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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

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