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Seroepidemiological study of *Pneumocystis jirovecii* infection in healthy infants in Chile using recombinant fragments of the *P. jirovecii* major surface glycoprotein

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Summary

Objectives—To characterize the seroepidemiological features of *Pneumocystis jirovecii* infection in healthy Chilean children using overlapping fragments (A, B, C) of the *P. jirovecii* major surface glycoprotein (Msg).

Methods—Serum antibodies to MsgA, MsgB, and MsgC were measured every 2 months by enzyme-linked immunosorbent assay (ELISA) in 45 Chilean infants from about age 2 months to 2 years.

Results—Peak antibody levels (usually reached at age 6 months) and the force (or rate) of infection were somewhat greater for MsgC than for MsgA. Significant seasonal variation in antibody levels was only found with MsgA. Respiratory infections occurred in most children, but nasopharyngeal aspirates were of limited value in detecting the organism. In contrast, serological responses commonly occurred, and higher levels only to MsgC were significantly related to the number of infections.

Conclusions—Serological responses to recombinant Msg fragments provide new insights into the epidemiological and clinical features of *P. jirovecii* infection of early childhood. MsgA, the amino terminus fragment, is more sensitive in detecting seasonal influences on antibody levels, whereas MsgC is better able to detect changes in antibody levels in response to clinical infection.

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Keywords

Serological responses; Pneumocystis; Major surface glycoprotein (Msg); Children

Introduction

Pneumocystis jirovecii remains an important cause of serious pneumonia ('Pneumocystis pneumonia' or PcP) in HIV-infected patients and other immunocompromised hosts.[1] Serological studies have shown that primary *P. jirovecii* infection (as defined by the development of antibody responses to Pc antigens) is acquired in early childhood; so by 2–3 years of age, 70–90% of healthy children have serum antibodies to the organism.[2-5] It is thought that this infection is mild or asymptomatic.[4,6-8] One study found that the presence of *P. jirovecii* DNA detected by the polymerase chain reaction (PCR) was more commonly associated with upper respiratory tract infection (URI) symptoms than lower respiratory tract infection (LRI) symptoms.[9]

These serological studies mainly used antigens that consisted of crude extracts obtained from Pneumocystis-infected human or rodent lung.[10] Attention was focused on the time when antibodies were first detected and cumulative seropositivity over time. Little was known about the specific antigens that were recognized by serum antibodies, the rate or force of infection, or whether antibody levels varied over time.[10]

The development of recombinant *P. jirovecii* antigens has begun a new era in serology, with attention focused mainly on two moieties: the major surface glycoprotein (Msg) and kexin (Kex1).[11,12] We selected Msg as our target antigen because it elicits a strong immune response, contains protective epitopes, and plays a major role in the interaction of Pneumocystis with its mammalian host.[1,13-15] We developed three overlapping recombinant fragments (Msg A, B, and C) that span the entire length of a single Msg isoform: MsgA, the amino terminus, which is quite variable; MsgB, the mid portion; MsgC, the carboxyl terminus, which is the most conserved fragment. We analyzed reactivity with serum antibodies in prevalence studies and in HIV patients and other adult populations, and found that MsgC was best able to distinguish (1) HIV patients hospitalized with PcP from patients with pneumonia due to other causes, (2) HIV patients who had previous PcP from patients who never had PcP, and (3) healthcare workers who had clinical contact with patients from workers who did not.[16-18] We then developed variants (Msg C3, C8, and C9) of the parent MsgC (C1) in order to better delineate the reactivity of this antigen.[19-21]

Similar to the prominent surface proteins of other eukaryotic pathogens, such as the variant surface antigen (VSA) of Plasmodium and the variant surface glycoprotein (VSG) of Trypanosoma,[22,23] Msg is encoded by multiple genes and is thus capable of antigenic variation.[24] Serological surveys of young children, who experience most of the malaria fatalities, and of adults in an endemic area provide information about the host immune response to VSA, which is important for vaccine development.[25-27] Analysis of serum antibody responses of populations in different geographic areas, climates, and seasons can enhance understanding of the host and environmental effects on the expression of VSA epitopes and their recognition by host antibodies.[28-30] Our studies so far have revealed geographic, but not seasonal differences in the serological antibody responses to Msg in adults.[21]

One of us (SLV) previously followed healthy infants during the first 2 years of life with regular visits and with nasopharyngeal aspirates (NPAs) taken when there were respiratory symptoms.[6] [Au?1] *P. jirovecii* DNA was detected in NPAs in 32% of these episodes, and at

a significantly younger age than the NPAs that were negative for *P. jirovecii*. Yet, detection of *P. jirovecii* did not identify a specific pattern of symptoms. Serum antibodies to *Pneumocystis murina* extracted from the mouse with PcP, developed in 53% of the infants at 8 months of age and in 85% of the infants by 20 months of age; seroconversion occurred in the presence of respiratory symptoms in 79% of the subjects. Thus, *P. jirovecii* or an immune response to the organism could be frequently detected in healthy infants with mild respiratory disease.

Little is known about the serological responses of infants and young children to recombinant *P. jirovecii* antigens. In the present study, we examined the sequential serum antibody responses to Msg A, B, and C over a 2-year period in 45 infants from this pediatric cohort. We wanted to compare the serological responses to these antigens for their value in determining the force or rate of infection; in looking for seasonal differences in antibody levels; and in examining the host immune response to respiratory infections.

Materials and methods

Patient population

In 1997, a total of 107 healthy infants were enrolled by 1 month of age in a 2-year prospective cohort study in Santiago, Chile.[6] [Au?2] Follow-up occurred monthly at a well-baby clinic and serum specimens were obtained every 2 months. Parents were asked to report infections via the telephone or to bring the child to the clinic. NPAs were obtained for *P. jirovecii* amplification when a respiratory tract infection was diagnosed, especially when cough was reported. An infection was diagnosed as a URI if a cold (rhinitis), nasopharyngitis, or pharyngitis were present. An LRI consisted of bronchitis, bronchiolitis, or pneumonia. Other manifestations such as tonsillitis, tonsillopharyngitis, or otitis media were considered separate disorders. Serum specimens and NPAs (which were mixed with saline) that were obtained were stored at −70 °C. Chest radiographs were obtained when clinically indicated. An informed consent form for study participation was signed by both parents and approval for the study was given by the University of Chile Ethics Commission.

The 45 subjects in the present report were chosen because they had many sequential serum specimens taken, reliable clinical data, and exhibited a broad range of antibody responses to *P. murina* extracted-purified antigen in the previous study. Serum samples from the previous study were available from 2 to 24 months of age. However, for the analysis of antibody peaks, rate or force of infection, seasonal variation, and episodes of respiratory infection, only serum specimens obtained at or after 6 months of age were used in order to control for maternal antibodies.

DNA amplification

NPAs were examined for *P. jirovecii* by nested-DNA amplification of the large subunit mitochondrial ribosomal RNA gene of *P. jirovecii*.[4] NPA samples were digested with proteinase K (20 mg/ml) at 60 °C in the presence of 10 mM ethylenediaminetetraacetic acid (EDTA) and 0.5% sodium dodecyl sulfate (SDS). Total DNA was purified and concentrated with use of the Pharma-Gen ‘clean up’ system (Pharma-Gen) and recovered in a volume of 50 μl. Five microliters of this DNA preparation were used for DNA amplification with *P. jirovecii* oligonucleotide primers pAZ102-E (5′-GATGGCTG-TAGG-3′) and pAZ102-H (5′GTGTACGTTGC-AAAGTACTC-3′), which are internal to the first set of primers and specific for *P. jirovecii*, in a second round of PCR take 1 μl from the first-round PCR product [Au?3]. Amplified *P. jirovecii* had a predicted length of 267 bp. Negative controls with no added template DNA were included after each sample to monitor for cross-contamination.

ELISA

Serum antibody levels to the recombinant Msg fragments were analyzed in a blinded manner by an ELISA.[16,17] We later made slight modifications to the ELISA.[19-21] Analysis of the same specimens by both methods showed good correlation of the results. The small amounts of serum in the specimens available to us precluded repeating the analysis of the specimens in this study by the new method. For similar reasons, we were only able to measure IgG antibodies in this study.

All serum specimens and the standard reference serum were diluted 1/100 and tested in duplicate wells of a 96-well plate against the following antigens: MsgA, MsgB, and MsgC; *Escherichia coli* extract expressing the pET vector without insert (vector control); phosphate buffered saline (PBS) without antigen (negative control); and tetanus toxoid (TT) (positive control). The plates were washed, followed by the addition of horseradish peroxidase (HRP)-labeled goat anti-human IgG; the plates were washed again and tetramethylbenzidine (TMB) substrate was added. The reaction was stopped by the addition of 0.18 M H_2SO_4 and the plates were read at a wavelength of 450 nm. The reference serum specimen was obtained from a single individual with known reactivity to Msg. This serum was run each day as an additional control. HRP-labeled S-protein was used as a positive control and to correct for antigen loading. The antibody level or reactivity of each serum specimen to Msg was expressed as the ratio of reactivity: (mean OD Msg_{test} serum − the mean OD $PBS_{test\ serum}$)/ (mean OD $pET_{test\ serum}$ − mean OD $PBS_{test\ serum}$).

Statistics

Antibody levels to Msg A, B, and C (C1) across time were evaluated for each child. The median age at first visit and median duration of follow-up were calculated. Analysis of variance (ANOVA) was performed to determine the differences in serum antibody levels to MsgC and MsgA among seasons and among the number of respiratory infections. Season was defined according to the climate in Chile as follows: summer (December–February), fall (March–May), winter (June–August), and spring (September–November). The number of respiratory infections was categorized as 0 (none), <3 (1–2 illnesses), and ≥3 illnesses. The maximum (peak) antibody level to each antigen was obtained for each child. The median and 25^{th} and 75^{th} percentiles of the set of all peak values were calculated to describe the distribution of these values. Similar percentiles of the distribution of ages when antibody levels peaked were calculated. The average age when *P. jirovecii* infection occurred was defined serologically as the median age at which the maximum of peak antibody levels occurred. The force (rate or incidence) of infection was defined as 1/median age of infection. The relationship between peak antibody levels and age at time of peak was graphically assessed to provide visual summaries of trends over time. SAS for Windows, version 9.1 (SAS Institute, Cary, NC, USA) was used for all the analyses and the level of statistical significance was based on 5% level (two-sided) unless stated otherwise.

Results

General demographic and serological findings

The median age at first visit was 2 months, the visits were about 2 months apart, and the median follow-up time was 19 months. There were 37 and 42 infants included in the analysis of MsgA and MsgC distributions, respectively (Table 1). Although we had initially planned to examine serum specimens for all 45 subjects, we found that there were missing specimens or specimens with insufficient amounts of serum for analysis. Since antibody levels to MsgB were very low and showed few sequential changes, they were excluded from the analyses. The median peak of antibody level to MsgC was 48.5 vs. 21.2 to MsgA. As defined by peak antibody levels, the median age when primary *P. jirovecii* infection

occurred was 10 months for MsgC vs. 12 months for MsgA. The force of infection measured by antibodies to MsgC was 10% per month compared with 8% per month for antibodies to MsgA. Thus, MsgC tended to elicit higher antibody levels and greater force of infection than MsgA, but the differences were not statistically significant.

Seasonal changes in antibody levels

The overall patterns of serological responses to MsgA and MsgC were quite different (Table 2). The lowest level of antibodies to MsgA was found in the fall (10.9), whereas the highest level was in the spring (33.2). Levels in the summer were in between. The seasonal differences in antibody levels to MsgA were significant ($p = 0.03$). In contrast to MsgA, high antibody levels to MsgC ranging from 46.8 to 53.7 were found in summer, fall and winter; the lowest antibody level was found in spring (28.3). However, these results were not statistically significant.

Antibody reactivity over the 2-year period

Most children had at least one major peak in their antibody levels to MsgA or MsgC, some had several peaks, while others had little or no peaks. The aggregate data also suggested that there were distinct antibody peaks in the antibody levels that tended to be grouped around specific ages rather than being uniformly distributed (Figure 1). Antibody peaks to MsgC were usually higher and had a shorter median interval between them (3.2 months) than the peaks to MsgA (3.7 months).

Antibody reactivity in individual subjects

Examples of the sequential antibody changes in individual children exhibited considerable variation in the responses to MsgC and MsgA (Figures 2 and 3). The most common pattern was one or more antibody peaks to MsgC after clearance of the maternal antibodies, which usually occurred at 6 months or the third clinic visit. Changes in antibody responses to respiratory infection were easy to find. Yet, since these episodes occurred in the presence or absence of detectable *P. jirovecii* DNA on the NPAs, and detection of *P. jirovecii* by PCR is highly dependent on the amount of sample (DNA content) analyzed,[31] it was difficult to correlate these specific antibody changes with nested PCR results.

Relationship of antibody levels to number of infections

In order to determine if there was an association between antibody levels to MsgC or MsgA and the number of respiratory infections developed by the children, we compared the geometric mean (95% confidence interval) of antibody levels of all visits for all subjects with the same number of respiratory infections (Table 3). The data showed that there was a progressive rise in mean antibody levels to MsgC with increasing number of respiratory infections; children experiencing three or more episodes had significantly higher antibody levels to MsgC than children who had experienced no episodes or one to two episodes. By contrast, no such difference was seen in the antibody levels to MsgA. We also examined these antibody levels without controlling for maternal antibodies, and again significantly higher antibody levels were found in the children with three or more respiratory infections (data not shown).

In addition, we attempted by several different approaches to correlate antibody levels to MsgC or MsgA with the specific types of respiratory infection (URI or LRI), but the results were inconclusive (data not shown).

Discussion

The present report is the first seroepidemiological study of *P. jirovecii* infection in children using recombinant antigen preparations. As in our previous studies of adults in the USA,[16-19] the consistently higher antibody levels elicited by MsgC suggest that it is the predominant antigen recognized by serum antibodies in Chilean infants and young children. MsgC also tended to elicit higher and more rapid antibody peaks than MsgA, as illustrated in the force of infection, which is an epidemiological tool that has been helpful in studying the dynamics of infection, both in populations and in models of infection.[32-34]

Previous reports have noted geographic differences in the occurrence and *P. jirovecii* colonization in HIV patients.[35,36] One characteristic of multi-gene antigens used in serological surveys is geographic variation in the antibody responses. Our serological study of HIV subjects enrolled in the Multicenter AIDS Cohort Study (MACS), which compared the reactivity of the four recombinant MsgC fragments, demonstrated significant differences in the antibody levels to these MsgC fragments among the cities (Baltimore, Pittsburgh, Chicago, and Los Angeles) where the study was conducted.[21] We also found that MsgC8 was the predominant Msg fragment recognized in Seville, Spain, and that MsgA was the major fragment recognized in Cameroon.[20,37]

The impact of climate on the occurrence of Pneumocystis infection in humans as well as in animals has been a subject of considerable investigation.[38-43] A complex and sometimes contradictory relationship has been found: some studies have shown an increased occurrence associated with warmer temperatures, whereas other studies have found an increased association with colder temperatures or no seasonal association at all. Our study of MACS subjects extended this debate to serological responses, but found no seasonal differences in serological responses to the MsgC fragments.[21]

The present report also found no significant seasonal differences in antibody responses to MsgC in young Chilean children; however, the report did reveal a different pattern of reactivity to MsgA that was associated with significant differences among the seasons. The reasons for these findings are not clear. Perhaps host recognition of MsgA, which is the most exposed part of Msg, is more easily influenced by changes in the environment (e.g., temperature, humidity) than recognition of MsgC. In addition, the exposures of the young children in this study differed from exposures in adults in that many of these exposures were the children's initial encounter with the organism. These findings support our previous data, which suggest that the Msg fragments are recognized independently by the host.[19-21] The results also emphasize the need for further investigation of the effects of environmental factors on host responses to *P. jirovecii*.

While previous reports have focused on the time at which children first develop serum antibodies to *P. jirovecii*,[4-6] the present study examined the sequential changes in antibody levels during the first 2 years of life. Most subjects developed at least one major peak to MsgC and/or MsgA, but there was considerable variation in the individual responses. Perhaps the tendency for the peaks to be grouped or clustered around specific ages was related to maturational changes in the immune response or to age-related environmental exposures. Although one study of *P. jirovecii* colonization in families did not find clustering,[44] clustering of serological responses in other infectious diseases has been observed.[28-30,45,46]

Respiratory infections occurred in most of the children at some point during the study, and were accompanied by antibody responses to MsgC and/or MsgA. The children who had more infections had significantly higher mean antibody levels to MsgC (but not to MsgA) than individuals who had fewer infections. These data suggest that MsgC antibody levels are

a more sensitive marker of more frequent or more intense clinical exposure to *P. jirovecii*. The results are also consistent with our previous reports of the value of MsgC in discriminating present from past infection or active disease from colonization.[16,17,21]

While the data in this report extend the results of the previous study of these children,[6] [Au? 4] both studies have limitations. The serological analysis was performed from an epidemiological point of view and the small quantities of serum prevented analysis of IgM antibodies; thus, the results should not be extrapolated to the diagnosis of *P. jirovecii* infection. NPAs to detect *P. jirovecii* were only performed when respiratory symptoms were present; testing for other pathogens by culture, PCR, or serology was not performed. Without this information, caution must be used in interpreting the results. In addition, recent data show compartmentalization of *P. jirovecii* colonization in the upper respiratory tract and that the yield of noninvasive respiratory sampling increases if more than one niche is sampled.[31]

In summary, this study shows that recombinant Msg fragments are sensitive new tools that can provide new information about the epidemiological and clinical features of *P. jirovecii* infection in early childhood. MsgA, the amino terminus fragment, appears to be more sensitive in detecting seasonal influences on immune responses, while MsgC, the carboxyl terminus fragment, is more effective in detecting changes in antibody levels in response to clinical infection.

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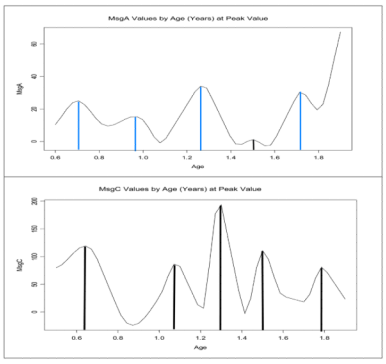

Figure 1.

MsgC and MsgA antibody levels by age. The vertical lines show the magnitude of the peaks. Note that the antibody peaks to MsgC are generally higher and have a shorter interval between them than the peaks to MsgA.

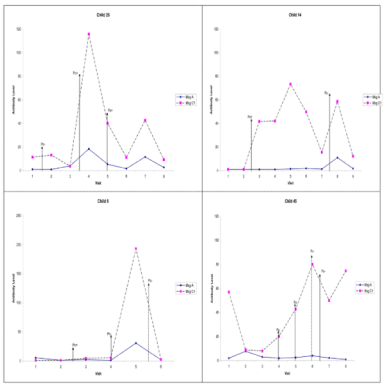

Figure 2.

Examples showing the changes in antibody levels to MsgC over time in four children. Pc+ indicates nasopharyngeal aspirate (NPA) positive for *Pneumocystis jirovecii* DNA, and Pc− indicates NPA negative for *P. jirovecii* DNA. The vertical arrows show the visits when the NPAs were obtained. Child 26 developed the first major peak at clinic visit 4 (8 months of age), followed by a smaller peak at clinic visit 7. The first respiratory illness that required medical attention occurred between clinic visits 1 and 2; NPA at that visit was negative for Pneumocystis (Pc−). Subsequent episodes of illness occurred between clinic visits 3 and 4 and at clinic visit 5; in both cases, the NPA was Pc+. Antibody levels to MsgC did not change after the first illness, increased after the second illness, and declined after the third illness. Child 14 developed the first MsgC antibody peak at clinic visit 5 and the second peak at clinic visit 8. Illness occurred between visits 2 and 3 (NPA Pc+), and between visits 7 and 8 (NPA Pc−). Serum antibody levels to MsgC increased after both illnesses. Child 6 developed the first and only MsgC antibody peak at visit 5. Illness occurred between visits 2 and 3 (NPA Pc+), at visit 4 (NPA Pc−), and between visits 5 and 6 (NPA Pc−). There was no change in antibody levels after the first illness, a rise after the second illness, and a fall after the third illness. Child 45 displayed the first antibody peak at visit 6 and second peak at visit 8. Illness occurred at visit 4 (NPA Pc−), visit 5 (NPA Pc−), visit 6 (NPA Pc−), and between visits 6 and 7 (NPA Pc−). Serum antibodies to MsgC rose after the first three illness episodes and fell after the fourth illness.

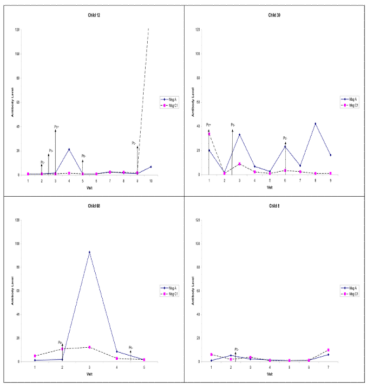

Figure 3.

Examples showing the changes in antibody levels to MsgA over time in four children. Pc+ indicates nasopharyngeal aspirate (NPA) positive for *Pneumocystis jirovecii* DNA, and Pc− indicates NPA negative for *P. jirovecii* DNA. The vertical arrows show the visits when the NPAs were obtained. Child 12 exhibited the first and only antibody peak to MsgA at visit 4. Illness occurred at visit 2 (NPA Pc−), between visits 2 and 3 (NPA Pc−), at visit 3 (NPA Pc +), and at visit 9 (NPA Pc−). Serum antibodies to MsgA rose after the illness at visit 3. Child 39 developed the first antibody peak at visit 3 and subsequent peaks at visit 6 and visit 8. Respiratory illness occurred at visit 1 (NPA Pc+), between visits 2 and 3 (NPA Pc−), and at visit 6 (NPA Pc−). Serum antibody levels rose after the first illness, fell after the second illness, and rose again after visit 7 though there was no illness reported. Child 68 exhibited the first and only MsgA antibody peak at visit 3. Illness occurred at visit 2 (NPA Pc−) and between visits 4 and 5 (NPA Pc−). The only serum antibody rise occurred after the first illness. Child 8 demonstrated no antibody peak to MsgA. Illness occurred between visits 2 and 3 (NPA Pc−).

Table 1

Percentiles (%) of the distributions of maximum peak antibody levels to MsgA and MsgC and the age (months) when these levels were reached

	No. of peaks	Median (25^{th}, 75^{th} percentiles)	Age (25^{th}, 75^{th} percentiles)
MsgA	37	21.2 (11.0, 34.8)	12 (8, 17)
MsgC	42	48.5 (12.3, 110.7)	10 (8, 14)

Table 2

Percentiles (%) of the distributions of peak antibody levels to MsgA and MsgC by season; median peaks (25^{th}, 75^{th} percentiles)

	Spring (Sept–Nov)	Summer (Dec–Feb)	Fall (Mar–May)	Winter (Jun–Aug)
MsgA[a]	33.2 (12.0, 68.5); *n* = 14	19.3 (13.1, 27.6); *n* = 8	10.9 (3.9, 15.7); *n* = 10	22.9 (10.4, 77.8); *n* = 11
MsgC	28.3 (5.4, 139.4); *n* = 13	53.7 (10.0, 84.9); *n* = 10	46.8 (25.3, 80.0); *n* = 6	52.3 (27.3, 110.7); *n* = 13

n is the number of peaks.

[a] Seasonal median estimates were significantly different ($p = 0.03$ by Chi-square test) among the seasons.

Table 3

Serum antibody levels to MsgA and MsgC in the children by the number of episodes of respiratory infection detected during the study

No. of episodes	No. of serum specimens	Mean ratio (percentiles 25–75)	
		MsgC	MsgA
0	38	3.74 (2.18–6.40)	3.92 (2.41–6.38)
<3	147	5.59 (4.25–7.35)	3.21 (2.56–4.03)
≥3	125	12.44 (9.46–16.35)[a]	4.49 (3.59–5.61)

[a] Children who had three or more clinical diagnoses had significantly higher mean MsgC levels compared to those who had no diagnosis and those who had one or two episodes ($p < 0.05$ by ANOVA).