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Efficacy of recombinant chimeric lectins, consisting of mannose binding lectin and L-ficolin, against influenza A viral infection in mouse model study

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Abstract

Influenza A virus infection could result in fatal complications. Although immunization is the most effective prevention it is not effective to pandemic infection and is less effective or not approved for certain age groups. Some influenza virus strains have developed resistance to antiviral agents. Thus, new therapeutic agents are urgently needed. We focused on innate immune molecules, including mannose-binding lectin (MBL). In order to optimize its antiviral activities, we have previously generated three recombinant chimeric lectins (RCL), by introducing portions of L-ficolin, another innate immune lectin. Our in vitro characterizations previously selected RCL2 and RCL3 for further investigations against viruses, including influenza viruses. Here, we examined efficacy of these lectins against infection with PR8 (H1N1) influenza A virus using mouse model studies and a human tracheal epithelial cell system. Our results provide in vivo evidence that RCL3 is effective agent against influenza virus infection. The therapeutic mechanisms are in part by providing host protective responses mediated by cytokines. We conclude that RCL3 is a potential new innate immune anti-influenza virus therapeutic agent.

Keywords

Influenza A virus; inflammation; innate immunity; mannose-binding lectin; ficolin; host response

1. Introduction

Infection with influenza virus, an RNA virus, is common and is normally self-resolving. However, influenza virus infection could result in fatal complications, even in individuals who are appeared to be healthy (Lynch and Walsh, 2007; Munoz, 2003). Mortality is estimated to exceed annually more than 30,000 in the United States alone (Lynch and Walsh, 2007). Prevention is currently relied upon immunization, however vaccines are less effective against pandemic infections. Immunization is also less effective in elderly and is not approved by the FDA for infants younger than 6 months old (Bouree, 2003; Munoz,

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2003). Some seasonal and pandemic influenza viruses have already developed resistance to antiviral agents, like tamiflu (Lynch and Walsh, 2007; Saito et al., 2010). Thus, there is a need for new effective anti-influenza virus therapeutic and prophylactic agents.

The first line of host defense system is the innate immune mechanisms, including lectins, like MBL, which recognizes pathogens through carbohydrate recognition domain (CRD) (Ip et al., 2009). MBL, a serum protein, is present in lungs of healthy mice (Chang et al., 2010). Mice genetically lacking MBL are susceptible to infection with a common strain of Philippine 82 (H3N2), but are relatively resistant to a pandemic strain of H1N1 (pH1N1) influenza A virus (Chang et al., 2010; Ling et al., 2012). These results suggest that MBL is less effective against H1N1 influenza A virus infection and that optimization of MBL is required.

Therefore, we have previously generated three recombinant chimeric lectin (RCL)s by replacing various length of the collagenous domain of MBL with that of L-ficolin (Michelow et al., 2010). These RCLs are superior to MBL for several antiviral activities, including inhibition of hemagglutination and viral aggregation; and binding to other viruses, such as Nipah, Hendra and Ebola (Chang et al., 2010; Michelow et al., 2010). Importantly, all RCLs have reduced interference with the coagulation system. Such characteristic is a significant advantage as a therapeutic agent because infectious diseases can cause coagulation disorders (Nesheim, 2003).

Other important aspects of infectious disease outcome are host inflammatory responses, which are mediated by cytokines and are also modulated by lectins, including MBL (Chang et al., 2010; Moller-Kristensen et al., 2006). Uncontrolled inflammation due to infection cause tissue injury and obstruction while asymptomatic infection can be observed in commensalisms and symbiosis without illness (Casadevall and Pirofski, 2000).

Our previous studies selected RCL2 and RCL3 for further investigations (Chang et al., 2011). Here, we investigated efficacy of these recombinant lectins against PR8 (H1N1) influenza A virus infection using murine lung infection model studies and human tracheal epithelial cells, natural targets of influenza viruses in humans (van Riel et al., 2007).

2. Materials and methods

2.1. Recombinant chimeric lectins

Chimeric lectins were produced as previously described (Michelow et al., 2010). RCL2 and RCL3 corresponded to L-ficolin/MBL76 and L-ficolin/MBL64, respectively in our previous study (Chang et al., 2011; Michelow et al., 2010). In both RCLs, MBL-collagenous domain was replaced with 76 or 64 amino acids of L-ficolin's collagenous domain, resulting in total amino acid length of 255 or 254, respectively (Michelow et al., 2010). Recombinant human MBL was a gift from Enzon (Piscataway, NJ).

2.2. Virus preparations

influenza A virus strain A/Puerto Rico/8/34 (PR8, H1N1) was prepared as previously described (Hartshorn et al., 2000). Briefly, PR8 was grown in the chorioallantoic fluid of chicken eggs and purified on a discontinuous sucrose gradient (Sigma-Aldrich, St. Louis, MO). Virus stocks were dialyzed against PBS (Sigma-Aldrich, St. Louis MO) and aliquots were stored at -150°C . Virus titers (fluorescent foci counts, ffc) were obtained by infection assay of Madin-Darby canine kidney (MDCK) cells (Hartshorn et al., 2000).

2.3. Mice

C57Black/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and used at ages between 6 and 10 weeks old. All animal experiments were performed under a protocol approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital, Boston, MA.

2.4. In vivo PR8 infection experiments

Infection experiments were performed as described previously (Chang et al., 2010). Briefly, mice were anesthetized with avertin (250 mg/kg, i.p.) and were then intranasally inoculated with 5×10^6 ffc of PR8 in 20 μ l PBS. One hour after viral inoculation, mice were intraperitoneally injected with 75 μ g of recombinant lectins or saline in 0.2 ml volume (Shi et al., 2004). The dose of 75 μ g was chosen because it fully restored MBL functions in MBL knockout mice (Moller-Kristensen et al., 2006; Shi et al., 2004). Survival, clinical observations and body weight were recorded every 2–3 days till day 14. Survival curves were generated by the product-limit survival fit using JMP software (SAS Institute, Cary, NC).

Lung homogenates were prepared on day 3, 7 and 14 as previously performed (Chang et al., 2010). Briefly, lungs were harvested, weighed, and homogenized with 0.5 ml of PBS using a polytron tissue homogenizer. Homogenates were centrifuged and supernatants were stored in the -80° C freezer.

2.5. In vitro human tracheal epithelial cell infection experiments

Human tracheal epithelial cells (ATCC, Manassas, VA) were plated at 4×10^4 cells/well in 100 μ l of RPMI1640 media, supplemented with 10% fetal bovine serum in 48 well plates. After incubation for 1 h in a 5% CO₂ incubator the wells were washed with PBS. The wells were then incubated with PR8 (5×10^6 ffc/40 μ l/well in RPMI1640), which were preincubated with recombinant lectins or PBS at 2 μ g/ml in the CO₂ incubator for 1 h. 60 μ l of culture media was added and incubated over night in the CO₂ incubator. Cell survival at 48 hr was assayed using a WST8 cell counting kit (Dojindo Molecular Technologies, Inc., Bethesda, MD) by reading A₄₅₀ with a Spectramax M5 plate reader. Cell survival (%) was calculated as media alone 100%. Supernatants and cell lysates were prepared at 24 and 48 hr and stored in the -80° C freezer.

2.6. Viral titrations by quantitative polymerase chain reaction (QPCR)

Total RNA from cell lysates, supernatants or lung homogenates were prepared using TRI agent (Sigma-Aldrich, St. Louis, MO), according to the manufacture's instructions. For cell lysates and supernatants, 6 wells were pooled into duplicate (3 wells x 2 data points), in order to obtain sufficient materials. cDNA was prepared using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The RT-PCR primers and the probe were designed specific to H1N1 and were: Sense, 5'-CCAGGAAATGCTGAGATCGAAGAT-3'; Antisense, 5'-GGCAAGACTTGTGAGCAACTGA-3'; the probe, 5'-CACGGTCTGCACTCAT-3', the latter was labeled with a reporter dye 6-carboxyfluorescein. PCR was performed in the Realplex² thermal cycler (Eppendorf North America, Hauppauge, NY). PCR program was one cycle of 95°C for 10 min followed by 40 cycles of 95°C for 15 sec then 60°C for 1 min. Standard curves were generated from serially diluted virus cDNA with known viral titers in each experiment. PR8 particles were normalized to the total RNA (μ g).

2.7. Cytokine assays

Three supernatants of human tracheal cell culture were pooled in each test group to have sufficient sample volumes. Similarly, supernatants of lung homogenates from three mice

were pooled. These samples were assayed using membrane array kits (human cytokine array C-series 4000 for 274 cytokines and mouse cytokine array C-series 2000 for 144 cytokines) (Raybiotech inc., Norcross, GA). Developed membranes were scanned using a Chemi-Doc scanner (Bio-Rad, Horcuris, CA). Relative chemiluminescence intensity of each spot, corresponding to each molecule, was recorded using the Quantity One software provided by the scanner. Arbitrary units for each molecule were derived by normalizing the relative intensity against an average of the positive controls in each membrane (supplemental data). Using these arbitrary units, fold changes were calculated against those from control lungs (no viral infection). Positive molecules were defined as those with more than 3-fold increase at any time point.

2.8. Statistical analysis

All data were analyzed for statistical significance using JMP software. P values less than 0.05 were considered to be significant and no values were provided where there was no statistical significance. Statistical methods applied were indicated in each figure legend.

3. Results

3.1. RCL3 improves PR8 infection

Recombinant lectins were examined for therapeutic efficacy against PR8 infection using a murine lung infection. Without infection, all mice survived following administrations of recombinant lectins or saline. In contrast, upon viral infection, mean survival time was 8.7, 9.4, 11.1, and 11.9 days for saline control, MBL, RCL2, and RCL3, respectively although there was no statistically significance. These results demonstrated that RCLs extended survival by 3 days compared with the saline control.

Illness is commonly assessed by weight loss. Without infection, mice treated with recombinant lectins gained weight similar to saline treated mice, indicating that these recombinant lectins had no adverse effect (Fig. 1A). In contrast, following viral infection, the saline group lost body weight continuously till day 7 and then recovered toward day 14 (Fig. 1A). Body weight changes of the MBL group were consistently lower than the saline group (no statistical difference) (Fig. 1A). Compared with both saline and MBL groups, mice treated with RCL2 or RCL3 lost less body weight with statistical significance for RCL3 (Fig. 1A). Taken together, these results showed efficacy of RCL3, in improving survival and illness from PR8 infection. Thus, we focused on RCL3 in following experiments.

3.2. Limited effect on viral clearance by RCL3

We next examined viral loads in the lungs. Compared with MBL and saline groups, RCL3 treatment reduced viral titers on days 3 and 7 (not statistically significant, Fig. 1B). These observations demonstrated that RCL3 had little effect in viral clearance from the lungs. The combined results with reduced body weight loss and extended survival suggested that RCL3 might have enhanced host protective mechanisms, which improve the host resistance against viral infection.

3.3. RCL3 modulates cytokine production differently from saline and MBL during PR8 infection in the lung

The host responses are important part of infectious disease outcome. Cytokine productions, as indices of lung responses, were examined for 144 cytokines. Twenty-eight proteins were positively identified using the criteria defined for the study (Fig. 2 and supplemental data). Of these, chemokines: CCL1, CCL2, CXCL13; and proteases: MMP-3 and MMP-9; and an inflammatory modulator, IL-12 p40/p70 were markedly upregulated by RCL3 treatment on

day 3 and/or day 7 compared with both saline and MBL groups. In contrast, RCL3 treatment did not increase sTNF RII, IL17RB and CD32 compared with saline and/or MBL groups. RCL3 treatment also did not upregulate HGF R on day 7 while the treatment significantly increased the molecule on day3 compared with both saline and MBL groups.

3.4. Effects of recombinant lectins on viral infection and survival of human tracheal epithelial cells

We next examined effects of recombinant lectins in PR8 infection of human tracheal epithelial cells because these cells are natural targets of influenza viruses in humans (van Riel et al., 2007). Viral titers in cell lysate were similar among three groups at 24 and 48 hr (Fig. 3A). In contrast, MBL and RCL3 significantly reduced viral titers in culture supernatants at 48 hr (Fig. 3B). These results suggested that although recombinant lectins have little effect in reducing cell-associated viruses they either limit virus in the solution or inhibit virus shedding.

Next, we examined effects of recombinant lectins in cell survival at 48 hr post infection. Viral infection reduced survival, less than 20% and MBL had little effect (Fig. 3C). In contrast, RCL3 significantly increased cell survival by 5% compared with MBL and Medium (Med) control (Fig. 3C). Cell survival with recombinant lectins alone was also increased by RCL3 treatment while MBL treatment was similar to Med control, indicating that recombinant lectins did not have an adverse effect on cells (Fig. 3D).

3.5. Effects of lectins in cytokine responses of tracheal cells during PR8 infection

Similar to in vivo studies, we also examined cytokine responses at protein expression levels from human tracheal epithelial cells. Eighteen molecules increased more than 3 fold compared with uninfected cells with or without recombinant lectins (Fig. 4). Of these, CCL2 and CCL5 chemokines were markedly increased by viral infection and were inhibited by both MBL and RCL3. Similar responses were also observed for CCL8 with much less intensity. In contrast, there was noticeable enhance in CXCL11 by both recombinant lectins. CCL2, CCL5 and IL-1 α were also upregulated in murine lungs during PR8 viral infection (Fig. 2A).

4. Discussion

Our Results show that RCL3, a chimeric lectin of MBL and L-ficolin, is effective in improving PR8 (H1N1) infection. In contrast, MBL has little or detrimental effect against this strain of influenza A virus, as survival and weight loss was similar to saline group and viral titers were higher than saline groups. These observations support our recent findings that MBL gene knockout mice were protected from a pH1N1, indicating that MBL negatively affects antiviral host defense against H1N1 viral infections (Ling et al., 2012). Taken together, these results suggest that RCL3 is potentially a new anti-influenza virus therapeutic agent.

In infectious diseases, appropriate regulations of host inflammatory responses are important aspects of the host protective mechanisms. During PR8 infection, RCL3 treatment down regulated pro-inflammatory molecules, sTNF RII and IL17B R and upregulated, IL-12 p40/p70 anti-inflammatory molecule, suggesting that RCL3 reduces inflammation. These results support our previous findings that during pH1N1 viral infection, inflammation is down regulated in mice those are protected from viral infection (Ling et al., 2012). It is understandable that exacerbated inflammation may have adverse effects in viral infection. Uncontrolled inflammation would initiate unresolving inflammation, resulting in weakened host protection. Taken together, these observations suggest that RCL3 is able to maintain appropriate inflammation during influenza virus infection.

Controlled cytokine production plays an important role in maintaining tissue integrity. Lungs from RCL3 treated mice have increased levels of MMP3 and MMP9, which are bifunctional. While both enzymes may cause tissue injury by digesting extracellular matrices they are also involved with wound healing as a chemoattractant and a mitogen of epithelial cells (Chen and Parks, 2009; Lefebvre et al., 1992; Legrand et al., 1999). CCL2, which was greatly enhanced by RCL3 treatment at the early stage of infection, also participates in wound healing through HGF, another epithelial cell mitogen (Amano et al., 2004; Mason et al., 1994; Shiratori et al., 1995). Incidentally, its receptor, HGFR is upregulated by RCL3 at the early stage of infection, suggesting that CCL2 and HGFR may be collaborating. Well-orchestrated cytokine productions are cause of host tissue protection as a part of the anti-infective mechanisms. Taken together, these observations suggest that early initiation of wound healing process may be a key factor in protecting lungs from infection-induced tissue injury.

Although it was modest, RCL3 enhances human tracheal epithelial cell survival during viral infection, suggesting that RCL3 attenuate viral infection-associated cell death. Such cell survival may contribute to increases cellular protective mechanisms. While the cytokine profiles were similar between MBL and RCL3 group, CCL2, CCL5 and IL1 α were also detected in the lungs, suggesting that tracheal epithelial cells may produce these cytokines. The cytokine profiles of tracheal epithelial cells differ from those of lungs. Such difference is expected as lungs also contain pneumocytes, endothelial cells and immune cells. Appropriate concert of these cells are required for virus clearance and tissue protection/repairing, resulting in host defense. Thus, the precise anti-viral mechanisms are likely complex, particularly in vivo. Nevertheless, these results suggest that RCL3 may protect tracheal epithelial cells from viral infection.

In this study, we chose intraperitoneal injection over an intranasal route in order to examine efficacy of systemic administration because such systemic route is practical in patients whose lungs and trachea are inflamed due to severe infection. Our unpublished observations show that recombinant human MBL, as an example of recombinant lectin, is detectable in BALF only following PR8 infection. The observations support previous findings that endogenous MBL in BALF increases following PR8 lung infection {Reading, 1997 #6}. These observations demonstrate that recombinant lectins leak into alveolar space of infected lungs. Another consideration is treatment regimen to increase efficacy. For example, Ebola viral infection was reduced when recombinant MBL was given 10 times of our study (Michelow et al., 2011). Thus, escalating dose and administration route may increase therapeutic efficacy of RCL3.

Previously, all recombinant lectins effectively inhibited viral infection to MDCK epithelial cells (Chang et al., 2011). In contrast, both MBL and RCL3 show little effect in inhibiting viral infection to human tracheal epithelial cells, natural targets of influenza viral infection in humans (van Riel et al., 2007). On the other hand, these recombinant lectins neutralize virus and/or inhibit viral shedding because there is significant viral titer reduction in culture supernatant. We note that such viral reduction in vitro is not significantly observed in vivo although RCL3 group tends to have reduced viral titers in the lungs compared with saline and MBL group. Other considerations for the in vivo system is that the in vitro infection assay is targeting only tracheal epithelial cells while there are other cell types, such as pneumocytes and phagocytes, thus in vivo system is complex and is multifactorial. Taken together, any viral reducing functions, including viral neutralization and inhibition of viral shedding, are also important as antiviral mechanisms to prevent viral propagation.

The lack of anti-cellular viral infection of recombinant lectins in vitro correlates with their little antiviral infection efficacy in vivo. These findings support our recent findings that

while MBL gene knockout mice did better for body weight and inflammatory scores than wild type mice in pH1N1 infection viral titers in lungs were similar (Ling et al., 2012). These observations suggest that viral clearance may be a partial to the antiviral mechanisms of lectins and that host responses to viral infection may play significant roles in fighting viral infection in vivo.

Although RCL3 treatment did not reduce viral titers in the lung it upregulates CXCL13 and CCL2, which have an anti-influenza viral immunity (Dessing et al., 2007; Rangel-Moreno et al., 2007; Wang et al., 2011). RCL3 treatment also reduces expression of CD32, a down regulator of B cell functions and proliferation (Malbec et al., 1999). These observations suggest that RCL3 is involved with enhancing the host anti-influenza virus immunity although further investigations are required to elucidate the mechanisms.

Lastly, H1N1 strains are less glycosylated compared with seasonal viral strains, thus MBL was believed not to recognize H1N1 viruses, including PR8 (Hartley et al., 1992; Hartshorn et al., 2008). We have recently demonstrated that MBL binds to PR8 through the collagenous domain and/or cysteine-rich domain (Larvie et al., 2012). Another recent study has shown that H-ficolin, a homologue of L-ficolin, has anti-H1N1 influenza A virus activities in vitro (Verma et al., 2012). Taken together, these observations suggest that recognition mechanisms of RCLs are not limited to the MBL CRD but also the collagenous domain.

In conclusion, we propose the idea that well-orchestrated cytokine responses to viral infection are important antiviral mechanisms. These host responses would provide appropriate inflammation and tissue protection, resulting in the successful host defense system. Our investigations provide the in vivo evidence that RCL3, a novel recombinant chimeric lectin, promoting these host protective mechanisms, is a potential new anti-influenza viral therapeutic agent.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We investigated efficacy of lectins in a mouse model of influenza A viral infection
- Inflammatory responses are important aspects of host defense mechanisms
- We provide a potentially new therapeutic agent against influenza A virus infection.

Fig. 1.

Efficacy of recombinant lectins in PR8 infection in vivo. 1A. Survival was shown with (PR8) or without (No virus) infection. RCL2, RCL3, MBL and saline indicated treatments. Two experiments were combined. Numbers in parenthesis indicated animals in each group. There was no statistical difference (Log-Rank methods). 1B. RCL3 treatment attenuates body weight loss. Experiments were performed as part of Fig. 1A. Percent body weight changes were plotted with mean $\pm$ SEM. *, $p < 0.05$ against saline group on day 2 and against MBL group on indicated days, including day 2 (Student's *t* test). 1C. Viral loads in the lung. Viral titers in lung homogenates were determined by Q-PCR (duplicate) and expressed as viral particles per μ g of lung RNA on indicated days. Bars indicated mean $\pm$ SD of 3 mice in each group. Experiments were performed as in Fig. 1A.

Fig. 2.

RCL3 treatment modulates lung responses differently from MBL and saline treatments during PR8 infection. 28 molecules were increased compared with no virus infection control, according to our criteria defined for this study as detailed in the materials and methods. Refer to supplementary data for raw data.

Fig. 3.

Human tracheal epithelial cell studies. PR8 viral titers (viral particles per μg of RNA) at 24 (open bars) and 48 (closed bars) hr were shown for lysates (3A) and supernatants (3B). Tracheal epithelial cells were cultured with medium control (Med) or recombinant lectins at $2 \mu\text{g}/\text{ml}$ for 48 hr. Bars indicated mean $\pm$ SD. Representative results of three experiments were shown. Statistical significance was analyzed against Med control.

*, $p < 0.0001$ (Student's t -test). Cell survival was shown with (3C) or without (3D) PR8 infection. Experiments were performed as in Fig. 3A. At 48 hr, cell survival was assayed using WST8 and was expressed as % survival of Med. Representative results of three experiments were shown. Bars indicated mean $\pm$ SD of 6 or 4 samples per group for 3C and 3D, respectively. *, $p < 0.005$ against Med and MBL group (Student's t -test).

Fig. 4.

Inflammatory responses of tracheal epithelial cells during PR8 infection. Culture supernatants at 48 hr were assayed by human cytokine antibody array. The experiments were performed as in Fig. 3. Supernatants of 3 wells were pooled and assay was performed in duplicate and expressed as arbitrary units $\pm$ SD against uninfected cell controls. Underlined molecules were also identified in lungs (Fig. 2).