Protective Levels of Neutralizing Antibodies to Influenza are Present in an IVIG (RI-002) Containing Standardized and Elevated Levels of Neutralizing Antibodies to RSV and Can Protect Influenza Infected Cotton Rats

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Introduction

Patients with primary immune deficiency disease (PIDD) have less than ideal responses to many vaccines including influenza vaccines and many vaccines are contraindicated in the immune compromised patient population. RI-002 a novel IVIG that completed a successful phase III trial in patients with PIDD, contains standardized, elevated levels of neutralizing anti-RSV antibodies as well as elevated levels of antibodies to other respiratory viruses. The virus neutralizing activity present in the serum of those vaccinated with the flu vaccine has been shown to correlate with clinical efficacy. Hemagglutination inhibition (HAI) dilutions of greater than 1:40 have been shown to correlate with protective activity against influenza virus. It was of interest to determine whether RI-002 also contained protective levels of antibody to different strains influenza and if they could mediate protection in an animal model of influenza.

Objective

To evaluate whether the elevated levels of binding antibody to influenza strains of virus present in RI-002 correlate with elevated levels of neutralizing activity and whether it would have protective activity in an animal model.

Method

Hemagglutination Inhibition Assay (HAI)
HAI assays were performed using infectious virus stocks. Sera were initially treated with receptor-destroying enzyme (RDE) from Vibrio cholerae overnight at 37°C. The enzyme was then inactivated at 56°C for 30 min and PBS was added to the sera for a final dilution of 1:10. The HAI assay was performed in V-bottom 96-well plates using 4 hemagglutinating units (HAU) of virus. The HAI titer was reported as the reciprocal of the highest dilution of serum in which complete inhibition of hemagglutination occurred. Hemaglutination inhibition titers to the following strains of influenza virus was performed: Influenza A (H1N1)pdm 09 control antigen (A/California), Influenza A (H3) (A/Texas/50/2012), Influenza B, Yamagata lineage (B/Phuket/3073/2013), Influenza B Victoria lineage (B/Brisbane/60/2008), A/Anhui/1/13 (H7N9):PR8, E3, HA:256, H5 SVP from Medign, A/Viet Nam/1203/2004, H9 SVP from Medigen, A/Hong Kong/33982/2009,0, H10 VLP from Medigen, H10N1 (A/teal/Egypt/12908-NAMRU3/2005 H10N1).

Cotton Rat Model of Influenza
Influenza/A California 07/2009 (H1N1) was propagated in embryonated chicken eggs. Animals were inoculated intraperitoneally with 1.5 g/kg RI-002 one day prior to infection with 106 TCID50 Influenza/A California 07/2009 (H1N1) per animal and sacrificed for analysis 1 and 4 days after infection.

Pulmonary Histopathology
Lungs were dissected and inflated with 10% neutral buffered formalin to their normal volume, and then immersed in the same fixative solution. Following fixation, the lungs are embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). Four parameters of pulmonary inflammation are evaluated: peribronchiolitis (inflammatory cell infiltration around the bronchioles), perivascultis (inflammatory cell infiltration around the small blood vessels), interstitial pneumonia (inflammatory cell infiltration and thickening of alveolar walls), and alveolitis (cells within the alveolar spaces).

Real-Time PCR
Total RNA is extracted from homogenized lung tissue using the RNeasy purification kit (QIAGEN). One µg of total RNA was used to prepare cDNA using Super Script II RT (Invitrogen) and oligo dT primer (1µl, Invitrogen). For the real-time PCR reactions the Bio-Rad iQ™ SYBR Green Supermix is used in a final volume of 25 µl, with final primer concentrations of 0.5 µM. Reactions are set up in duplicates in 96-well trays. Amplifications were performed on a Bio-Rad iCycler for 1 cycle of 95ºC for 3 min, followed by 40 cycles of 95ºC for 10 seconds (s), 60ºC for 10 s, and 72ºC for 15 s.

Results

Six different lots of RI-002 were tested by HAI to six strains of influenza virus. Titers ranged from 1:160-1:640 (Table 1)

Table 1: Hemagglutination Inhibition Titers in RI-002 to Various Strains of Influenza

Samples/Antisera	pdm H1N1	H3N2 (Tex)	H3N2 (Sw)	B-Vic	B-Yam	H5N1 (VN/1203)	H9N2 (HK/2009)	H7N9 (A/Anhui/1)	H10N8	H10N1 (Teal/Egypt)
RI-002 Lot #1	640	640	160	320	320					
RI-002 Lot #2	640	320	160	320	320	20	<10	20	<10	<10
RI-002 Lot #3	320	320	80	320	160	40	<10	20	<10	<10
RI-002 Lot #4	640	640	160	320	320	20	<10	20	<10	<10
RI-002 Lot #5	640	320	160	320	320	20	<10	40	<10	<10
RI-002 Lot #6	640	320	160	320	320	20	<10	20	<10	<10
Positive sera	>1280	>1280	>1280	>1280	>1280	320	1280	320	1280	1280
Negative control	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

HAI inhibition titers were as described in methods. Six lots of RI-002 that were manufactured at different times were tested. Negative controls were different strains of avian viruses.

PK Based Modeling of Hemagglutination Inhibition Titers
With the assumption that the dilution effect of the anti-influenza HAI titer after infusion of RI-002 would be the same as observed in the anti-RSV antibody concentration, the final concentration of neutralizing anti-influenza antibody that would be present in patients infused with RI-002 was calculated. In order to estimate the amount of neutralizing antibodies that would be present in the serum of patients infused with RI-002, the anti-RSV titer data collected during pharmacokinetic analysis of the phase III trial was used. To calculate the dilution observed for the RSV antibody after infusion in PIDD subjects, the anti-RSV concentration in RI-002 was divided by the RSV concentration in the serum of the 30 patients infused with RI-002 and who were participating in the PK wing of the trial.

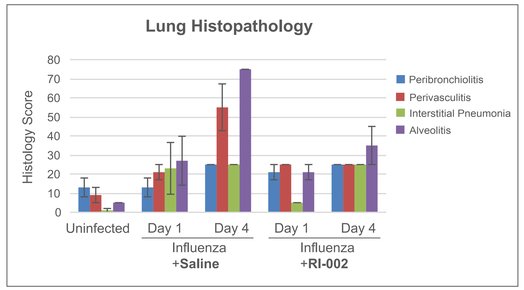
Table 2: PK Based Modeling of Hemagglutination Inhibition Titers to 5 Strains of Influenza Virus After Infusion of RI-002 into Patients with PIDD (protective titer is ≥1:40)

	pdm H1N1		H3N2 (Tex)		H3N2 (Sw)		B-Vic		B-Yam	
	<500 mg/kg	>500 mg/kg	<500 mg/kg	>500 mg/kg	<500 mg/kg	>500 mg/kg	<500 mg/kg	>500 mg/kg	<500 mg/kg	>500 mg/kg
Times post infusion										
Prior to infusion	33	33	23	23	9	9	18	19	21	21
60 min	**96**	**124**	**67**	**87**	26	34	**54**	**70**	**62**	**80**
2 hours	**77**	**105**	**54**	**74**	21	29	**43**	**59**	**50**	**68**
24 hours	**83**	**118**	**58**	**83**	23	32	**46**	**66**	**53**	**76**
48 hours	**70**	**90**	**49**	**63**	19	25	39	**50**	**45**	**58**
4 days	**65**	**75**	**46**	**52**	18	20	37	**42**	**42**	**48**
7 days	**76**	**91**	**53**	**64**	21	25	**42**	**51**	**49**	**58**
14 days	**56**	**63**	**39**	**45**	15	17	31	35	36	**41**
21 days	**51**	**43**	**36**	30	14	12	29	24	33	28
28 days	39	32	28	22	11	9	22	18	25	21

Bold numbers reflect concentrations of antibody that would be deemed to be protective for influenza

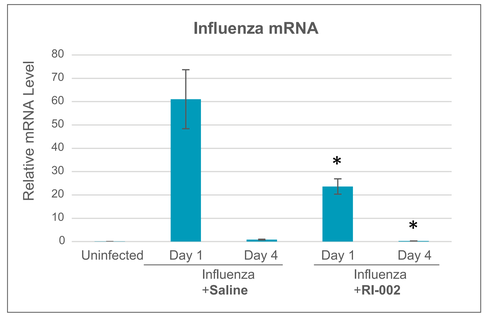
Lung histopathology of cotton rats given RI-002 followed by influenza infection, are shown for one and four days following infection. Infection induced a significant inflammatory response in the lungs four days after infection which was significantly reduced in the lungs of cotton rats given RI-002, but not those given saline.

Figure 1



RNA was measured in the pulmonary tissue of control and infected rats and there was a significant reduction in the animals that were pretreated with RI-002.

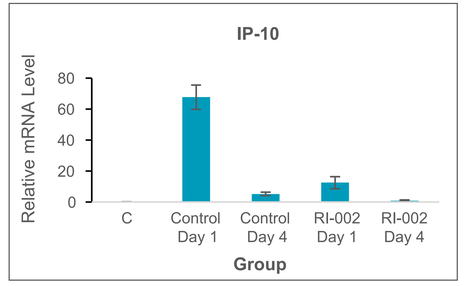
Figure 2



*p<0.05 comp to same-day samples from influenza-infected, saline treated animals

IFN-gamma-inducible protein 10 (IP-10, CXCL10), is a chemokine secreted from cells stimulated with type I and II IFNs and, is a chemo-attractant for activated T cells. Expression of IP-10 is seen in many Th1-type inflammatory diseases, where it is thought to play an important role in recruiting activated T cells into sites of tissue inflammation. Animals pretreated with RI-002 prior to infection with influenza showed a reduction in the expression of this chemokine in their pulmonary tissue. (FIG 3)

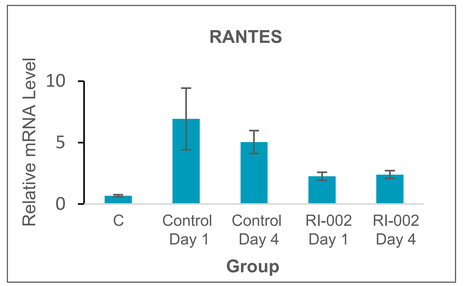
Figure 3



*p<0.05 comp to same-day samples from influenza-infected, saline treated animals

RANTES or CCL5 is a chemotactic cytokine or chemokine, which is chemotactic for T cells, and plays an active role in recruiting leukocytes into inflammatory sites. Animals pretreated with RI-002 prior to infection with influenza showed a significant reduction in the expression of this inflammatory chemokine. (FIG 4)

Figure 4



Conclusion

RI-002 used in this study, which uniformly contain standardized, elevated levels of neutralizing antibodies to RSV, was also found to contain elevated levels of neutralizing antibodies to contemporary strains of influenza virus in quantities that may be sufficient to confer passive immunity to infused subjects based on PK modeling calculations. Neutralizing titers were found to all 5 strains of influenza that were tested and showed consistent levels and results in all of the manufactured lots of RI-002 evaluated in this study. The in vivo efficacy of RI-002 was tested in a cotton rat animal model infused with RI-002 which demonstrated that infusion of RI-002 prior to infection could prevent influenza mediated pulmonary inflammation, suppression of pulmonary influenza mRNA, and suppression of influenza induced inflammatory cytokine formation. RI-002 is an IVIG with a unique antibody composition that may contain high levels of neutralizing activity against respiratory viruses other than RSV. Additional studies in humans will be needed to determine whether activity against RSV, influenza, and other viruses may improve clinical outcome in patients infused with RI-002.

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