FMF assay for assessing vaccine generated antibodies in a biomimetic manner

2015

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FMF ASSAY FOR ASSESSING VACCINE GENERATED ANTIBODIES IN A BIOMIMETIC MANNER

by

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M.S., University of Central Florida, 2008

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science
in the Department of Biomedical Science
in the College of Medicine
at the University of Central Florida
Orlando, Florida

Spring Term
2015

Major Professor: Annette Khaled

ABSTRACT

Traditional functional assays such as hemagglutination inhibition (HAI) and micro-neutralization (MN) assays have been routinely used for assessing the vaccine response, since influenza vaccine has been administered in people (1940). Such assays are not always predictive regarding the protection conferred by the influenza vaccine and are not able to monitor neutralization related to stem region of influenza hemagglutinin responsible for virus membrane fusion in the endosomes. In order to study Influenza vaccine response in a more biomimetic manner and overcome the deficiencies of the traditional functional assays, we developed a fluorescent membrane fusion assay (fMF). The assay uses viruses labeled with Octadecyl Rhodmaine B Chloride (R18) to monitor two major neutralization pathways: blocking the attachment of virus to the target cells and blocking of virus membrane fusion in the endosomes. The latter was tested using endosomal acidification inhibitor Bafilomycin a1 which blocked membrane fusion by 85%. Specificity of the assay was tested using two different subtypes of viruses H1N1 (A/Puerto Rico/8/1934 and A/Brisbane/59/2007), and H3N2 virus (A/Aichi/68) with their respective subtype specific stem specific monoclonal antibodies: M145, Aca-1, Aca-2 (H1N1 specific) and Aca-3 (H3N2 specific). Subtype specific mAbs blocked membrane fusion, while a mismatch in virus subtype and the mAb resulted in lack of blocking. We also studied the effect of H1N1 head specific mAb Aca-4, which not only blocked attachment of the virus, but also demonstrated blocking of membrane fusion. Results were validated by testing pre- and post-sera from 2009 seasonal Influenza vaccination and to show that at higher Ab concentration the majority of virus (85%) was blocked from attaching cells, but at lower Ab concentration, where attachment could not be prevented, blocking of membrane fusion was still in effect - up to 50%.

Sera screening experiments showed that sera antibodies work beyond just blocking attachment. They also may neutralize the already attached virus by blocking fusion of the viral membrane in the endosomes. The assay has the capacity to monitor blocking of attachment and fusion in a single run. Therefore, it is more representative regarding the natural process of infection and the corresponding neutralization pathways. The assay is unique in terms of assessing stem specific antibodies; stem specific response and its measurement are relevant for the advancement of a universal influenza vaccine.

This thesis is lovingly dedicated to Siddharth and Vinayak.
Thank you for the joy and love you both bring in each day of my life.

ACKNOWLEDGMENTS

I would like to acknowledge my employer Sanofi-Pasteur for supporting my graduate studies. Being a Sanofi employee, I am privileged to be part of a company that fosters innovation through its employees and invests in their development.

I am deeply grateful to my supervisor to Dr. Anatoly Kachurin (Director, Sanofi-Pasteur VaxDesign campus) for mentoring my project and providing his intellectual input. Thank you for always encouraging me to move forward and believing in my capabilities.

I would like to sincerely thank Dr. William Warren (Vice President, Sanofi-Pasteur VaxDesign campus) for his support and guidance. Thank you very much for taking time from your busy schedule to review my work and teaching me how to give better presentations. Your complete attention to detail and demand for excellence have made a big impact on my scientific acumen.

I am truly obliged to my mentor at UCF, Dr. Annette Khaled (Associate Professor, College of Medicine UCF). Thank you very much for accepting me as your student, reviewing my work from time to time and keeping my work on track. I would also like to thank Dr. William Self (Associate Professor, College of Medicine UCF) for serving on my thesis committee and providing his inputs and insights.

Finally, I would like to thank my friends and colleagues Atul Asati, Jyoti Gangur and Olga Kachurina for their kindness, support and from time to time their very useful suggestions.

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LIST OF ACRONYMES

A/Aichi/68 H3N2	X-31
A/Brisbane/59/2007 H1N1	Brisbane H1N1
A/Puerto Rico/8/1934 H1N1	PR8
fMF	Fluorescent membrane fusion
HA	Hemagglutinin
HI; HAI	Hemagglutination inhibition
mAb	Monoclonal antibody

INTRODUCTION

Influenza is a respiratory disease caused by influenza virus. The virus causes seasonal outbreaks every year and pandemics are caused sporadically. The burden of disease is tremendous, worldwide every year there are hundreds of millions of influenza cases, with 3 to 5 million cases of severe illnesses and 250,000 to 500,000 people die from the illness[1]. The disease readily attacks and spreads easily in immunocompromised individuals such as older population, younger children and people with ongoing medical conditions[2].

Influenza vaccine is the most widespread method of protection against influenza virus. Inactivated seasonal Influenza vaccines came into practice in 1940's followed by live attenuated influenza vaccine in 1960's[3]. The response to the vaccine is mounted in form of serum antibodies. These antibodies are directed mainly towards the globular head of virus's surface glycoprotein Hemagglutinin (HA). In order to escape the immune response of its host, the virus frequently mutates epitopes on the head region of its HA[4]. For this reason, influenza vaccines need to be reformulated every year, based on the currently circulating strain recommendations made by WHO's influenza surveillance programs[5]. This approach to formulating vaccines is very well established and saves human lives and resources every year. On the other hand, this process of influenza vaccine production costs $1-2 billion every year. In addition to the high cost associated with yearly vaccine reformulation, pandemic strains are unexpected, difficult to predict, and seasonal strain matching with vaccine is not always accurate[6]. In the wake of these issues, the influenza vaccines portfolio is evolving.

There have been many new advances made to existing influenza vaccine such as using higher dose of HA to generate a better antibody response for elderly population. Recently,

quadrivalent flu vaccine was approved to provide broader protection as it contains two A strains (H1N1 and H3N2) and two B strains, to cover both most probably expected B lineages in the vaccine. In addition, modifications such as intradermal vaccine, using oil in water emulsion adjuvant have also been made to existing licensed vaccine[7]. In order to alleviate the need for reformulating vaccine every year, current scientific efforts are enthusiastically directed towards development of a Universal influenza vaccine. The universal vaccine approach is based on targeting conserved epitopes on influenza virus proteins such as stem region of HA, nucleoprotein and M2 channel protein[8].

The serum antibody response towards globular head of HA is accepted as primary correlate of protection for influenza vaccine. This antihead response is universally tested by hemagglutination inhibition (HI, or HAI) assay. HI assay is based upon agglutinating ability of influenza virus towards red blood cells. In presence of HA head specific antibodies the agglutination of RBCs by influenza virus is inhibited. An HI titer 40 is generally recognized as measure of 50% protection[9]. With the evolving influenza vaccine portfolio the correlates of protection are changing[7]. For example, with universal flu vaccine being based mainly on stem region of HA, measuring stem specific response is becoming crucial and the stem specific response has become a new correlate of protection.

The HA protein on influenza virus is a trimeric molecule consisting of three identical HA molecules. The protein appears as spikes on the surface of influenza virus envelope. The HA spike is divided into three domains: globular head responsible for virus attachment to host cells, stem domain which is responsible for membrane fusion in endosomes of host cells and a transmembrane domain which holds the HA trimer into viral membrane[10]. The globular head

is the most antigenic and variable region. The epitopes on head are 98% variable among different subtypes and strains of influenza virus. On the other hand, the stem region has epitopes which are more than 98% conserved amongst different influenza viruses. The highly conserved structure of HA stem amongst various subtypes of influenza virus make it a very attractive target for developing universal flu vaccine[6]. Scientists have shown that natural infection and seasonal vaccine are able to generate stem specific antibodies in humans. Many stem specific monoclonal antibodies (mAbs) have been isolated from humans that are able to neutralize a wide range of influenza viruses of different subtypes and strains[11-13]. Now the efforts are being made to boost up the stem specific antibody response after vaccination. Approaches towards such vaccines include stem based vaccine constructs without globular head[14] and chimeric hemagglutinin based vaccine constructs[15]. There are studies that have shown that stem based vaccine constructs are able to generate heterosubtypic neutralizing antibodies in animal studies[15, 16]. Although there is a definitive amount of progress that has been made in direction of stem based vaccines, still there are few substantive methods to assess general anti-virus immune response, and no universally accepted laboratory methods that can be used to measure stem specific response. Therefore, there is need to develop a method that can measure this new correlate of protection i.e., functional effect of stem specific antibodies.

On another note, a study in neonatal pigs showed that mismatch between vaccine and infecting virus has been reported to generate stem specific antibodies that increase endosomal membrane fusion, thereby increasing the morbidity of the disease[17]. Therefore, it is important to monitor membrane fusion and stem specific response not just in context of universal flu vaccine, but also to test the safety of vaccine that generates stem specific antibodies.

Summary Of Thesis:

The functional assays currently used for measuring antibody response generated by influenza vaccine provide only fragmentary information about the mode of protection of the antibodies. For example, HI assay only measures titers in terms of blocking of attachment of virus to RBCs. MN assay cannot differentiate between blocking of attachment and blocking of membrane fusion by neutralizing antibodies. The aim of this project was to develop an assay that can assess functional characteristics and mechanisms of protection provided by influenza vaccine generated antibodies, i.e. blocking of virus attachment to the cells (pre-attachment neutralization pathway) and blocking fusion of viral membrane in the cell endosomes (major element of post-attachment neutralization).

Influenza virus attaches to its target cells by binding through globular head of HA trimer on its surface to the Sialic acid receptors present on glycolipids or glycoproteins of cellular membranes. Once the virus is attached to target cells, it gets internalized into endosomes due to cycling of cellular membranes. In the endosomes, the acidic environment splits apart the HA trimer, opening up the globular head and exposing the stem region. The stem region of the protein then turns itself inside out and exposes its hidden fusion peptide at its N terminus. Fusion peptide then inserts itself into endosomal membrane, bringing endosomal membrane and viral membrane closer together and fuse. The fusion of two membranes leads to stalk pore formation and release of viral RNA in cellular cytoplasm through the pore[18]. fMF assay monitors the process of virus attachment to the cells and endosomal membrane fusion by kinetically following interaction of target cells (vero cells) with Octadecyl Rhodamine B Chloride (R18) dye labeled Influenza virus.

Use of R18 dequenching for studying kinetics of fusion between biological membranes was first described by Hoekstra, D. et al in 1984[19]. Since then it has become a widely used label for studying fusion kinetics. R18 dye is lipophilic in nature; it enters the envelope of the virus and remains in the envelope in self quenched state. When the labeled virus fuses with another membrane such as host cell endosomal membrane, the dye gets diluted and becomes fluorescent. This increase in fluorescence due to dye dilution in membrane is indicative of viral endosomal membrane fusion[20].

The ability of fMF assay to monitor membrane fusion was tested using Bafilomycin a1 a specific endosomal acidification inhibitor. As Influenza needs acidic environment for membrane fusion to occur, the inhibitor blocked membrane fusion by 85%. Specificity of the assay was tested using two different subtypes viruses H1N1 (A/Puerto Rico/8/1934 and A/Brisbane/59/2007), and H3N2 virus (A/Aichi/68) with their respective subtype specific stem specific monoclonal antibodies. As stem region on HA is key participant in membrane fusion, stem specific mAbs effectively blocked membrane fusion. In addition, only subtype specific mAbs were able to block membrane fusion. There was no cross neutralization between different subtypes and their mAbs. In order to study effect of antibodies on attachment of virus, one H1N1 head specific mAb was also included in the study. The head specific antibody blocked both attachment of the virus and membrane fusion. The study was concluded by testing pre- and post-sera from 2009 seasonal Influenza vaccination. The sera showed characteristics similar to head specific antibodies, at lower sera dilution (higher Ab concentration) majority of virus (85%) was blocked from attaching cells, but at higher sera dilution (lower Ab concentration) where attachment could not be prevented, blocking of membrane fusion was still in effect - up to 50%.

In conclusion fMF assay is a method that is able to monitor interaction of influenza virus and its target cells in a biomimetic manner. It is an assay that can functionally characterize response of post vaccination sera into two major neutralization pathways: blocking of attachment of virus and blocking of viral endosomal membrane fusion. By monitoring both the pathways in the same run the assay is able to demonstrate the interplay of these two neutralization pathways in presence of antibodies. With conventional assays only being able to either monitor blocking of attachment or altogether neutralization, fMF assay opens the door for monitoring an important new correlate of protection, the membrane fusion. Monitoring membrane fusion is very important in context of universal flu vaccine and pandemic threats.

METHODOLOGY

Labeling Influenza Virus

Influenza viruses PR8 (A/Puerto Rico/8/1934 H1N1), X-31(A/Aichi/68 H3N2) (Charles River laboratory) and Brisbane H1N1 (A/Brisbane/59/2007 H1N1) (Sanofi Pasteur) were labeled with Octadecyl Rhodamine B chloride dye (R18) (Life Technologies). Viral protein content was determined using BCA kit (Pierce Technology) using high grade Bovine Serum Albumin (BSA; Sigma-Aldrich) as internal standard. 100µg of viral protein was labeled with 5µg R18 while vortexing. Labeled virus was then incubated at room temperature for one hour on orbital shaker. The labeled virus was stored at 4ºC. The virus was used within one week after labeling.

Vero Cell Culture

Vero cells were obtained from ATCC, USA. Cells were grown in Iscove's Modified Dulbecco's Medium (IMDM, ATCC) containing 4mM L-glutamine, 4500mg/L glucose and 1500mg/L sodium bicarbonate with fetal bovine serum (10%) and 1% penicillin-streptomycin-glutamine (Life Technologies). Cells were maintained at 37ºC, 5% CO_2 in a humidified incubator.

Fluorescent Membrane Fusion Assay

1µg of labeled virus was incubated with 500,000 vero cells on ice in BSA buffer ,1% Bovine serum albumin (Sigma-Aldrich) in Dulbecco phosphate buffered saline (DPBS) (Lonza) for 45 minutes. The cells with virus were then washed two times with cold BSA buffer. The cells were then resuspended in BSA buffer pre-warmed at 37ºC. The kinetics of interaction of virus

with the cells was then read on ELISA plate reader (Biotek instruments) in fluorescence mode. The excitation wavelength of 530 nm was used and emission at 590 nm was observed. The kinetic study was carried out for 30 minutes with an interval of 10 to 20 seconds. At the end of kinetics study, 10µl of 10% Triton X (Invitrogen) was added to cells and fluorescence generated after addition of Triton X was recorded.

In order to assess the ability of fMF assay to monitor endosomal fusion, an endosomal acidification inhibitor Bafilomycin a1 (Sigma Aldrich) was used. For the experiments carried out in presence of Bafilomycin a1, the BSA buffer in fMF assay was replaced with a 100 nM solution of Bafilomycin a1 prepared in BSA buffer.

In order to assess the subtype specificity of fMF assay, a group of subtype specific mAbs were tested in the fMF assay. Table 1 shows the list of all the antibodies used in this study.

For the experiments carried out in presence of monoclonal antibodies (mAbs), the virus was incubated overnight at 4°C with the mAbs diluted at appropriate concentration in BSA buffer. The viruses preincubated with mAbs were then incubated with cells and the fMF assay was carried out in the same way as described above.

Table 1: List of monoclonal antibodies

mAb	Epitope specificity	Source
M145	H1N1 stem	Takara Bio Inc
Aca-1		Sanofi Pasteur Cambridge Campus
Aca-2		
Aca-3	H3N2 stem	
Aca-4	H1N1 head	

Pre and post vaccination sera obtained from human donors who received 2008 influenza vaccine were analyzed using fMF assay. The donor sera were diluted appropriately in BSA buffer. The viruses were preincubated with diluted sera for one hour at 4°C. The viruses were then incubated with cells and the fMF assay was carried out in the same way as described above.

RESULTS

Scheme Of Study

The study was divided into five stages, and their deliverables are described below. Figure 1 shows the breakdown of these stages.

Stage 1: Show that the presence of endosomal acidification blocker blocks membrane fusion.

Stage 2: Test the effect of presence of stem specific mAbs on membrane fusion. Demonstrate the specificity of fMF assay by testing two different subtypes of viruses PR8 H1N1 and X-31 H3N2 with their subtype specific mAbs.

Stage 3: Transition to a vaccine strain influenza virus, Brisbane H1N1. Use fMF assay to study blocking of membrane fusion of Brisbane H1N1 virus in presence of stem specific antibodies

Stage 4: Use fMF assay to study blocking of attachment of Brisbane H1N1 virus in presence of head specific antibodies

Stage 5: Use fMF assay to study effect of presence of pre and post vaccination on attachment and membrane fusion of Brisbane H1N1 virus.

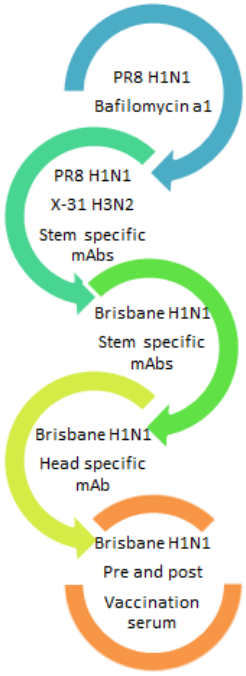

Figure 1 Scheme of study

Effect of Blocking Membrane Fusion on fMF Read Out

In order to assess whether fMF assay that is based on dequenching of R18 fluorescent probe can monitor endosomal processing of influenza virus, an endosomal acidification blocker Bafilomycin a1 was used. Bafilomycin a1 is an H+ VATPases blocker. V-ATPase is a proton pump which is responsible for pumping protons into endosomes and acidifying the endosomal compartments.

For fusing with endosomal membrane, the influenza, virus needs presence of acidic environment in the endosomes. Therefore, presence of bafilomycin a1 should have a blocking effect on membrane fusion.

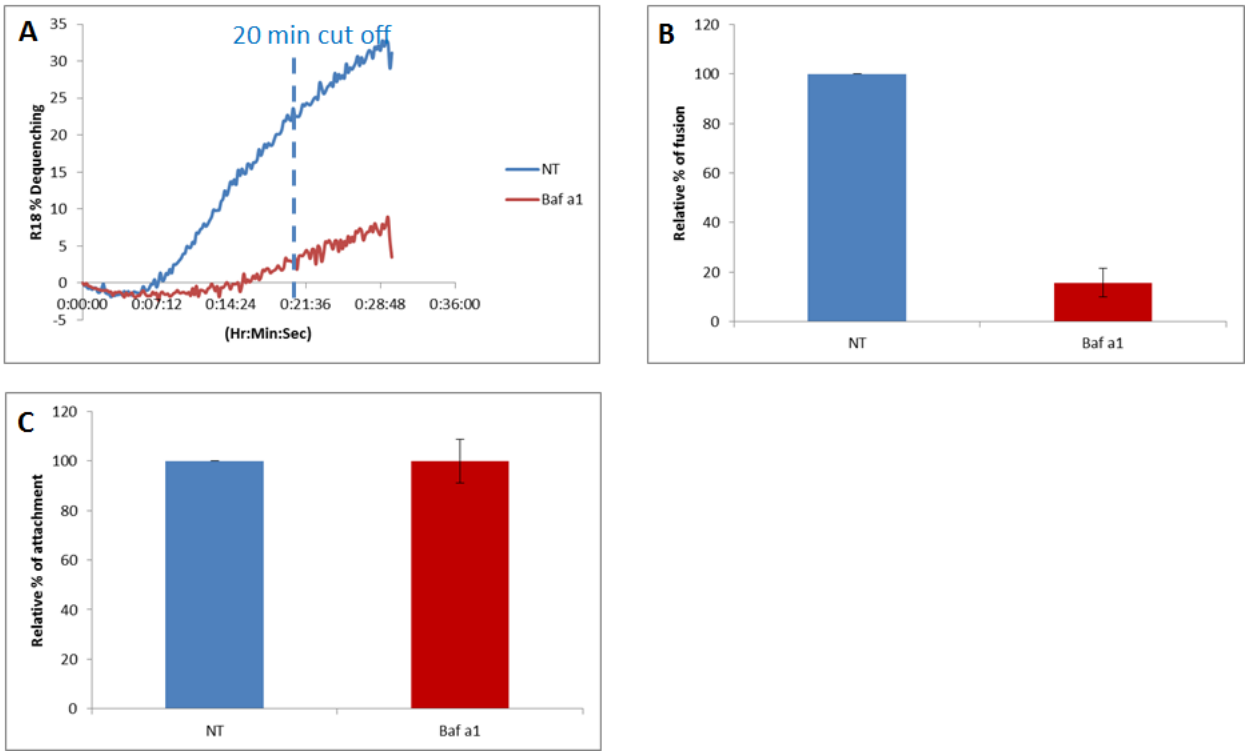

Figure 2 Endosome acidification inhibitor blocks R18 dequenching

A: R18 dequenching %, relative to total fluorescence.

B: Fusion at 20 minutes, relative to No Treatment.

C: Total virus attachment relative to No Treatment, determined by measuring total fluorescence after adding detergent that disperses membranes and releases the dye.

Figure 2 shows that in comparison to no treatment, using endosomal acidification blocker Bafilomycin A1 reduced the relative fusion by ~ 85% without affecting attachment of virus to cells. This shows that the dequenching of R18 observed in our experiments is indicative of endosomal fusion of influenza virus. We also observed a lag of ~ 7 minutes before the beginning of dequenching, this lag possibly corresponds to endocytosis of virus by the cells. This experiment was a first step to show that the fMF assay that uses R18 dequenching can address influenza virus endocytosis and endosomal fusion.

Effect Of Stem Specific Antibodies on Membrane Fusion Of Two Different Subtypes Of Influenza Viruses

In order to prove that fMF assay can represent endosomal membrane fusion, stem specific mAbs were used to test the system. Since stem region of hemagglutinin plays an important role in the membrane fusion process, stem specific antibodies should block membrane fusion.

Two viruses belonging to two different subtypes, PR8 (H1N1) and X-31 (H3N2) were tested with subtype specific stem related mAbs in order to to demonstrate the ability of fMF assay to differentiate between different subtypes of viruses and their respective antibodies.

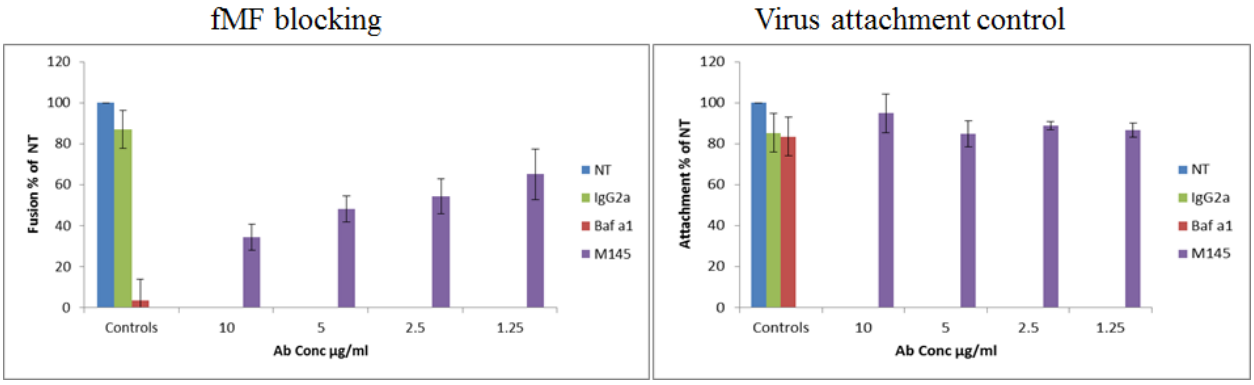

Figure 3 H1N1 specific mAb M145 blocks fusion of PR8 (H1N1) virus

Figure 3 shows effect of stem specific murine mAb M145 on membrane fusion and attachment of PR8 virus. M145 is known to be a H1N1 stem specific mAb and it blocked fusion of a H1N1 virus. 50% titer for M145 with PR8 virus was 2.6 µg/ml. M145 blocked fusion of the virus without affecting its attachment to vero cells. Stem region of the HA plays important role in fusion of viral and endosomal membranes. Blocking of fusion in presence of stem specific mAb shows that fMF assay is indicative of endosomal fusion process. In most of the following experiments, M145 was used as a positive control at 10 mg/ml.

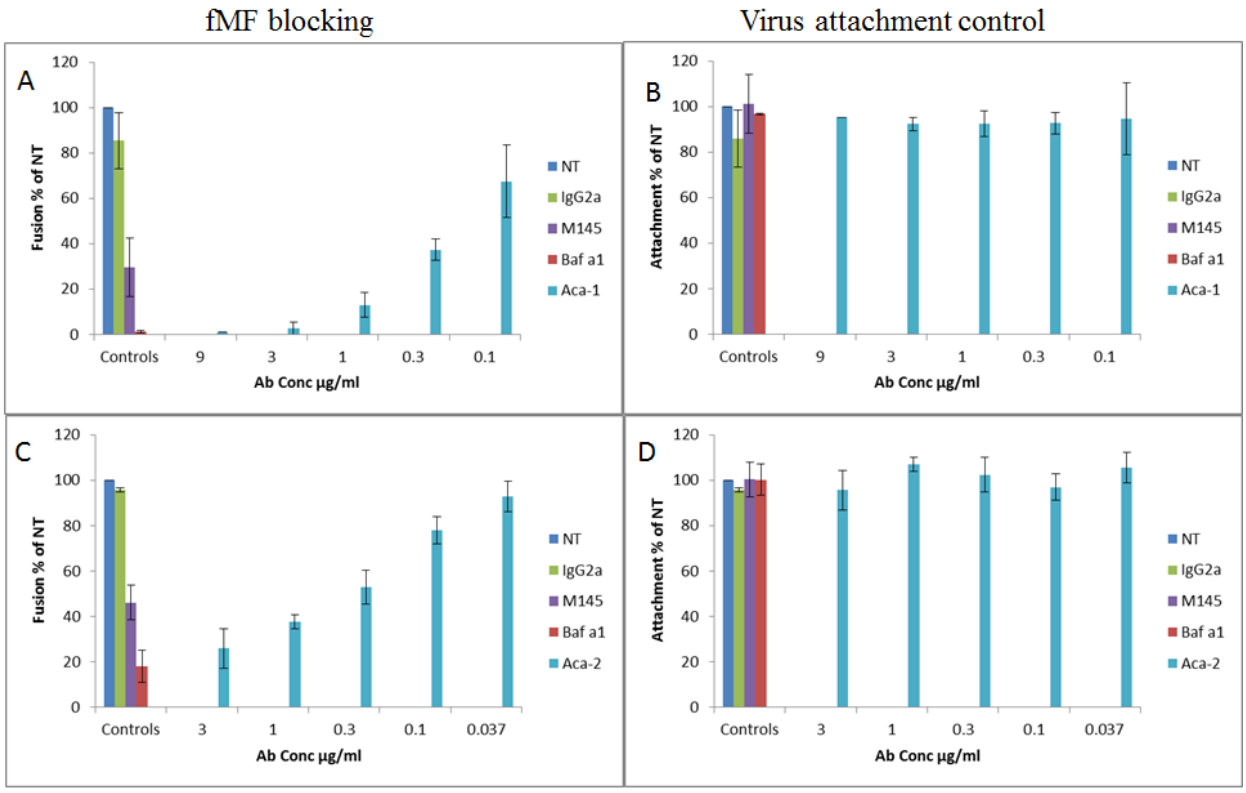

Figure 4 H1N1 specific mAbs Aca-1 and Aca-2 block fusion of PR8 (H1N1) virus

A and B: Effect of mAb Aca-1 on blocking membrane fusion and attachment of PR8 virus.

C and D: Effect of mAb Aca-2 on blocking membrane fusion and attachment of PR8 virus.

Figure 4 shows effect of human derived mAbs Aca-1 and Aca-2 on membrane fusion and attachment of PR8 virus. Both antibodies blocked membrane fusion in dose dependent manner without affecting the attachment of virus to vero cells. Aca-1 and Aca-2 are H1N1 stem region specific mAbs, their blocking of membrane fusion of an H1N1 virus without affecting its attachments shows that fMF assay is able to observe endosomal membrane fusion in fMF assay. The 50% titer of the Aca-1 and Aca-2 mAb's were 0.2 and 0.5 µg/ml respectively.

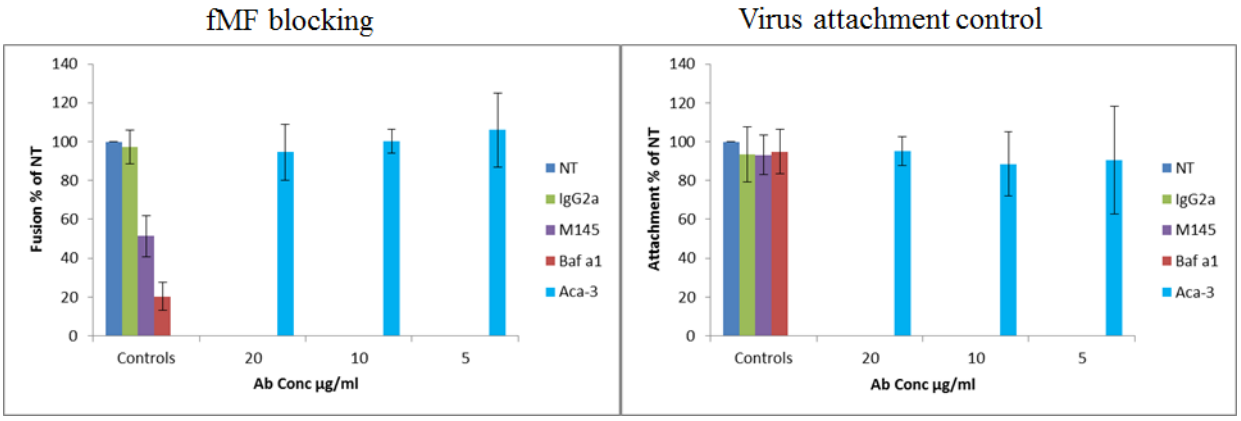

Figure 5 H3N2 specific mAb Aca-3 does not block fusion of PR8 (H1N1) virus

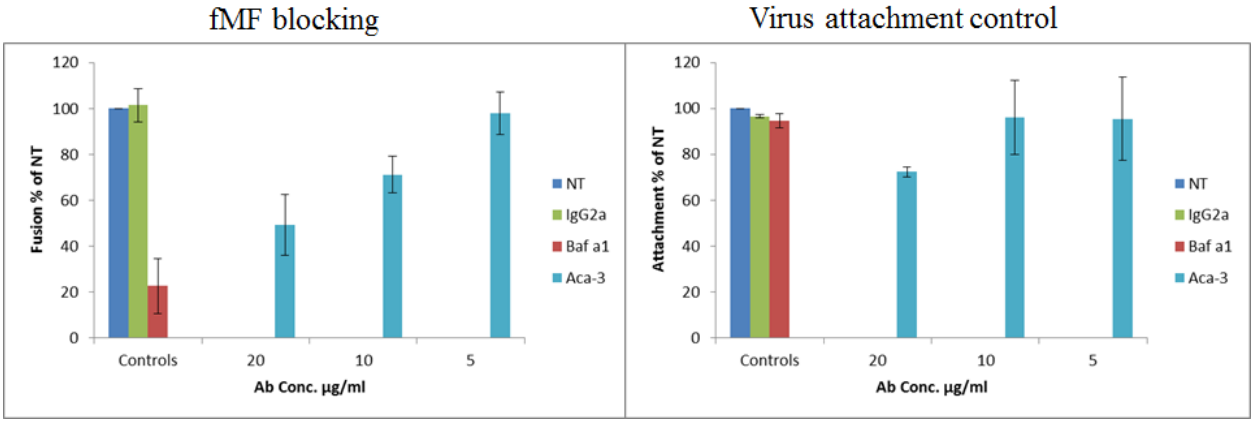

Figure 6 H3N2 specific mAb Aca-3 blocks fusion of X-31 (H3N2) virus

Figure 5 shows that an H3N2 stem specific mAb Aca-3 does not block membrane fusion of PR8, an H1N1 virus. The experiment was carried out to demonstrate the specificity of fMF assay. The membrane fusion of H1N1 virus can only be blocked by a H1N1 specific mAb, not with a H3N2 specific mAb.

Figure 6 shows that Aca-3 which is a H3N2 stem specific mAb blocks membrane fusion of X-31 H3N2 virus in dose dependent manner. This corroborates the capacity of fMF assay to differentiate between H1N1 and H3N2 specific antibodies and between corresponding viruses.

At 20 µg/ml Aca-3 showed a slight and possibly nonspecific effect on attachment as well, this could be due to high Ab concentration.

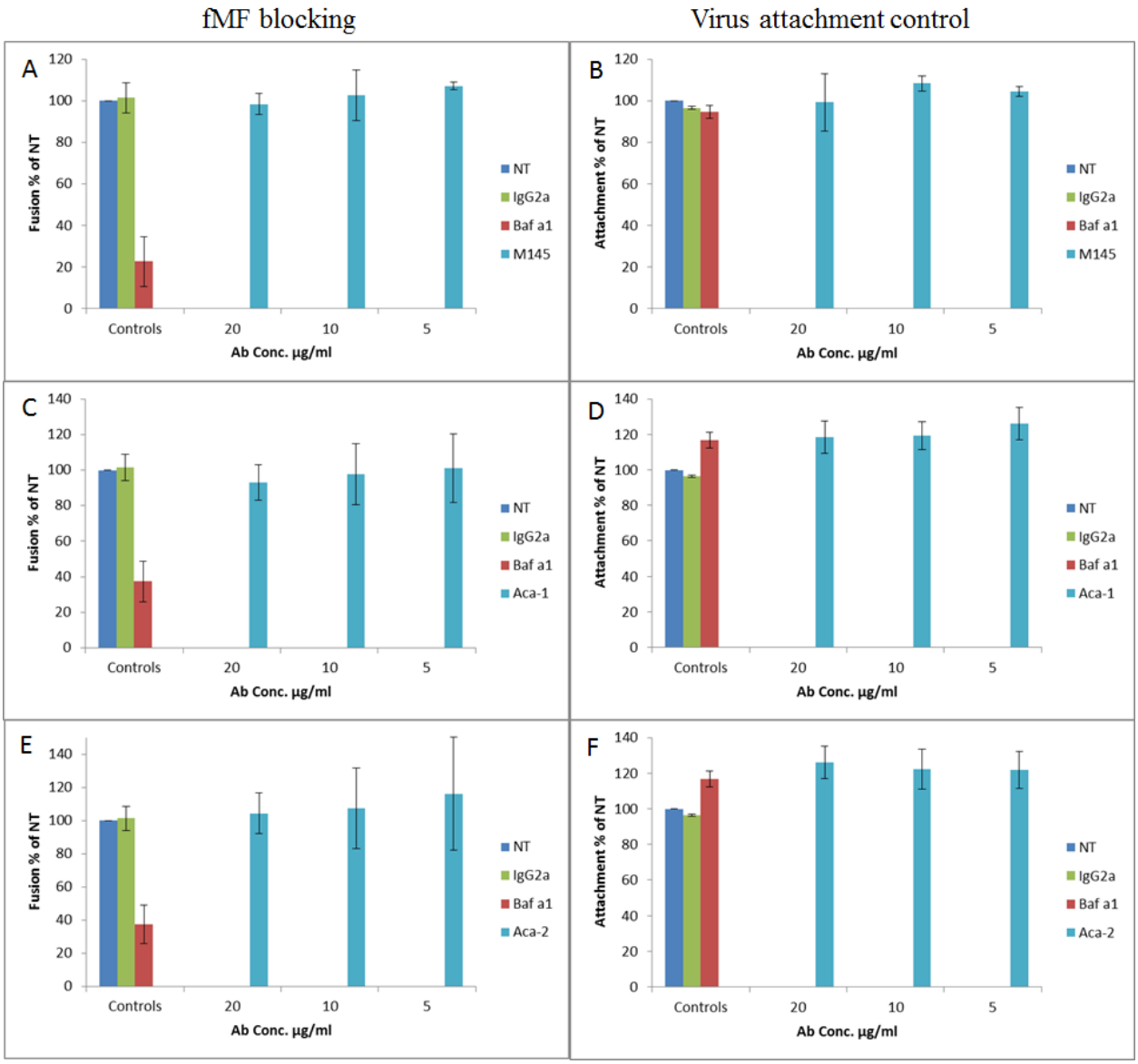

Figure 7 H1N1 specific mAbs do not block fusion of X-31 (H3N2) virus

A and B: Effect of mAb M145 on blocking membrane fusion and attachment of X-31 virus.

C and D: Effect of mAb Aca-1 on blocking membrane fusion and attachment of X-31 virus

E and F: Effect of mAb Aca-2 on blocking membrane fusion and attachment of X-31 virus

Figure 7 shows the effect of H1N1 stem specific antibodies on membrane fusion and attachment of X-31 H3N2 virus. H1N1 stem specific mAbs M145, Aca-1 and Aca-2 were not able to block membrane fusion and attachment of X-31 virus to vero cells. This set of experiments served as negative control for H3N2 subtype of virus and were carried to demonstrate specificity of fMF assay.

The series of experiments with mAbs showed that membrane fusion can be blocked by stem specific mAbs. This further supported capacity of fMF assay to monitor membrane fusion and its blocking. The subtype specific antibodies showed that fusion of influenza viruses of two different subtypes (PR8 and X-31) could only be blocked by mAbs specific to their serotypes. These results show that fMF assay is very specific and able to differentiate between two very distinct subtypes of influenza viruses.

Applicability Of fMF Assay To A Seasonal Influenza Strain A/Brisbane/59/2007 H1N1

In order to achieve the main objective of this project "Use fMF assay to test vaccine generated Abs in biomimetic manner", a more recent vaccine strain Brisbane H1N1 was tested in the fMF experiments.

Table 2 Summary of the fMF assay titers using Brisbane H1N1 virus and stem specific mAbs

mAb	Epitope specificity	Titer µg/ml
Aca-1	H1N1 stem	1.25
Aca-2		1.25
Aca-3	H3N2 stem	Subtype mismatch hence does not block fusion of H1N1 virus

Aca-1, Aca-2 and Aca-3 were used to test applicability of fMF assay to Brisbane H1N1 virus. Table 2 shows the summary of results from dose titration experiments of mAbs with Brisbane H1N1 virus. H1N1 stem specific mAbs Aca-1 and Aca-2 were able to block fusion of Brisbane H1N1 virus. Aca-3 which is H3N2 specific mAb did not have an effect on the fusion of Brisbane H1N1 virus. This shows that vaccine specific strain Brisbane H1N1 virus performed well in fMF assay.

An H1N1 head specific mAb Aca-4 was used in order to test the capacity of fMF assay to monitor blocking virus attachment. Brisbane H1N1 virus was incubated with different dilutions of Aca-4 and the effect of presence of Aca-4 antibody on virus attachment and membrane fusion was monitored.

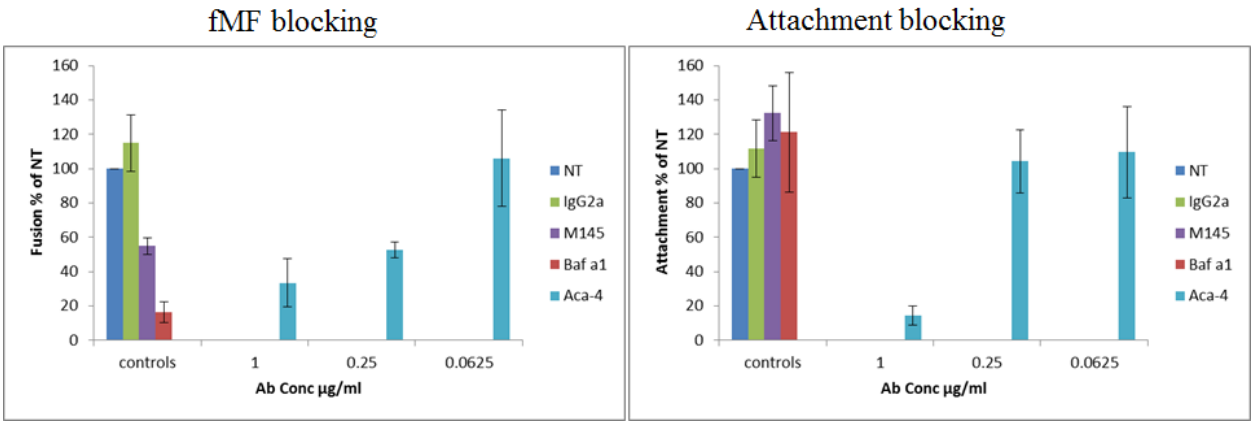

Figure 8 Effect of head specific mAb Aca-4 on attachment and membrane fusion of Brisbane H1N1 virus

Figure 8 shows that at a higher antibody concentration 1µg/ml head specific mAb Aca-4 blocks attachment of the virus to cells by 85%. For the remaining 15% of the attached virus, 70% of fusion is blocked. Fusion % for each condition was calculated using total fluorescence generated by virus attached at that condition. Therefore, at 1 µg/ml, fusion blocking was

calculated using fluorescence corresponding to ~15% virus attached. At a lower antibody concentration 0.25 µg/ml, 50% fusion was blocked and there was no effect on attachment of virus to the cells.

Blocking of fusion in presence of head specific mAb was an unexpected outcome. However, there are some previous studies that have shown that head specific mAbs have membrane fusion blocking effect[21, 22]. These studies have shown that mAbs directed towards different antigenic sites on HA head are capable of post attachment neutralization and this post attachment neutralization is accomplished by blocking membrane fusion.

Presence of head specific antibody Aca-4 blocked attachment of virus and membrane fusion. fMF assay was able to detect both effects in a single run. Therefore, the assay was turned to study effects on attachment and fusion of Brisbane H1N1 virus in presence of sera from vaccinated donors.

FMF Assay To Monitor Blocking Of Fusion and Attachment Of Influenza Virus By Vaccine Generated Antibodies In Donor Sera

The objective of this set of experiments was to test ability of fMF assay to screen pre- and post-vaccination human sera for their capacity to block fusion and attachment, in a single run. Pre- and post-vaccination sera of four human donors immunized with 2009 seasonal flu vaccine was run through the fusion assay using Brisbane H1N1 virus. This strain of virus was used since 2009 seasonal flu vaccine contained Brisbane H1N1 virus antigen.

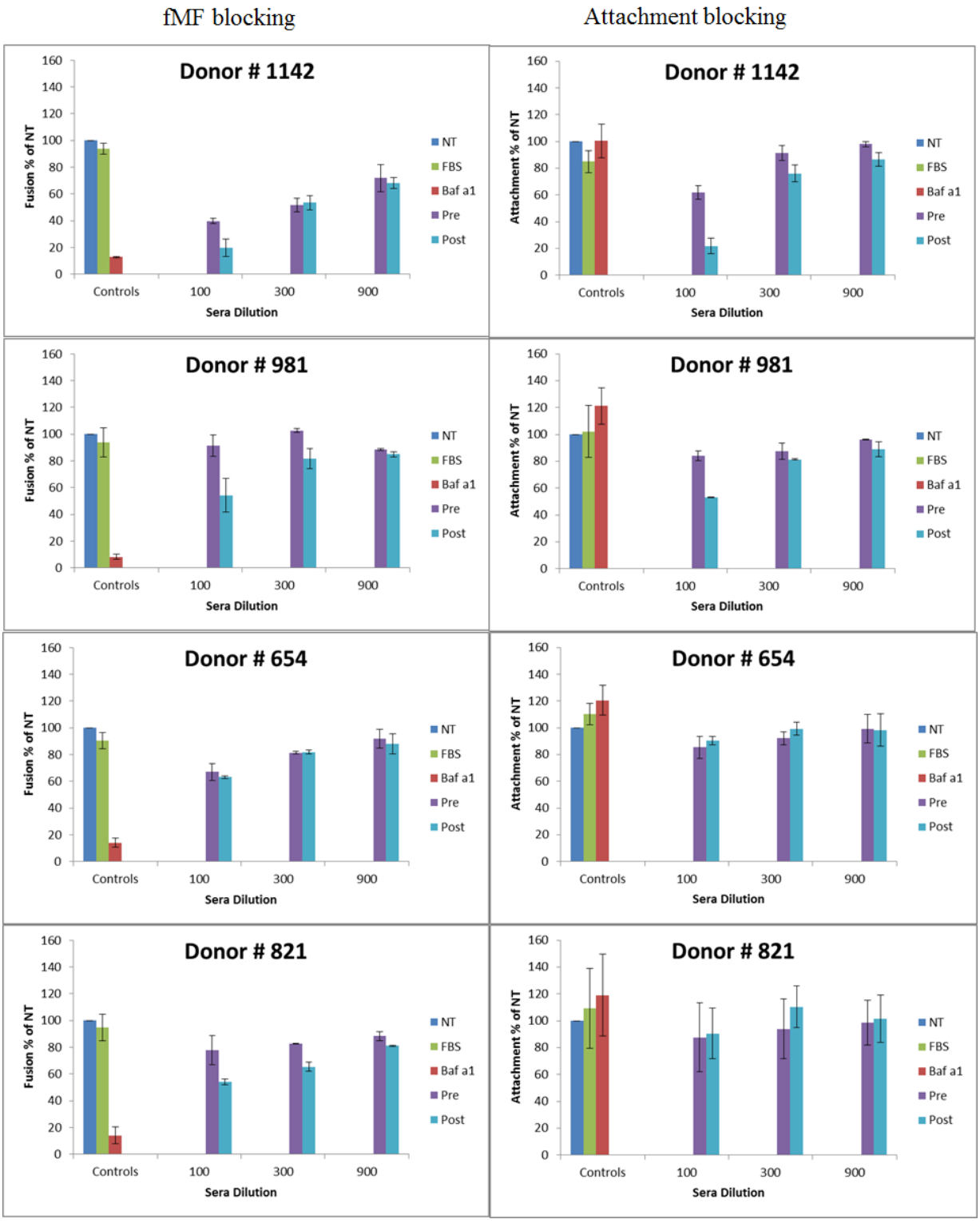

Figure 9 Monitoring the capacity of pre and post vaccination sera to block attachment and fusion

Figure 9 shows fMF assay data from four donors. Pre and post vaccination sera from four human donors were run through fMF assay. Donor #1142 and donor #981 were high responders, the other two donors, #654 and #821 were low responders. High responder sera showed blocking profile similar to head specific mAb Aca-4. At higher sera concentration, 80-50% virus was blocked from attaching cells. For the remaining attached 20-50% of the virus, fusion was blocked by 50-80%. At lower sera concentration, when blocking of attachment was no longer effective, blocking of fusion was still in effect up to 50%. A low responder post vaccination serum, donor #821, showed insignificant capability of blocking of virus attachment. On the other hand, the same donor showed an effective 50% blocking of membrane fusion. The blocking of membrane fusion in the absence of blocking of attachment shows the value of fMF assay. The donors which appear to be nonresponder in attachment blocking tests such as HAI can show response in a post attachment step such as blocking membrane fusion.

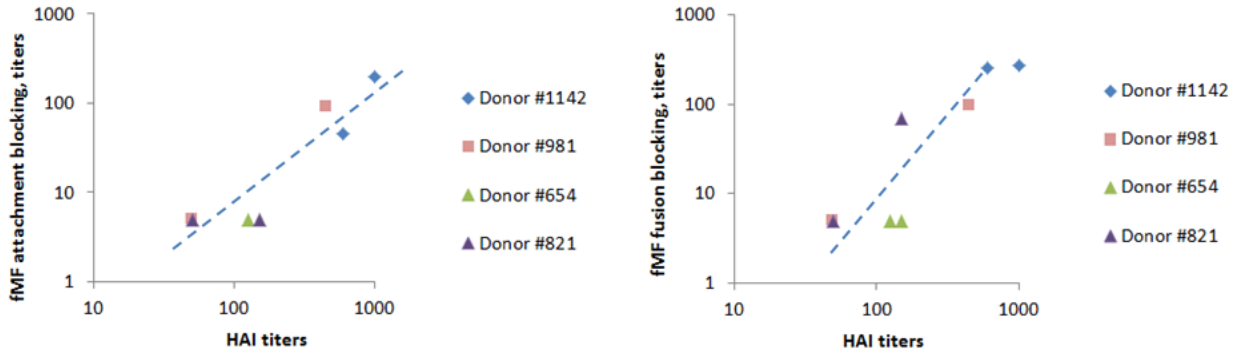

Figure 10 Correlation of fMF assay titers and HAI titers

Titers from fMF assay were compared with titers from classical HAI assay. Figure 10 shows that blocking of attachment and membrane fusion observed in the fMF assay positively correlated with the data of classical HAI assay.

In the same time, blocking of membrane fusion has been observed for a serum which showed insignificant capacity to block attachment (#821), which may signify importance of neutralization via membrane fusion blocking pathway.

DISCUSSION

fMF assay was developed to meet two unmet needs of influenza vaccine assessment, first to assess a new correlate of protection of influenza vaccine i.e. the stem specific response and second to simultaneously analyze immune sera for pre attachment neutralizing and post attachment neutralization antibodies. The method ties together the two major neutralization pathways: blocking attachment of virus to host cells and blocking of membrane fusion of the internalized virus in the endosomes.

fMF assay was tested for its ability to monitor membrane fusion by using Bafilomycin a1, a very specific endosomal acidification blocker. By demonstrating that Bafilomycin a1blocks 85% of membrane fusion without affecting the attachment of virus, the fMF assay was shown to be representative of endosomal fusion process. The specificity of the assay and its capacity to measure stem specific response was tested by using subtype specific stem related antibodies. It was shown by using two subtypes of viruses H1N1 and H3N2 and their respective stem specific antibodies, that fMF assay is able to monitor blocking of membrane fusion in presence of stem specific antibodies. The assay is also able to distinguish between two distinct subtypes of viruses H1N1 and H3N2, as their membrane fusion was blocked only in presence of their subtype specific antibodies. Experiments with stem specific antibodies further corroborated the capacity of fMF assay to measure stem specific response and the subtype specificity of the assay.

A head specific mAb was used to study its effect on attachment and fusion of Brisbane H1N1 virus. Expected outcome of this experiment was that head specific mAb should block attachment of Brisbane H1N1 virus and fusion should remain unaffected. On the contrary, the head specific antibody not only blocked attachment of virus, it also had a significant effect on

blocking membrane fusion. Similar results with head specific antibodies have been observed in some previous studies where they have shown that head specific mAbs neutralize influenza virus by preferentially blocking attachment of virus. However, when at 50% neutralization the blocking of virus attachment was minimal. Therefore, there was some other mechanism that blocked infectivity of virus. It was shown in the same study that there is a very strong correlation between infectivity and membrane fusion. Therefore, it was proposed that head specific antibodies along with blocking attachment can also block membrane fusion by either sterically interfering with fusion process or by crosslinking two adjacent HA trimers[21].

After substantiating fMF assay with mAbs, sera screening experiments were performed. fMF assay was able to detect blocking of both fusion and attachment in the presence of immune sera in a single run. The high responder sera showed characteristics similar to a head specific mAb. At higher sera concentration, blocking of attachment was most effective, i.e 85% of virus was prohibited from attaching the cells. At lower sera concentration when blocking of attachment was not effective, the sera were still able to block post attachment membrane fusion of virus. Sera screening experiments showed that sera antibodies work beyond just blocking attachment. They may also neutralize the already attached virus by blocking fusion of the viral membrane in the endosomes. This could mean that in order to achieve protection from influenza, lesser quantity of antibodies may be needed than what can be required based on HAI data alone. Therefore, for in vitro vaccine testing, blocking of membrane fusion needs to be taken into consideration as a very important neutralization pathway.

The assay has demonstrated ability to monitor blocking of attachment and fusion in a single run. Therefore, it is more representative regarding the natural process of infection and the corresponding neutralization pathways.

For future applications, fMF assay can be developed for other enveloped viruses, such as dengue and filoviruses to study the neutralization pathways and functional characteristics of antibodies generated by vaccine candidates. Other cell types other, such as monocytes, dendritic cells or NK cells can also be used in fMF assay to study effect of antibodies on protection of different types of target cells.

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