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No association between mannose-binding lectin deficiency and H1N1 2009 infection observed during the first season of this novel pandemic influenza virus

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ABSTRACT

Genetic variations in host immunity may influence susceptibility to novel infections like the recently emergent pandemic influenza virus. Prior studies demonstrated that mannose-binding lectin (MBL) inactivates influenza. Furthermore, MBL deficiency is common and appears to predispose to respiratory virus infections. Therefore, we studied whether MBL deficiency played a role in infection with the novel H1N1 2009 influenza strain in exposed health care workers. In a nested case–control study, we observed no association between phenotypic MBL deficiency, variously defined, and predisposition to H1N1 2009 influenza in 63 pairs of seropositive and seronegative participants. MBL appears to currently have little impact on innate immune responses to H1N1 2009 influenza.

1. Introduction

Emerging infectious diseases may afford an opportunity to examine variations in host susceptibility caused by the absence of widespread prior immunity. The recent pandemic influenza strain H1N1 2009 emerged in Mexico in April 2009 and produced a late surge in northern hemisphere influenza cases. This virus then rapidly became the dominant influenza strain in the southern hemisphere. Although infections were generally mild, there was marked morbidity in pregnant and obese patients [1]. Determining host genetic factors that influence the innate immune response and susceptibility to novel infections may lead to therapeutic interventions for this and future emerging infections with higher overall morbidity.

Mannose-binding lectin (MBL) is a pattern recognition protein of the innate immune system that recognizes pathogen-associated molecular patterns [2]. MBL binds to microbial cell surface polysaccharide residues that have characteristic chemical and spatial features that distinguish them from animal cell walls. Having bound to microbial pathogens, MBL mediates killing through lectin complement pathway activation and opsonophagocytosis. MBL has been observed to bind to and neutralize the influenza virus in studies involving nonpandemic H3N2 strains [3]. In this study, the efficacy of MBL-mediated inhibition of these influenza strains that have multiple exposed glycosylation sites on the hemagglutinin head was proportional to the number of glycosylation sites available for MBL binding. MBL null mice have increased susceptibility to H3N2 influenza A viruses [4]. Most recently, however, it has been demonstrated that in contrast to previous, seasonal influenza strains, MBL plays no role in murine models of influenza caused by H1N1 2009 infection [5].

MBL deficiency resulting from polymorphism in the *MBL2* gene is one of the most common genetic influences on immune responses to infection. Approximately 25% of the many human populations studied have lowered levels of MBL [6] and this state has been associated with predisposition to numerous infectious diseases, including those of the respiratory tract [7]. Although MBL is predominantly a serum protein, significant amounts are observed in the respiratory tract in the presence of inflammation [8]. Given the *in vitro* neutralization of influenza by MBL, we believed that MBL deficiency may predispose to infection with influenza. We tested this hypothesis in a population naive to the novel pandemic strain H1N1 2009.

2. Subjects and methods

2.1. Recruitment of cases and controls from a prospective cohort study assessing risk of H1N1 2009 infection in health care workers

As part of a prospective cohort study investigating H1N1 2009 infection risks in health care workers [9], we assessed MBL status

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among hospital workers who had been demonstrated to have (i) serologic evidence of infection (H1N1 2009 hemagglutination titer (HAT) ≥ 1:40) with or without an influenza-like illness or (ii) no infection (H1N1 2009 HAT < 1:10) and no influenza-like illness. This study was performed at 4 Melbourne tertiary referral hospitals during the influenza season of 2009 when H1N1 2009 was the predominant serotype. Approval for the study was provided by the human research ethics committees at all 4 hospitals and participants gave written informed consent.

2.2. MBL enzyme-linked immunosorbent assay (ELISA) and MBL phenotypic deficiency definition

MBL level was determined by mannan-binding ELISA and MBL function by C4 deposition ELISA as described previously [10]. We defined MBL deficiency in 3 ways, which have been used in previous infectious diseases association studies: (a) MBL level <0.5 μg/mL [11], (b) C4 deposition <0.2 U/μL [12], or, (c) most stringently, both levels below the preceding ranges. *MBL2* genotype was not determined in this study because we have previously demonstrated that MBL level is a more sensitive and specific marker of MBL deficiency because a considerable number of individuals bear wild-type *MBL2* with unmeasureable MBL [6].

2.3. Sample size calculation

To determine the sample size required for the MBL disease susceptibility study, we powered the study to detect 3-fold odds of influenza infection in MBL-deficient individuals compared with the odds of infection for MBL-sufficient participants. This odds ratio (OR) was selected as being representative of respiratory infection predisposition studies wherein *MBL2* polymorphisms encoding for low MBL levels were analyzed for associations with respiratory infection, including a meta-analysis in pneumococcal infection (OR = 2.57) [13] and respiratory infections generally (OR = 3.4) [14]. The required number of influenza-infected cases and uninfected controls was 65 in each group (two-sided p value = 0.05, 80% power), assuming a 25% frequency of MBL deficiency (as indicated by MBL level < 0.5 μg/mL) in the influenza-seronegative controls as is the case in healthy controls. To reach our predetermined study sample size, all seropositive and randomly selected seronegative participants from the health care worker study were invited to participate in this nested case–control predisposition study.

2.4. Statistical methods

Comparison of the nonnormally distributed results for MBL level and function was performed using Mann–Whitney tests and frequency of MBL deficiency was compared using χ^2 tests. Statistical calculations were performed using Minitab 14 (State College, PA).

3. Results

3.1. Matching of H1N1 2009 seropositive cases and exposed, uninfected (seronegative) control health care workers

A total of 231 health care workers and 215 nonclinical staff controls were enrolled in a study of the risk of infection conducted by the Melbourne H1N1 clinical research team [9]. The "parent" study aimed to determine the effect of occupation and personal protective equipment on H1N1 2009 acquisition. From this cohort we recruited subjects for the MBL study reported here, based primarily on their H1N1 2009 serology results.

A majority of the influenza-infected participants (63/79 H1N1 2009 HAT > 1:40) from the health care worker study consented to inclusion in this study and an equal number of randomly selected, uninfected controls were recruited. For the study participants, there were no symptomatic, laboratory-confirmed, influenza infections among health care workers, forcing a reliance on our serologic definition for H1N1 2009 infection. The median H1N1 2009 HAT among cases was 1:80 with a range of 1:40–1:640 and interquartile range of 1:40–1:80.

Controls were matched to cases in that they worked at the same hospital only. Our parent study demonstrated no association between age, sex, or number of children in the health care workers' household; on that basis, none of these factors was used in matching cases and controls. Additionally, MBL levels do not change significantly with age in adult life [15].

3.2. Comparison of MBL levels and frequency of deficiency in H1N1 2009–infected health care workers and exposed, uninfected controls

The overall frequency of MBL deficiency as defined by MBL level <0.5 μg/mL was 37%, which exceeds the predicted frequency used to power our study. There were no differences in the median levels of MBL (1.04 μg/mL in the seropositive group and 0.75 μg/mL in the seronegative group, p = 0.4) or C4 deposition (0.23 U/μL in the seropositive group and 0.18 U/μL in the seronegative group, p = 0.4) between the groups of influenza-infected and uninfected participants (Table 1). Additionally there was no significant difference in the frequency of MBL deficiency as defined by low MBL level (23/63 of seropositive participants vs 24/63 of seronegative participants, OR 0.93; 95% confidence interval (CI) 0.43–2.05), low MBL function (30/63 of seropositive participants vs 34/63 of seronegative participants, OR 0.78; 95% CI 0.36–1.66), or both low MBL level and function (21/63 of seropositive participants vs 24/63 of seronegative participants, OR 0.81; 95% CI 0.37–1.80). If a lower MBL level (<0.1 μg/mL) was used to define deficiency, there remained no difference between the frequency of MBL deficiency in seropositive (9/63) and seronegative (10/63) study participants (OR 0.88; 95% CI 0.30–2.59).

There were no participants with symptomatic, proven influenza to analyze the influence of MBL deficiency on disease severity.

Table 1
Comparison of mannose-binding lectin (MBL) levels, function (C4 deposition), and frequency of MBL deficiency in health care workers infected with H1N1 2009 and uninfected, exposed controls

	H1N1 2009 seropositive (n = 63)	H1N1 2009 seronegative (n = 63)	p value	Odds ratio	95% Confidence intervals
MBL level (μg/mL)	1.04	0.75	0.4		
C4 deposition (U/μL)	0.23	0.18	0.4		
Cases and controls with MBL deficiency variously defined					
MBL level <0.5 μg/mL	23	24		0.93	0.43–2.05
C4 deposition <0.2 U/μL	30	34		0.78	0.36–1.66
MBL level <0.5 μg/mL and C4 deposition <0.2 U/μL	21	24		0.81	0.37–1.80
MBL level <0.1 μg/mL	9	10		0.88	0.30–2.59

4. Discussion

Numerous well-established host factors predispose to influenza infection or severe disease. Recent studies of pandemic influenza strain H1N1 2009 disease have demonstrated that pregnancy and obesity were among important factors associated with disease severity [16]. A novel recent host factor for influenza severity appears to be IgG2 subclass deficiency [17]. Here we have sought information on the link between MBL deficiency and predisposition to influenza.

We have demonstrated no association between MBL deficiency states and infection with the novel pandemic influenza strain H1N1 2009. Numerous factors may potentially account for this lack of association. However, sample size is unlikely to have played a role. The study was powered to detect an OR of 3, and the CIs of the observed odds ratios are sufficiently narrow that a true OR of 3 is highly implausible. Whereas our sample size calculation was based on a background frequency of MBL deficiency of 25%, we actually observed a higher frequency, which would have improved the study power. Similarly, there were sufficient influenza infection events based on positive H1N1 2009 serology to analyze. The absence of symptomatic influenza is not expected to have altered the result of this study but means there was no opportunity to assess MBL's influence on the severity of disease manifestations.

Differences in exposure to the novel H1N1 2009 influenza strain between the infected and uninfected groups are a potential source of study error. In the health care worker "parent" study [9] from which subjects for this influenza predisposition study were drawn, we observed no significant difference in the rate of influenza seroprevalence in those health care workers involved in direct patient care compared with nonclinical staff who had a background risk that approximated that of the general community. The frequency of H1N1 2009 seropositivity in health care workers involved in the "parent" study varied among the 4 study sites because the geographic distribution of symptomatic cases presenting to Melbourne hospitals was notably uneven. Therefore, we matched the numbers of seropositive health care worker cases and seronegative controls from each study hospital site. Taken together, these points would support the argument that the overall level of exposure among the study population described here is equivalent.

The obvious conclusion is that MBL is not involved in innate immune responses to influenza or other respiratory viruses. This is countered, however, by the *in vitro* evidence of MBL's inhibition of influenza virus [3,18], recent evidence of MBL's role in the development of humoral immunity to influenza [19], and clinical association studies that demonstrate that MBL deficiency predisposes not only to respiratory tract infection in general, but also to respiratory tract infections with viral pathogens like severe acute respiratory syndrome in particular [7].

Our study's choice of MBL phenotyping over genotyping in defining MBL deficiency may have affected its results. However, phenotype has a more direct biologic link with MBL activity. Low MBL level is a more sensitive and specific marker of the biologic effect of MBL (MBL function as measured by C4 deposition) than *MBL2* genotype [10]. Large studies of healthy controls confirm that many patients with wild-type *MBL2* genotype have levels of MBL in the deficient range [6]. The choice of an MBL level of <0.5 μg/mL to define MBL deficiency is informed by published data [6]. When we used a lower level of MBL to define deficiency, we also observed no indication that extremely low MBL levels predisposed to H1N1 2009 infection. Although there were fewer individuals with MBL <0.1 μg/mL than with MBL levels <0.5, this only slightly reduced the power to detect our proposed OR of 3. Unfortunately, it was not possible to recruit the greater sample size required (168 participants) to achieve power using this MBL cutoff. However, given the observed OR and the CI (OR 0.88; 0.30–2.59), we can exclude with some confidence our *a priori* OR of 3 in this population.

Other studies have used MBL phenotyping rather than *MBL2* genotyping to determine whether MBL deficiency plays a role in disease susceptibility [20,21]. Concerns are sometimes raised as to the possibility that MBL levels may be elevated because of relatively limited acute-phase reactant changes observed in this protein [22]. However, because the patients studied here did not have clinical signs of influenza at the time of blood testing, there is no possibility of MBL levels being affected by acute-phase changes.

Potentially, the most potent factor that abrogates any role for MBL in protection against H1N1 2009 infection is specific features of the surface of this virus. Recent data indicate that there is only 1 glycosylation site exposed on the globular head of the hemagglutinin protein of this novel virus and that MBL does not bind to this strain of influenza [23] or contribute to mouse immune responses to H1N1 2009 [5]. The absence of association between MBL deficiency and predisposition to H1N1 2009 infection we have observed is in line with and highlights the biologic significance of these *in vitro* data. It may be predicted that future mutation of the H1N1 2009 virus to escape the human immune response will lead to more glycosylation sites becoming exposed on the head of the hemagglutinin and that MBL may then play a role in protection against infection. It may be useful to repeat this study after future influenza seasons involving H1N1 2009 transmission.

Because of MBL's broad range of activity against pathogens, it has the potential for activity and even therapeutic efficacy against emerging organisms. Indeed, it has recently been shown to inhibit severe acute respiratory syndrome [24], Ebola [25], and henipaviruses (unpublished), all highly virulent pathogens demonstrated to have multiple glycosylation sites. Although we have not demonstrated a clinical association between MBL deficiency and predisposition to the novel H1N1 2009 influenza strain, this may be because of the absence of exposed MBL-binding sites on this specific virus. There are no previous clinical studies on the role of MBL in innate immune defenses against influenza. The previously documented viral inhibitory activity of MBL against influenza viruses suggests that this arm of the immune system may indeed be active against influenza, including against H1N1 2009 after antigenic drift occurs.

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