serum reacted with the MSG-14Ca protein but not with either MSG-14Am or p11 (data not shown). We then used immunoblotting to examine reactivity of human serum samples with the recombinant proteins. Antibodies against MSG-14Am were detected in 14 of 32 human serum samples and against MSG-14Ca in 52 of 52 samples (figure 1). Of note,
many samples showed very weak, but clearly positive, reactivity with MSG-14Ca.

Because preliminary studies showed that virtually no samples reacted exclusively with MSG-14Am, we developed an ELISA that used the MSG-14Ca fragment, and we examined reactivity of a variety of human serum samples (figure 2A). For 53 healthy persons, the median antibody level was 4 U (range, 1–438 U); for 39 patients with AIDS with PCP, the median antibody level was 5 U (range, 1–247 U, with 1 additional patient with 10,000 U); for 6 patients with AIDS with no history of PCP, the median antibody level was 2 U (range, 0–32 U); for 3 patients with AIDS with PCP before diagnosis, the median antibody level was 2 U (range, 0–32 U); and for 1 patient with AIDS with PCP, the median antibody level was 2 U (range, 0–32 U). Non-AIDS immunocompromised patients with PCP had higher antibody levels than did either patients with AIDS with PCP (P = .01) or healthy persons (P = .005).

To examine antibody responses to acute P. jiroveci infection, we quantitated antibody levels longitudinally in serum from 8 patients with AIDS with a history of PCP. No consistent pattern of antibody response was seen among these patients. Three patients showed no substantial change in antibody levels during follow-up. Only 2 patients showed an increase in antibody levels before diagnosis of PCP, whereas 3 additional patients showed an increase only after the diagnosis. Figure 2B shows serial results for 2 representative patients.

To determine whether levels of anti-P. jiroveci antibody changed over time in healthy persons, we examined serum samples—obtained at 4 collection times, over a period of 1 year—from 35 healthy persons. All subjects were HIV-negative and were followed closely while they participated in a vaccine trial. Four subjects (11%) had ≥1 sample with a ≥4-fold change in antibody levels, compared with an earlier sample, and therefore we examined serum samples from additional collection times, when available, from these persons. Three subjects had antibody levels <10 U that subsequently and persistently increased >8-fold. Figure 2C shows serial results for 2 of these subjects. One subject initially had very high antibody levels (up to 471 U), which remained elevated for >6 months and then rapidly dropped to <10 U. In clinical terms, the 3 patients with increases in antibody levels were well, but they complained of upper respiratory symptoms (nasal congestion, sinusitis, or cold symptoms) within the 3 months before the increase. To ensure specificity of the titer changes in anti-MSG antibody in these 4 healthy persons, we measured their antibodies against CMV at the same collection times. Although 3 of the 4 persons did have antibodies to CMV, their titers did not significantly change over time or correlate with titers of anti-MSG-14Ca antibody.

**DISCUSSION**

We have developed a quantitative ELISA for detection of antibodies to a P. jiroveci-specific antigen, by use of recombinant reagents that can be produced easily and reproducibly in large quantities. This assay potentially can be an important tool to better understand the epidemiology of P. jiroveci infection in healthy persons, as well as in immunocompromised patients. Our preliminary data suggest that many immunocompromised patients, especially those with HIV infection, may not show an antibody response to acute P. jiroveci infection. To our surprise, we found that ~10% of healthy persons produced a ≥4-fold change in antibody titer to P. jiroveci MSG-14 during 1 year of observation, a finding suggesting that they had been acutely infected with P. jiroveci, either from reinfection or reactivation of latent infection, although, alternatively, these findings may represent cross-reactive or nonspecific changes.

In preliminary evaluations of antibody responses to the recombinant MSG proteins, we found, by immunoblot, that ~44% of subjects, including healthy and immunocompromised persons, reacted with MSG-14Am, and all subjects reacted with MSG-14Ca, although the reactivity was often faint. Because there are an estimated 100 copies of the msg gene in the Pneumocystis genome, copies that are related but unique, and because there is greater variability in the amino terminus of MSG, it is not surprising that fewer persons reacted with MSG-14Am.

In a recent study, Daly et al. [12] found, by immunoblot, that the majority of healthy persons (84%) reacted with any of 3 overlapping recombinant MSG fragments; 64% had antibodies against the middle region (approximately aa 243–760), but less reactivity (~40%) was seen in recombinant proteins.
Quantitation of Anti-*Pneumocystis* Antibodies

Figure 2. A, Cross-sectional results of ELISA for detection of antibodies to recombinant *Pneumocystis jiroveci* major surface glycoprotein (MSG) fragment MSG-14Ca. Human serum samples (1:100) were assayed in duplicate. Each circle represents an individual patient; bars indicate medians. Antibody levels of patients immunocompromised for reasons other than AIDS (non-AIDS) with *P. jiroveci* pneumonia (PCP) were significantly higher than were antibody levels of healthy persons (*P* = .005) and were higher than were antibody levels of patients with AIDS with PCP (*P* = .01). For patients from whom serial samples were available, the earliest sample was plotted and was used in statistical analysis. Antibody units >100 are not shown to scale. B, Levels (in units) of anti–MSG-14Ca antibody, over time, for 2 representative human immunodeficiency virus–positive patients with history of PCP. Arrows indicate timing of bronchoscopy; “Neg” indicates that *P. jiroveci* (PC) was not detected at that time. Patient 1 showed an increase in antibody titer after diagnosis of PCP, and patient 2 showed high antibody levels at early collection times, levels that decreased significantly (>4-fold) during recurrent episodes of PCP. Although there was a transient increase in antibodies after the last episode, antibody levels were minimal just 5 days before the patient’s death, when he presented with disseminated *P. jiroveci* infection. C, Levels (in units) of anti–MSG-14Ca antibody, over time, for 2 of 3 healthy persons who demonstrated a >4-fold increase in antibodies over time. Subject 1 had antibody levels up to 196 U, and subject 2 had antibody levels up to 53 U.

Although, overall, the number of patients in some of the subgroups we studies is small, it is noteworthy that only patients with PCP who were immunocompromised for reasons other than AIDS had higher titers than did healthy persons (figure 2A); HIV-infected patients with PCP had generally low titers, consistent with the severe B cell dysfunction that has been reported in this population. Analysis of serial samples from patients with AIDS with histories of PCP showed that there encompassing the amino or carboxy portion. We may have increased sensitivity by using a greater amount of antigen and a high concentration of secondary antibody, and, therefore, we would have been able to detect lower-titer reactivity. Because MSG proteins are highly variable (except for a few well-conserved regions), it is also possible that MSG-14 is more often expressed by *P. jiroveci* than are the fragments used by Daly et al. [12].
was no consistent correlation between increases in antibody levels and time of diagnosis of Pneumocystis infection, a finding suggesting that measuring antibody titers will not be useful in the diagnosis of PCP.

Of particular interest are the 4 healthy persons who demonstrated significant (>4-fold) changes in antibody titers over a period of 1 year. Given that 3 of these subjects had titers that were very low for months then persistently increased, the most plausible explanation is that these persons were exposed to a burden of P. jiroveci organisms substantial enough to induce an immune response, probably by a subclinical infection. These responses were specific, because similar changes to CMV were not seen.

This study illustrates the advantages of ELISA to quantitate antibody responses to Pneumocystis. Well-designed prospective studies making use of this assay should provide insights into the epidemiology of P. jiroveci infection in healthy persons, as well as in immunocompromised patients.

Acknowledgments

We thank the patients, for their willingness to donate blood samples for this study; Thomas Nutman, for his helpful suggestions in establishing the ELISA; Eric Gerstenberger, for statistical assistance; and Julie Metcalf, for her assistance.

References