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Geographical variation in serological responses to recombinant *Pneumocystis jirovecii* major surface glycoprotein antigens

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Abstract

The use of recombinant fragments of the major surface glycoprotein (Msg) of *Pneumocystis jirovecii* has proven useful for studying serological immune responses of blood donors and human immunodeficiency virus (HIV)-positive (HIV^+) patients. Here, we have used ELISA to measure antibody titres to Msg fragments (MsgA, MsgB, MsgC1, MsgC3, MsgC8 and MsgC9) in sera isolated in the USA (n=200) and Spain (n=326), to determine whether geographical location affects serological responses to these antigens. Blood donors from Seville exhibited a significantly greater antibody titre to MsgC8, and significantly lower responses to MsgC3 and MsgC9, than did Cincinnati (USA) donors. Spanish blood donors (n=162) also exhibited elevated responses to MsgC1, MsgC8 and MsgC9 as compared with Spanish HIV^+ (n=164) patients. HIV^+ patients who had *Pneumocystis* pneumonia (PcP^+) exhibited a higher response to MsgC8 than did HIV^+ PcP^- patients. These data show that geographical location plays a role in responsiveness to Msg fragments. Additionally, these fragments have utility in differentiating HIV^+ PcP and HIV^+ PcP^+ among patient populations.

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Introduction

Pneumocystis jirovecii is an opportunistic fungal pathogen of humans that causes *Pneumocystis* pneumonia (PcP) in immunocompromised individuals, including those infected with human immunodeficiency virus (HIV) [1–4]. Effective drugs for the treatment of PcP exist; however, the potential for resistance to these therapies, together with the longer survival of HIV-positive (HIV^+) patients, due to antiviral therapies, has spurred an interest in antigen-specific immunity to *Pneumocystis* infection in HIV^+ patients [5].

The role of antibodies in infection with *P. jirovecii* is not well understood, but there is a high frequency of reactivity to *Pneumocystis* antigens in healthy adults and children [6–10]. Much work has focused on using animal models of PcP infection, highlighting a potential role for antibodies in the prevention of PcP [11–18].

The majority of immunological studies on reactivity to *Pneumocystis* spp have used complex antigens derived from infected animal lungs [7,9,19–22]. These preparations of antigens are not well defined, and there are many limitations to their use: they contain many different antigens; the spectrum of variable antigens such as the major surface glycoprotein (Msg) can vary with the preparation; the absolute volume of a specimen is limited because, in the absence of an *in vitro* culture system, the only source of organisms is infected host lung; and samples may be contaminated with co-infecting pathogens. Taken together, these problems suggest that the use of recombinant antigens may be more appropriate for immunological studies.

Smulian *et al.* [9] used western blot to demonstrate significant geographical variation in serological responses to high molecular weight antigens from rat-derived *Pneumocystis carinii* in HIV-negative (HIV^-) people from five global locations. The nature of these antigens could not be determined in this study, given that multiple proteins may co-migrate in electrophoresis, and many immunoreactive proteins have not been

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definitively identified. Given the limitations of using crude preparations of antigen, it would be interesting to determine whether the serological response to a single antigen exhibits geographical variation. Such a study would require the use of recombinant antigens to provide a clear answer.

We and others have started using recombinant fragments of Msg to probe the immune responses of blood donors and HIV^+ patients [6,23–25]. Msg is a well-characterized antigen that is encoded by a family of genes in the *Pneumocystis* genome, and only one Msg is expressed at a given time [12,26–31], suggesting that the protein may have a role in immune evasion. Msg has B-cell and T-cell epitopes, and can give protective immunity in some animal models [32–36]; however, the relative roles of cell-mediated and humoral immunity to Msg are not well understood.

We have recently examined the serological responses of blood donors and HIV^+ patients in the USA to three recombinant fragments of Msg, which we called MsgA, MsgB, and MsgC. Our work has focused mainly on MsgC, the C-terminus of Msg, which is relatively conserved among different Msg molecules, and can be recognized by human serum in western blot and ELISA [24,25,37]. We have identified a panel of four MsgC clones that differ from one another in putative amino acid sequence. These clones behave differently from one another in serological assays; for example, in ELISA, there is a significantly higher level of reactivity to MsgC1 and MsgC3, but not to MsgC8 or MsgC9, in a cohort of HIV^+ patients who have had a previous bout of PcP as compared with either the HIV^+ PcP^- patient group or blood donors. The frequency of reactivity seen in western blot analysis also varies with the Msg construct and the patient populations tested [37].

Here we have performed a study to address global reactivity to a panel of recombinant Msg fragments and examined the potential geographical variation in reactivity to these proteins in ELISA. First, we examined blood donor sera isolated in the USA and Spain for reactivity to recombinant Msg antigens. Second, as antibody titres to *Pneumocystis* antigens have been shown to vary with onset and recovery from PcP [38–41], we tested HIV^+ Spanish patients who did or did not have PcP for reactivity to these recombinants, and compared the results with those obtained for Spanish blood donors.

Materials and Methods

Serum specimens

The serum specimens used in this study came from the USA and Spain. They included 200 samples from healthy blood donors at the Hoxworth Blood Center, Cincinnati, OH, USA, as well as 162 samples from blood donors and 164 samples from HIV^+ patients, 29 of whom had PcP, at the Virgen del Rocio University Hospital, Seville, Spain. PcP diagnosis was based on clinical data and microscopic examination of respiratory samples (sputum or bronchoalveolar) using immunofluorescence with specific monoclonal antibodies (Pneumo Cel IFA test; Cellabs, Brookvale, Australia).

Isolation and expression of Msg fragments

We have previously described the characterization of the Msg fragments used in this study [24,25,37]. Briefly, oligonucleotides were designed on the basis of conserved sequences of the known *msg* genes of *P. jirovecii*. These primers were used in PCR, using DNA isolated from *P. jirovecii*-infected human lung at autopsy or cloned *msg* genes as templates, and Amplitaq enzyme (Applied Biosystems, Foster City, CA, USA) to generate *msg* gene segments. The PCR products were cloned into the pET30 vector (Novagen, Madison, WI, USA) in the correct orientation for expression in *Escherichia coli*. The recombinant proteins were purified by affinity chromatography using HIS-binding resin (Novagen). The protein concentration was determined by absorbance at 280 nm ($A_{280\ nm}$), using a standard curve generated with bovine serum albumin.

ELISA

ELISA was performed similarly to previously reported procedures [25]. Briefly, the reactivity of each serum specimen to Msg was corrected by subtraction of the reactivity of that serum to phosphate-buffered saline (mean OD Msg – mean OD phosphate-buffered saline). The results were quantitated using a method similar to that of Bishop and Kovacs [23], using a standard curve specific for each construct. Test sera were assayed at dilutions that fit the linear portion of the standard curves, and units of reactivity were calculated. Samples whose values were below the standard curve were assigned the lowest possible value of 1 U.

Statistics

Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Data were log transformed to approximate normalcy, and statistical significance was calculated using either unpaired *t*-tests or ANOVA (Kruskal–Wallis) with Dunn's multiple-comparison test. p-Values <0.05 were considered to be significant.

Results

Reactivity in healthy blood donor sera

We first compared 200 blood donor sera from Cincinnati and 162 BD sera from Seville for reactivity to a panel of

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three recombinant Msg fragments that, together, span the full length of Msg (MsgA, MsgB, and MsgC1 (Fig. 1). Similar to our previously published findings [25,37], the overall reactivity to each of these three Msg fragments was low in the healthy blood donor populations, with no significant differences in the levels of reactivity to any of these constructs between sera from Cincinnati and from Seville (Table 1).

To determine whether clonal variability had an effect on reactivity of sera, we expanded the study to include three MsgC clones that differ from MsgC1 and from one another (Fig. 1) [37]. The magnitude of the responses to MsgC3, MsgC8 and MsgC9 varied with the population studied. The Cincinnati population exhibited higher responses than the Seville population to both MsgC3 and MsgC9 ($p < 0.0001$ and p 0.035, respectively) (Table 1). However, the response to MsgC8 was much greater in the donors from Seville (geometric mean value of 22.27 U) than in the donors from Cincinnati (geometric mean value of 3.42 U) ($p < 0.0001$) (Table 1).

Reactivity to Msg fragments in Spanish HIV$^+$ patients

We had previously shown that HIV$^+$ patients from Cincinnati responded more strongly to MsgA, MsgB and MsgC1 than did blood donors from Cincinnati [25]. We now tested 164 HIV$^+$ patients from Seville for reactivity to each of the Msg constructs, and compared the results with those obtained for the blood donors from Seville. In contrast to our results with US sera, the Spanish HIV$^+$ patients had responses similar to or lower than those of blood donors from Spain. In the Spanish population, blood donors had significantly higher responses than did HIV$^+$ patients to MsgC1 (2.10 U and 1.11 U, respectively), MsgC8 (22.27 U and 8.59 U, respectively), and MsgC9 (3.04 U and 1.33 U, respectively) ($p < 0.0001$ for all comparisons) (Table 2).

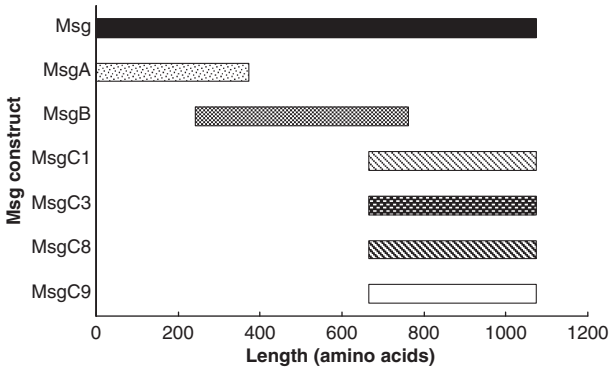

FIG. 1. A schematic of the constructs used in this study. Major surface glycoprotein (Msg)A, MsgB and MsgC span the length of Msg. MsgC1, MsgC3, MsgC8 and MsgC9 are different clones of the C-terminus of Msg.

TABLE 1. Geometric mean values and 95% CIs of ELISA by blood donor and geographical location for each antigen

Antigen	Cincinnati blood donors (n = 200)	Seville blood donors (n = 162)	p
MsgA	3.82 (2.99–4.886)	4.67 (3.65–5.96)	0.253
MsgB	1.81 (1.49–2.19)	1.84 (1.49–2.26)	0.911
MsgC1	2.05 (1.64–2.55)	2.10 (1.67–2.64)	0.883
MsgC3	4.42 (3.32–5.88)	1.90 (1.56–2.30)	<0.0001
MsgC8	3.42 (2.62–4.45)	22.27 (17.06–29.04)	<0.0001
MsgC9	4.70 (3.48–6.34)	3.04 (2.35–3.94)	0.035

Seroreactivity to Msg fragments in HIV$^+$ patients with or without PcP

We have shown that HIV$^+$ patients in Cincinnati who had a prior bout of PcP responded more strongly to MsgC1 and MsgC3 than did HIV$^+$ patients who did not have PcP [37]. To determine whether any of the Msg constructs were useful for differentiating between Spanish HIV$^+$ patients on the basis of prior PcP, the HIV$^+$ patients who had PcP (n = 29) were compared with those who did not (n = 135). With the exception of the responses to MsgC8, there were no significant differences in the reactivity to any of the Msg constructs between the PcP$^+$ and PcP$^-$ HIV$^+$ populations (Table 3). The

TABLE 2. Geometric mean values and 95% CIs of ELISA by blood donor and human immunodeficiency virus (HIV) status for each antigen

Antigen	Seville blood donors (n = 162)	Seville HIV$^+$ patients (n = 164)	p
MsgA	4.67 (3.65–5.96)	3.58 (2.88–4.46)	0.113
MsgB	1.84 (1.49–2.26)	2.36 (1.89–2.93)	0.101
MsgC1	2.10 (1.67–2.64)	1.11 (1.01–1.22)	<0.0001
MsgC3	1.90 (1.56–2.31)	1.69 (1.41–2.02)	0.385
MsgC8	22.27 (17.06–29.04)	8.59 (6.48–11.40)	<0.0001
MsgC9	3.04 (2.35–3.94)	1.33 (1.15–1.52)	<0.0001

TABLE 3. Geometric mean values and 95% CIs of ELISA by *Pneumocystis* pneumonia (PcP) status in human immunodeficiency virus-positive (HIV$^+$) patients from Seville for each antigen

Antigen	Seville HIV$^+$ PcP$^+$ (n = 29)	Seville HIV$^+$ PcP$^-$ (n = 135)	p
MsgA	3.19 (1.90–5.34)	3.67 (2.87–4.69)	0.629
MsgB	1.93 (1.19–3.15)	2.46 (1.92–3.14)	0.4088
MsgC1	1.15 (0.86–1.53)	1.10 (1.00–1.20)	0.676
MsgC3	1.54 (0.99–2.41)	1.72 (1.41–2.10)	0.64
MsgC8	19.41 (10.12–37.24)	7.21 (5.30–9.83)	0.0079
MsgC9	1.65 (1.02–2.65)	1.27 (1.10–1.45)	0.1536

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response to MsgC8, however, was significantly higher in the PcP^+ population (19.41 U) than in the PcP^- population (7.21 U; p 0.0079).

Hierarchy of responses to MsgC8

We have previously shown that there is a hierarchy of reactivity to each of the Msg constructs across different patient populations in Cincinnati [25,37]. The lack of strong reactivity to many of the constructs used here precludes the assignment of such a hierarchy, except for reactivity to MsgC8. Blood donors and PcP^+ patients had very similar levels of reactivity to MsgC8 (22.27 U and 19.41 U, respectively), and PcP^- patients exhibited a lower level of response (7.21 U) (Fig. 2). PcP^+ patients showed the widest range of responses (1–614 U), and the PcP^- patients had the smallest range (1–172 U) (data not shown). The range of values obtained for the other Msg constructs also changed with the population tested, with the PcP^- patients consistently having the lowest ranges of reactivity to all MsgC constructs (data not shown).

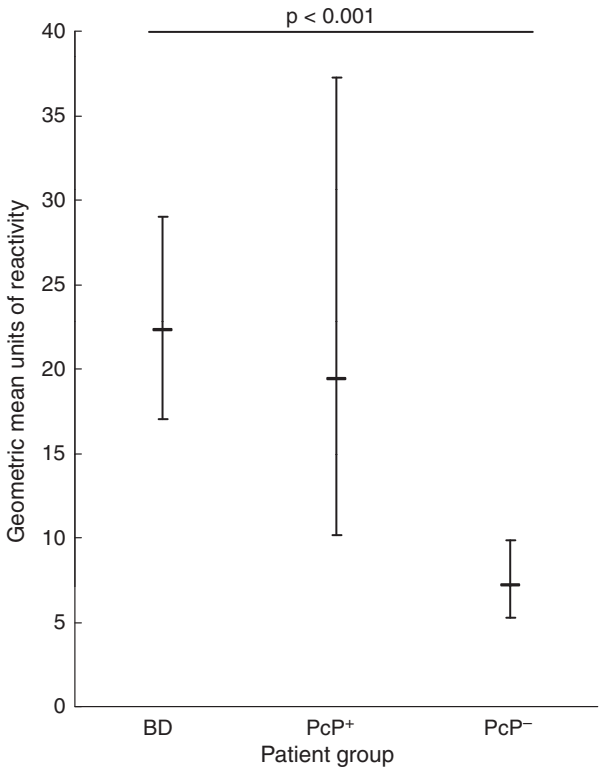

FIG. 2. Comparison of blood donor (BD) with *Pneumocystis* pneumonia (PcP^+) and without *Pneumocystis* pneumonia (PcP^-) patient groups, showing geometric means and 95% CIs for reactivity to major surface glycoprotein (Msg)C8 in ELISA. Comparisons between groups were made using ANOVA. Significant values ($p < 0.05$) are shown.

Discussion

In our previously published work, we described the use of recombinant fragments of Msg in probing serological reactivity of blood donors and HIV^+ patients in the USA [25,37]. In this study, we have analysed blood donors from Seville with these constructs, and found a geographical difference in the reactivity to three of the constructs, MsgC3, MsgC8, and MsgC9. Spanish blood donors, as a group, exhibited significantly higher reactivity to MsgC8, and lower reactivity to MsgC3 and MsgC9, than did blood donors from Cincinnati. This regional difference may indicate that Msg isoform expression is geographically controlled. Extending the analysis to include a cohort of Spanish HIV^+ patients shows that those who had PcP have higher responses to MsgC8 than do those who did not have PcP.

The level of recognition of MsgA, MsgB and MsgC1 is very similar in blood donor populations from Cincinnati and from Seville. This is consistent with our previous data showing no difference in the frequency of recognition of these constructs in western blot analysis of serological reactivity, using blood donor samples from the USA, Haiti, South Africa, and South Korea [24]. However, as reported here, the antibody titres to MsgC3, MsgC8 and MsgC9 are significantly different in the Cincinnati and Seville blood donor populations. MsgC8, which is recognized poorly by sera from the USA, was recognized very strongly by the Spanish sera.

There are three potential reasons for the increased response to MsgC8 in the Spanish sera. First, the rate of *P. jirovecii* infection or colonization may be higher in Spain than it is in the USA, with the increase in exposure to *P. jirovecii* antigens having a boosting effect on antibody responses to Msg. We feel that this is unlikely, as the seroprevalence of *Pneumocystis* infection in Spanish children is similar to that found in other global regions, including North America, Africa, and northern Europe [7,10,42–44]. Second, the expressed repertoire of antigen-specific receptors on B-cells, T-cells and antigen-presenting cells may be different in the Spanish and North American populations. Third, the spectrum of Msg molecules expressed in *P. jirovecii* from Spain may be different from that expressed in midwestern USA isolates. It is likely that both immune repertoire and Msg repertoire play a role in the expression of Msg epitopes. Presumably, the antibodies that we are measuring arose as a consequence of exposure to *P. jirovecii* expressing MsgC8-like molecules. This would indicate that MsgC8-like molecules are expressed more prevalently in Spanish populations of *P. jirovecii* than in American populations of the organism. Variation in *msg* genes among different isolates of *P. jirovecii* has

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recently been shown by Kutty *et al.* [45], highlighting the possibility of global differences in expressed Msg antigens.

It is unclear why the spectrum of Msg molecules expressed by *P. jirovecii* would vary according to the geographical location of its host, but factors potentially affecting such expression might include selection of antigens within the infected host population, strain variation in *P. jirovecii* between Seville and Cincinnati, and geographical factors such as temperature, humidity, and elevation. The localized expression of Msg molecules within a population may be directly related to the mode of transmission. Person-to-person transmission is likely to be a very important mechanism of dissemination of *P. jirovecii*. However, the antigenic complexity of the Msg fragments precludes their use as tools for clearly tracking transmission of the organism.

Interestingly, there were two Msg fragments (MsgC3 and MsgC9) that showed a significantly higher level of reactivity in the Cincinnati population than in the Seville population. It is unclear whether these MsgC molecules represent antigens with localized expression, as the difference in response to these antigens between European and American populations was not as great as the difference seen for MsgC8. It is possible that an isotype of MsgC that could be selectively recognized in American populations does exist; however, such a molecule is not present in our small library of Msg fragments.

The hierarchy of recognition of MsgC8 showed that HIV^+ PcP^- patients had a significantly lower level of reactivity than did healthy blood donors or HIV^+ PcP^+ patients. Variation in recognition of the Msg fragments according to different patient populations has been seen before. However, the variation seen in the Spanish populations is very interesting. The high level of reactivity in the blood donors and PcP^+ patients suggests that MsgC8 is expressed during *P. jirovecii* exposure, either in infection or colonization, and this drives the immune response up. Furthermore, the higher titre of antibodies in the PcP^+ patient group suggests that these antibodies are relevant to recovery from disease and that MsgC8 has protective epitopes in a geographical location where expression of MsgC8 is high. The lack of a strong response in the PcP^- patients may be due to impaired immunity.

Finally, the data presented here show that MsgC clones must be regarded as individual entities and not as generic antigens. The isoform-specific antibodies seen in this study underline the potential for Msg as a variant antigen that may play a role in immune evasion. It is likely that Msg molecules are composed of shared and unique epitopes. However, the contribution of each type of epitope to the overall immune reactivity to *P. jirovecii* and the ability to counteract *P. jirovecii* infection/colonization remain unknown.

Transparency Declaration

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Each author has disclosed a lack of conflict of interests.

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