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Production and Characterization of Human Monoclonal Antibodies from the Cells of A(H1N1)pdm2009 Influenza Virus Infected Indian Donors

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Abstract

Analysis of human monoclonal antibodies (mAbs) developed from influenza infected donors have enormously contributed to the identification of neutralization sensitive epitopes of influenza virus. The HA protein is a crucial target of neutralizing antibodies and at monoclonal level only Abs binding to HA have been able to neutralize the virus. In this study, eight A (H1N1)pdm 2009 seropositive patients within the age range of 20-50 years (median = 36 years) were recruited. Two anti-HA mAbs secreting stable clones, 2D8 and 2F12 were established under optimized conditions from the peripheral blood mononuclear cells (PBMCs) of the volunteers. These antibodies efficiently neutralized the homologous laboratory isolated strain of the pandemic virus as well as the reference strain. Our study suggests that the anti-HA antibodies derived from infected Indian patients display neutralization potential against the A(H1N1)pdm 2009 virus. This is the first ever study of generation of mAbs against the pandemic influenza virus involving the immune repertoire if Indian patients. Molecular characterization of the target regions will help in identifying potential immunogens in the Indian pandemic isolates and confer protective immunity against this virus.

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1. Introduction

Influenza A virus is a member of the Orthomyxoviridae family and it is one of the main causes of prevalent infection of the respiratory tract in humans. The susceptibility to influenza illness is most common in immunologically naive infants, immunocompromised individuals and elderly[1]. The virulence of influenza A virus is because of its easy spread

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by aerosol; the frequent changes in the viral antigens by antigenic drift and antigenic shift that enables it to escape from protective immunity. Influenza infection in humans is self-limiting, but the virus is known to cause substantial mortality and morbidity worldwide[2]. During the most devastating influenza pandemic of 1918 the global mortality reached to 50 million individuals in one year. In a stark reminder to the 1918 pandemic, the population that is considered fittest was the most vulnerable during the recent 2009 H1N1 pandemic[3].

Despite intensive efforts, the threat to influenza persists as there are many limitations to the use of existing vaccines and antiviral therapies. The protection provided by vaccines that contain killed or recombinant viral glycoprotein is weak and may last as little as 6 months. Moreover, the vaccine efficacy in immunocompromised and elderly individuals is only 39% [4]. Above all the vaccines have to be formulated annually as the existing year's vaccine may not provide protection to the newly emerging strains. Currently four antiviral drugs are approved for use against influenza virus but, their use is restricted due to possible side effects and rapid emergence of resistant strains in the recent years. Hence, the development of new therapeutic targets and strategies to control pandemic and seasonal influenza virus infection in humans is urgently needed.

Passive immunotherapy against influenza has been reported since the 1918 influenza pandemic, where convalescent sera was used for treating patients[5]. However, use of convalescent plasma is being largely replaced with monoclonal antibody preparations owing to the recent advances in monoclonal antibody engineering. There are several reports where antibody therapy using polyclonal and monoclonal antibodies has been used effectively as prophylaxis against varicella, hepatitis A, hepatitis B, rabies, and respiratory syncytial virus infections[6].

The use of human mAb or humanized mAb to key epitopes of infectious pathogens may help in defining the humoral responses with significant therapeutic potential. The use of human mAbs may also lead to more effective post exposure prophylaxis including their use intranasally in viral diseases[7]. Human monoclonal antibody technology has seen various advances recently, like the use of Epstein Barr virus (EBV) to transform human B cells, development of several new heteromyeloma cell lines and CpG oligonucleotides that further enhance the efficiency of B cell transformation[8].

In this study, we generated strongly neutralizing novel human monoclonal antibodies that were selected from the immune repertoire of influenza infected seropositive patients. We generated two stable monoclonal antibody secreting fusion clones that produced antibodies specific against A(H1N1)pdm09 virus. We further investigated the in vivo prophylactic and therapeutic efficacy of these monoclonal antibodies against influenza A virus infection in the in vivo model. This study supports the fact that fully human mAbs with neutralization activity can be successfully generated from the peripheral blood of convalescent patients under optimized conditions.

2. Materials and Methods

2.1 Cells and viruses

Madine Darby Canine Kidney (MDCK) cells were procured from the National Centre for Cell Science (NCCS), Pune, India. EBV transformed marmoset leukocyte cell line (B95-8) was a kind gift, from Dr. Rahul Pal, NII, Delhi. The HMMA2.5 human mouse heteromyeloma was provided by Dr. Lisa Cavacini, Beth Israel Deaconess Medical Centre, Boston, USA. Pandemic virus reference strain A/Cal/07/2009 (H1N1) was procured from Victoria infectious disease reference laboratory (VIDRL), Australia.

2.2 Collection and processing of the clinical samples

Eight A(H1N1)pdm 2009 seropositive patients (5 males and 3 females) within the age range of 20-50 years (median = 36 years) were recruited in this study. RNA was isolated from the nasal and throat swab samples and influenza virus infection was detected by real time RT PCR. All the volunteers had low Ct (cycle threshold) values (below 25) for influenza approximately 3-4 weeks after onset of the disease. 15 ml blood samples were collected in heparanized vacutainers from these donors. 1-2 ml plasma samples were collected from each sample and stored at - 80°C for serological examination. The remaining blood was used for PBMC isolation.

2.3 Hemagglutination inhibition (HAI) assay of the plasma samples

Plasma samples were treated with Receptor destroying enzyme (RDE) for the destruction of non-specific inhibitors. Serial two fold dilution of the plasma samples was prepared in PBS in a microtiter plate.The plasma samples (diluted 1:10) were added in equal volume (25 µl) of 4HA units of A/Cal/07/2009(H1N1). Plates were covered and incubated at room temperature (RT) for 30 min. The contents of the plate were mixed by gently agitating the plates manually.50 µl of the 0.75% guinea pig RBCs were added to each well. Plates were covered and incubated at RT for 30 min. The HAI titre was the highest dilution of the serum that showed hemagglutination activity.

2.4 Generation of monoclonal antibodies from PBMCs

The monoclonal antibodies were generated from the PBMCs of infected patients by the method as per Gorny M, 1994[8], with minor modifications. Mononuclear cells were isolated from the blood of the seropositive patients by density gradient centrifugation and were resuspended in the EBV transformation medium (B95-8 culture supernatant that was diluted 1:2 with complete medium (RPMI+ 20% FCS) to a concentration of 2 x 10^6 cells/ml in the presence of CpG ODN-2006 (1µg/ml)[9] and were cultured overnight at 37°C. After three weeks of culture, proliferating transformed colonies of B lymphocytes were observed using an inverted microscope[10]. The positive clones were screened by microneutralization[11] and expanded to T-25 tissue culture flasks. The positive clones were fused with HMMA2.5 (at a ratio of 1:3) cell lines by adding 1 ml of warm PEG/DMSO solution dropwise for 1 min. The cell culture supernatant of the stable clones was collected and the monoclonal antibodies were subsequently purified by affinity chromatography by Protein G columns (Sigma Aldrich, USA).

2.5 Immunoflourescence

Madin Darby Canine Kidney (MDCK) cells were grown in 24 wells tissue culture plates, at a density of 0.1 x 10^6 cells per well for 24h.Cells were infected with 100 $TCID_{50}$ units of A (H1N1) pdm2009 virus. 24 h after infection, cells were fixed with acetone: methanol (1:1) for 1 min, followed by permeabilization with Triton X 100. Fixed cells were blocked with 2% BSA in PBS for 1 h at 4 °C and washed twice with PBS. Monoclonal antibodies were added at a concentration of 5 µg/ml and the plates were incubated for 2h at 4 °C. Plates were washed thrice with PBS. Bound antibodies were detected with FITC labeled goat anti-human IgG and observed under an immunofluorescent microscope[3].

2.6 Hemagglutination inhibition (HAI) assay

Hemagglutination inhibition assay of the purified monoclonal antibodies was performed by the end point dilution method[11]. Two fold serial dilutions of the purified monoclonal antibodies were taken, with the maximum concentration of 2.5µg/ml. The HAI titers were expressed as the lowest concentration of the antibody that completely inhibited 4HA units of the pandemic virus.

2.7 *Microneutralization assay*

Microneutralization assay of the purified antibodies was performed by the end point dilution method[11]. Briefly, two fold serial dilutions of the antibodies were prepared in PBS starting with the highest dilution of 2.5µg/ml in 96 well tissue culture plates. 100 $TCID_{50}$ of the pandemic isolate as well as reference strain[12] were incubated with equal volume of the antibody for 1 h at 37°C. 100 µl MDCK cells (1.5 x 10^5 cells/ml) were added to each well. The plates were incubated for 3-4 days at 37 °C in 5% CO_2 and examined for CPE. The residual infectivity was tested in four wells per dilution. The neutralizing titer was determined as the lowest concentration of the antibody at which the infectivity of 100 $TCID_{50}$ of the respective virus for MDCK cells was completely neutralized in 50% of the wells. Infectivity was identified by the presence of cytopathic effect on day 4 and the titer was calculated by the Reed-Muench method[12].

2.8 Immunoblot analysis

The infected cell lysates were first resolved on 12% SDS-PAGE and then transferred to PVDF membrane using Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, CA, USA). The membrane was blocked with 5% non-fat milk in TBST for 2 h at room temperature and was washed thrice with TBST. The blot was then cut into strips and denatured antigens were probed by incubating each strip with a single purified monoclonal Ab at a concentration of 1µg/ml. Anti-human IgG was taken as the negative control. The strips were then washed thoroughly with 1X TBST for three times, 10 min each, followed by incubation with 1:1000 dilution of horse-radish peroxidase (HRP) conjugated goat anti-human IgG as secondary antibody (diluted in 5% milk-TBST) for 1 h at room temperature. After washing, the immuno-reactive bands were visualized by the addition of PBS containing 10 µl/ml of 30 % H_2O_2 and 0.5 mg/ml

of DAB (Sigma Aldrich, USA).

2.9 Characterization of the antibodies in vivo

To evaluate the degree of protection of monoclonal antibodies, 6-8 weeks old female BALB/c mice (n=8 per group) were intraperitoneally injected with purified monoclonal antibodies in three different concentrations (2µg, 20µg, 200 µg), 2h before (pre), 24 h after (24 h post) and 48 h after (48 h post) challenge with 2 X 10^4 PFU A/Cal/07/2009 (H1N1) virus. Mice were monitored daily for sickness, weight loss and death for 21 days.

2.10 Statistical analysis

Significance of the differences between means was determined by Student t test using Graph Pad Prism software version 3.02 (San Diego, CA, USA). The results were presented as mean + S.D**.**

3. Results

3.1 Profiling of the patient plasma

Plasma samples of all the donors showed serologic reactivity with the HA of the reference pandemic strain as was evident with the hemagglutination inhibition assay and showed a titer of ≤1:40.The individual titers of the plasma samples are mentioned in table I.

Table I: Serologic testing of the eight volunteers (reciprocal titers are mentioned)

Donor number	1	2	3	4	5	6	7	8
Plasma titer	640	160	640	160	1280	320	320	160

3.2 Generation of antibodies from the PBMCs

PBMCs from all the eight seropositive volunteers were transformed with EBV and the supernatants of the transformed colonies were screened by microneutralization assay for the influenza virus specific antibodies (Table II). The positive clones were fused with the HMMA2.5 heteromyeloma cells. After fusion only two stable fusion clones secreting neutralizing monoclonal antibodies against the pandemic virus were established.

Table II: Characteristics of the screening of EBV transformed B-cell cultures with A(H1N1)pdm09 virus.

Number of influenza infected patients	8
Total number of PBMCs isolated (million) [1]	64.1
Number of wells plated[2]	672
Number of wells positive for antibodies against influenza virus[3]	53
Number of influenza virus antibody secreting stable fusion clones[4]	4

1 PBMCs of all 8 patients were successfully transformed with EBV. The mean of PBMCs isolated from patients was 8.0 million per individual (range 3.9-10.2).
2 PBMCs were plated in 96 well tissue culture plates at 80,000—100,000 cells per well.
3 approximately 7.2% wells (49/672) were positive for neutralizing antibodies against their autologus virus.
4 Both the clones 2D8 and 2F12 clones were derived from separate donors (donor 7 and donor 5 respectively).

Most of the transformed colonies lost reactivity in subsequent rounds of screening due to possible out-growth of non-secretors and instability in antibody generation. The mAbs were purified by affinity chromatography on protein G columns.

3.3 Binding of monoclonal antibodies to the whole virus by ELISA

The monoclonal antibodies were analyzed in vitro by a series of assays.2D8 and 2F12 bound to the pandemic virus A/Cal/07/2009 on ELISA in a dose dependent manner. 2F12 showed lesser binding compared to the 2D8 antibody (Figure 1). Similar results were obtained when the two monoclonal antibodies were tested for their binding to the HA of a panel of viruses selected for the HAI assay. The mAbs showed binding to the reference pandemic strain as well as the laboratory isolate but did not show binding to any other H1N1 or H3N2 virus they were tested against.

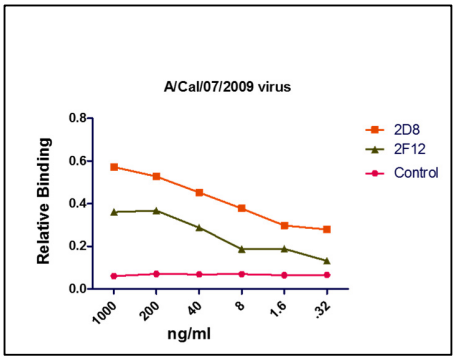

Figure 1: Relative binding of monoclonal antibodies 2D8 and 2F12 to A/Cal/07/2009 (H1N1) influenza virus.

Table III: HAI specific activity of the monoclonal antibodies (µg/ml) against a panel of H1N1 and H3N2 viruses.

Influenza virus used	Subtypes	Concentrations of antibodies used (µg/ml)	
		2D8	2F12
pdm09 (H1N1) isolate	H1N1	0.04	1.8
A/Cal/07/2009	H1N1	0.08	1.3
A/Udorn/307/1972	H3N2	>	>
A/PR/8/34	H1N1	>	>
A/Perth/16/2009	H3N2	>	>

>Indicates that HAI activity was not detected at any concentration that was tested up to 2.5µg/ml.

The immunofluorescence data further supported the above findings that 2D8 bound more strongly to influenza virus infected MDCK cells compared to the 2F12 mab (Figure 2).

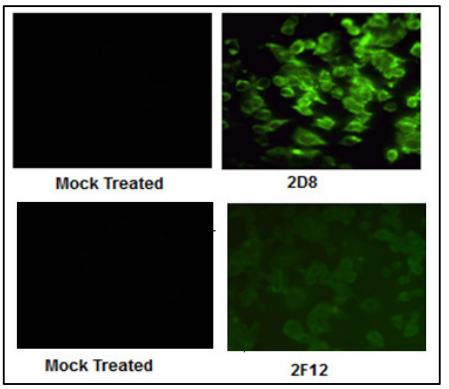

Figure 2: Immunofluorescence Assay of the A/Cal/07/2009 infected MDCK cells showing the binding of monoclonal antibodies 2D8 and 2F12.

3.4 Western blot analysis of the purified antibodies

The gel was run in the denaturing condition, to allow HA protein to denature to HA1 and HA2. Antibody 2D8 reacted with the denatured viral HA1 protein as was evident by a band at 49kDa (Figure 3). In contrast antibody 2F12 showed no binding activity to the denatured HA1.It may be hypothesised that 2F12 that did not show binding to any region in the denatured HA protein, as it might be recognizing a conformational epitope.

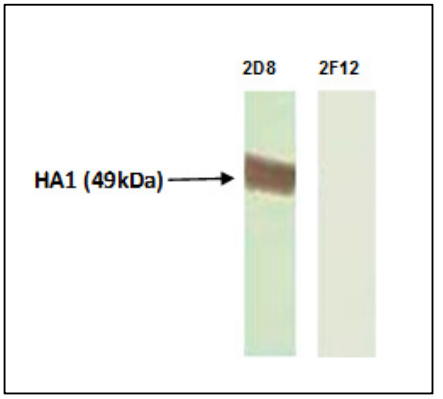

Figure 3: Western blot analysis of monoclonal antibodies. Band corresponding to the HA1 region were observed in the lysate of MDCK cells infected with A(H1N1)pdm09 virus with monoclonal Abs 2D8 but was not observed with 2F12 antibody.

3.5 Characterization of the monoclonal antibodies in vivo

Mice were treated with graded doses (200µg, 20 µg, and 2 µg) of the two mAbs (2D8 and 2F12), 2h pre, 24 h post and 48 h post infection with the respective strains of influenza viruses as mentioned in the methods section. The antibodies were evaluated for their prophylactic or therapeutic efficacy by the assessment of morbidity (loss of weight), mortality, viral load and histopathologic examination of the lungs. The survival and weight loss experiments suggested that the mAb 2D8 achieved considerable protection at the doses 20 µg and 200 µg when administered prophylactically and therapeutically

(table IV). The 2F12 mAb was more effective at the dose of 200 µg. Viral load analysis was performed in the subgroups 2h pre and 24 h post challenge with 20 µg mAb. The lung virus titers of the treated mice confirm that upon treatment with the mAbs, the virus titer considerably reduces in the lungs (Table V).

Table IV: Therapeutic and prophylactic efficacy of the monoclonal antibodies against A(H1N1)pdm09 influenza virus.

Antibody	Virus inoculated	Dose per mouse (µg)	Weight loss (%)			Survival (no. of protected/total no.)		
			2h pre*	24 h post#	48 h post^	2h pre	24 h post	48 h post
2D8	A(H1N1)pdm09	200	2.5	4.3	5.0	5/5	5/5	5/5
		20	10	11	14	5/5	5/5	5/5
		2	14	14	15	0/5	0/5	0/5
2F12	A(H1N1)pdm09	200	4.5	5.5	6.6	4/5	5/5	5/5
		20	14	15	14	3/5	3/5	2/5
		2	16	15	18	0/5	0/5	0/5
Control human IgG	A(H1N1)pdm09	200	10	12	11	0/5	0/5	0/5
		20	15	12	16	0/5	0/5	0/5
		2	18	17	19	0/5	0/5	0/5

*Monoclonal antibody treatment was given 2 h before infection with the virus.
#Monoclonal antibody treatment was given 24 h after inoculation with the virus
^Monoclonal antibody treatment was given 48 h after infection with the virus

Table V: Pulmonary virus titers in the lungs of infected mice (2 X 10^4 PFU) after treatment with monoclonal antibodies.

Monoclonal antibody	Virus inoculated	Virus titers (PFU/ml)		
		2h pre	24 h post	48 h post
2D8	A(H1N1)pdm09	$(1.2\pm0.4)\times10^2$	$(1.5\pm0.6)\times10^2$	ND
2F12	A(H1N1)pdm09	$(1.5\pm0.4)\times10^3$	$(2.0\pm0.4)\times10^3$	ND
Control IgG	A(H1N1)pdm09	$(2.0\pm0.4)\times10^5$	$(2.02\pm0.4)\times10^5$	ND

*ND-Not determined

Subsets of the mice with therapeutic treatment (24 h) were killed on the fourth day of infection for histopathologic evaluation. Without treatment the lungs showed degenerative changes with dense neutrophilic 7nfiltrates and diffuse alveolar damage. Passive immunization with either of the two mAb considerably reduced the pathologic changes and offered protection to the lungs from the lethal challenge of influenza virus (Figure 4).

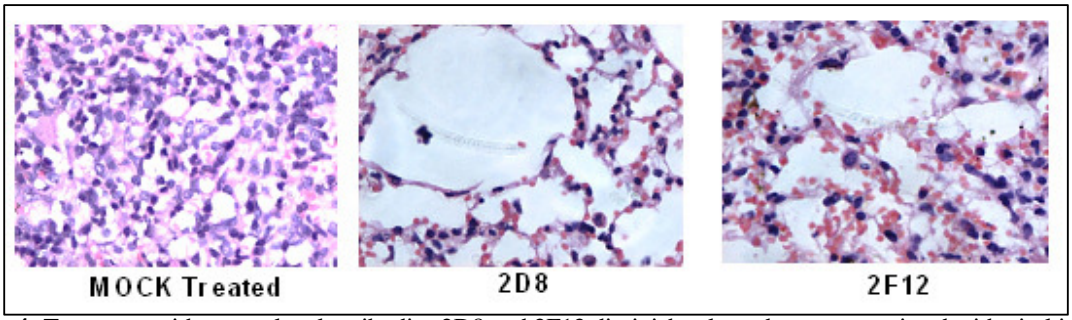

Figure 4: Treatment with monoclonal antibodies 2D8 and 2F12 diminishes lung damage associated with viral infection caused by A/Cal/07/2009 (H1N1) virus.

4. Discussion

The production of human monoclonal antibodies is a much more complex procedure than the production of murine monoclonal antibodies. Scarcity of antibody producing cells in the peripheral blood and the difficulty to obtain the blood from naturally infected patients at the right time is one of the major concerns. Moreover, EBV transformed B cells are hard to grow and secrete very low amount of antibodies[13]. The instability of antibody production by Epstein-Barr virus transformed cells or fused cells is another concern. These factors result in the decreased fusion efficiency and the chances of obtaining a human hybridoma against an antigen of interest is only of the order of 10^{-9} to$10^{-10.}$

The human hybridoma technology is based on two main methods: fusion of Ab producing B cells to myeloma cells [14] and transformation of B lymphocytes with EBV[15]. Combination of both the methods has proved to be more beneficial than either of them alone[16]. In this study, we report the generation of two stable fusion clones, secreting monoclonal antibodies against A(H1N1)pdm09 influenza viruses. The conditions of EBV transformation and fusion with the heteromyeloma cell line were optimised by overcoming the limitations of the current technologies.

The convalescent phase plasma of the eight influenza positive patients was screened for influenza virus specific antibodies, as the serum/plasma antibody titre is a critical factor for the generation of monoclonal antibodies from PBMCs. The PBMCs were EBV transformed in the presence of CpG oligonucleotides as it has been reported to increase the rate of EBV transformation[17]. An overall 7.8% transformation efficiency was achieved and the cells that were positive for antibodies against the pandemic H1N1 virus were fused with HMMA2.5 cells.

The best antibody secreting hybrids were cloned at one cell /well to ensure monoclonal antibody generation and 2 stable fusion clones were obtained- 2D8 and 2F12 that secreted mAbs against A(H1N1)pdm09 and showed neutralization in the *in vitro* as well as *in vivo* conditions.

Monoclonal antibody 2D8 showed the maximum binding in the *in vitro* assays and neutralized the human isolate of the pandemic strain as well as the reference strain at lowest concentrations when compared to the 2F12 antibody. MAb 2F12 showed the lesser binding in ELISA and immunofluorescence assay and although it showed HAI and neutralizing activity, but at a higher concentration compared to the 2D8 antibody. The western blot analysis of the monoclonal antibodies revealed that 2D8 bound to the HA1 region in the denatured HA protein. 2F12 did not show binding to any of the region in the denatured HA protein. Hence, it is hypothesised that 2F12 may be binding a conformational epitope in the HA1 region. However, the binding properties of 2F12 need to be investigated further to confirm the hypothesis.

The monoclonal antibodies did not show binding to the seasonal H3N2 or any other H1N1 or H3N2 virus against which its HAI activity was determined. The reference strains available in the laboratory were picked randomly for the HAI studies. Further studies regarding the mapping of the epitope of these antibodies can reveal more information on whether or not they exhibit cross reactivity to other influenza virus strains. The antibodies however, showed comparative neutralization and HAI activity between the laboratory isolates of the pandemic virus and the reference strain A/Cal/07/2009(H1N1). This data reflects the fact that these Indian pandemic isolates have not shown much deviation from the prototype pandemic strain in terms of antibody binding sites.

The prophylactic and therapeutic efficacy of these antibodies was determined in mice. The antibody treatment was given 2 h before, 24 h after and 48 h after challenge with the pandemic strain. The *in vivo* results were consistent with the *in vitro* study and all the antibodies were able to considerably protect mice from lethal influenza challenge. Both the

antibodies were protective prophylactically but, 2F12 could achieve only 60% protection in terms of survival assay at 20 µg, but achieved 100% survival rate at 200µg. The antibodies were less protective 48 h post infection as compared to 24 h, the fact that is supported by the survival assays and weight loss percentage. The histopathologic examination of the lungs was done in the group of mice that were treated therapeutically at 24h The hisptopathology data further supported the survival and morbidity results, as the lungs from the mice that were given therapeutic doses of the antibodies, showed considerably less signs of alveolar damage and neutrophilic infiltrates resulting from virus infection.

The antibodies 2D8 and 2F12 may be employed as therapeutic agents for at least 48 h after influenza infection as is indicated by the studies in mice. To, the best of our knowledge, these antibodies are the first fully human monoclonal antibodies generated from the immune repertoire of Indian patients infected with A(H1N1)pdm09 virus. Molecular characterization of their epitopes with Indian pandemic isolates will reveal more information of their cross-protectivity and binding characteristics. The strength of our approach for antibody generation lies in the fact that it has used human immune repertoire rather than animals and the antibodies have been generated in response to a natural infection of influenza virus. Since, the antibodies have originated from humans, self -reactivity against self- antigens is minimised in comparison to antibodies that have been generated in mice or through the technique of phage display.

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