Cross-Reactive Anti-Avian H5N1 Influenza Neutralizing Antibodies in a Normal ‘Exposure-Naïve’ Australian Blood Donor Population

Garry W. Lynch*,1,2, Paul W. Selleck3, Anna-Maree Axell1,3, Teena Downton1, Natalie M. Kapitza1, Ingrid Boehm2, Wayne Dyer1,2, Ying-Fan Yvonne Wang1, Sacha Stelzer-Braid4, William Rawlinson3 and John S. Sullivan1,2

1The Transfusion Immunobiology and Biosafety Group, Transfusion Medicine and Immunogenetics Research, The University of Sydney, Camperdown, Sydney, Australia
2Research & Business Development, The Australian Red Cross Blood Service, 153 Clarence St, Sydney, NSW 2000, Australia
3The Australian Animal Health Laboratories, CSIRO, Geelong, Victoria, Australia
4Virology Division, Department of Microbiology, South Eastern Area Laboratory Service, Prince of Wales Hospital, Randwick, NSW 2031, Australia

Abstract: It is necessary to understand whether some humans possess natural humoral-immune protection for avian-H5N1 influenza. To broadly assess an exposure naïve cohort we have examined intravenous immunoglobulins (IVIGs) isolated from pools of many thousands of normal Australian blood donations. In studies of the anti-H5N1 antibody potential of these highly purified IVIG therapeutics and of individual donor sera we have identified antibodies that bind to both H5N1 surface envelope and internal viral proteins and neutralize in vitro MDCK and Vero cell infections by highly pathogenic avian influenza clade I and II and human-derived H5N1 isolates. As this reactivity is removed by adsorption with purified H3N2 and H1N1 strains, anti-H5N1 cross-reacting hetero-typic antibodies are implicated. These findings support that some individuals do contain low levels of specific and neutralizing anti-H5N1 antibodies. The protective relevance of this in vivo remains yet to be determined.

Keywords: Avian H5N1 influenza, human anti-viral antibodies, infection neutralization, virus, IVIG.

INTRODUCTION

Fed by high mutation rates and gene re-assortments Influenza strains can escape host homotypic antibody defenses. Yearly, genetic drift in human influenza strains generates epidemics that mainly target the immune-compromised, particularly the very young and the elderly, and result in the deaths of approximately 0.1% of those infected with up to 250-500 thousand lives lost every year [1]. Three to four times a century, genetic shifts promote the emergence of avian influenza strains of altered phenotypes, cell tropisms and pathogenicities that enable them to transverse the species barrier and seed highly lethal human pandemics. Notable is that pandemic influenza particularly targets otherwise healthy youths as well as the immune-compromised. During the 1918 H1N1 pandemic, local mortalities of 30% and more were recorded, and an overall mortality of approximately 2.6% with up to 50 million lives lost [2, 3]. The epizoonotic expansion over the past decade of highly pathogenic H5N1 avian-influenza (HPAI) has so far resulted in a 61.8% mortality of the confirmed 348 human infections (WHO) identified up till the beginning of 2008 and continues to pose a realistic risk of also developing into a human pandemic [4]. As a significant proportion of infected individuals confirmed by laboratory tests have survived exposure to H5N1, as did the majority of the populations through the earlier 1918, 1957 and 1968 pandemics, we have asked whether there is some cross-reactive immune protection in the population at large. Since neutralizing antibodies remain the current best single protective correlate for influenza [5], we aimed to determine if there exists cross-reactive anti-H5N1 antibodies gained from broad lifetime exposures to various human influenza A strains that may provide a level of protection. Our findings challenge the conventional view that an exposure naïve population is void of neutralizing antibodies against avian H5N1 influenza.

MATERIALS AND LABORATORY PROCEDURES

Antibodies

Intravenous Immunoglobulins (IVIGs) derived from plasma collected by the Australian Red Cross Blood Service from thousands of non-re-numerated Australian blood donors were examined to broadly assess the potential repertoire of anti-H5N1 antibodies (Abs) in the Australian population. Each IVIG batch was prepared by large pool fractionation and purification by CSL Pty Ltd into the highly pure (≥ 98%) Intragam-F™ IVIG formulation. This antibody was sourced from the plasmas of up to 60,000 normal blood donors and provides a broad antibody sampling of the herd immunity of the donor population. Dilution of the IVIG for our experiments with TBS or PBS is indicated in the text. We have also examined the antibodies of individual and pooled serum obtained from children (aged 1-4 years) or adults of varying...
In eight separate micro-neutralization experiments we have consistently observed blanket inhibition of MDCK or Vero cells infections by HPAI clade I and II chicken-derived viruses and a clade I human-derived strain, when the viruses were first pre-treatment with Australian-derived IVIG (Table 1). This data represents a broader panel of experimental findings with the additional support of parallel neutralization of each of the H5N1 isolates using 3 other IVIG-Ab formulations, sourced from Europe, the US and SE-Asia (data not shown). This also clearly and consistently demonstrates neutralization of H5N1 cell culture infections by IVIG-Abs. It was noteworthy that in marked contrast to the H5N1 neutralization by the IVIGs they all have failed to inhibit Vero cell infection by a H7N7 HPAI virus (i.e. A/chicken/ Victoria/1985 cell infection by a H7N7 HPAI virus (i.e. A/chicken/ Victoria/1985). This also clearly and consistently demonstrates neutralization of H5N1 by the highly purified antibodies in IVIG instead of an indirect action of IVIG effects on cell mediated neutralization by the IVIGs they all have failed to inhibit Vero cell infection by a H7N7 HPAI virus (i.e. A/chicken/ Victoria/1985) (Table 1). This indicates a direct specific neutralization of H5N1 by the highly purified antibodies in IVIG instead of an indirect action of IVIG effects on cell mediated neutralization by the IVIGs they all have failed to inhibit Vero cell infection by a H7N7 HPAI virus (i.e. A/chicken/ Victoria/1985) (Table 1). This indicates a direct specific neutralization of H5N1 by the highly purified antibodies in IVIG instead of an indirect action of IVIG effects on cell mediated.
responses. Partial neutralization with breakthrough virus was found to extend for several dilutions beyond the listed titres that gave complete neutralization.

**Human Antibodies Bind to Denatured Surface Envelope and Internal H5N1 Proteins**

To biochemically identify the viral proteins targeted by IVIG-Ab, the purified viruses were solubilized by Sodium Dodecyl Sulfate (SDS) and the extracted viral proteins subjected to electrophoresis (PAGE). Followed by staining to profile the proteins of the human and avian influenza viruses (Fig. 1, middle panel lanes 2-6). Identification of only viral proteins [8] indicates the purity of the respective viruses isolated free of egg culture proteins. As predicted each of the viral proteins from the respective isolates migrate with equivalent mobility’s in SDS-PAGE. The hemagglutinins (HA) were the standout exception with relative gel mobility ranking differences of HA5 > HA1 > HA3. Observed apparent molecular weights of 72, 77 and 82 kDa for the respective hemagglutinin monomers of HA5, HA1 and HA3 were found and indicative of differential HA glycosylation between those strains [7]. SDS-PAGE fractionated viral proteins separated in the same gel were alternately electrotransferred to nitrocellulose and WB performed using Australian-sourced IVIG [7]. When matched against the viral protein signatures (Fig. 1, lanes 2-6) the WB profiles (lanes 8-9) revealed clear human-Ab binding to denatured (linear) epitopes in human influenza envelope HA and NA and internal NP2, M1 and NS2 viral proteins (Fig. 1, lanes 8 & 9). Most relevant was parallel antibody binding to the corresponding NP2, M1 and NS2 proteins of H5N1 (lane 7). Technical refinement, including altering the detergent in the blotting reaction, previously found to effect WB detection of viral proteins[9], subsequently enabled resolution of IVIG-Ab binding to H5N1 HA, NA and M2 proteins (lane 1). However, a caveat needs to be raised regarding the long held perception that antibodies bind in western blots to only linear epitopes, in the light of recent findings by Zhou et al. that challenge this view, with evidence that Ab-binding to discontinuous regions can occur. They have suggested that this is possible as a result of protein refolding of secondary structures during gel-blot processing [21].

**Table 1. Australian IVIG Antibody Neutralization of Avian H5N1 Cell Culture Infections**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Vero Cells Neutralization Titre</th>
<th>MDCK Cells Neutralization Titre</th>
</tr>
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<tbody>
<tr>
<td><strong>Avian H5N1 Influenzas</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chicken/Vietnam/8/2004* (Clade I)</td>
<td>8 – 32¹</td>
<td>8 – 16⁷</td>
</tr>
<tr>
<td>Chicken/Wates/1/2005 (Clade II)</td>
<td>32</td>
<td>1024</td>
</tr>
<tr>
<td>Human/Vietnam/1203/2004 (Clade I)</td>
<td>32¹</td>
<td>128</td>
</tr>
<tr>
<td><strong>Avian H7N7 Influenza</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chicken/Victoria/1985</td>
<td>&lt;2¹</td>
<td>ND</td>
</tr>
</tbody>
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The data from 8 separate neutralization experiments have been collated and the antibody inhibition titers are listed. For this Australian prepared IVIG was assayed in duplicates against chicken-derived H5N1 clade I and II strains, a human-derived strain and a duck H7N7 virus. The values obtained are given as the reciprocal of the highest dilution that gave complete neutralization against 100 TCID₅₀ of each of the low passage HPAI Influenza A isolates. Note: This was also supported in each experiment by parallel neutralization of each of the isolates by three other IVIG-Ab formulations, sourced from Europe, the US and SE-Asia. Those IVIGs also failed to inhibit H7N7 infections (data not shown).

¹Titre range from 6 separate infections.

**Fig. (1). Detection of Antibody binding to influenza antigen in Australian Intravenous Immunoglobulin (IVIG).** *Coomassie Stained Center Panel.* Viruses were subjected to SDS-PAGE under non-reducing conditions. Avian H5N1 lane 2: *A/chicken/Vietnam/8/04*; Human H1N1 viruses; lane 3: *A/Texas/36/1991* and lane 4: *A/Johannesburg/82/1996*; Human H3N2 viruses: lane 5: *A/Beijing/32/1992* and lane 6: *A/Sydney/5/1997* viral strains. The purity of each of the virus preparations was evident from coomassie blue gel staining which clearly revealed the well established profiles of most abundant proteins in the respective isolates; namely Hemagglutinin (HA) (and multimers) (500 copies per virion), Neuraminidase (NA) (100 copies), Nucleoprotein (NP) (1,000 copies), M1 (3,000 copies) and NS2 protein (130-200 copies) [8]. *IVIG Western Blot Panels.* Alternately WB with a 1/250 dilution of IVIG (50 mg/ml, 1 hr incubation at RT), revealed human antibody binding to the viral proteins HA, NA, NP, M1 and NS2 of both human (H1N1 (lane 8) and H3N2 (lane 9)) and avian (H5N1) influenza (lane 1 was blotted in TBS without tween-20; and lane 7 blotted in TBS with 0.1% tween-20). For direct comparison of the blotting and protein staining results these were performed in the same SDS-PAGE gel experiment (i.e. Fig. (1B) middle and right hand panels (lanes 2-9)).
Human Antibodies Bind to the Envelope Surface of Intact H5N1 Virus

To further evaluate the anti-H5N1 antibodies in neutralization competent IVIG we performed IVIG-WB assays on whole avian-H5N1 and human-H1N1 and H3N2 strains immobilized to nitrocellulose for evidence of antibody binding to native H5N1 virus (Fig. 2). Serial dilutions of purified human influenza are shown with IVIG antibody binding to intact virus titred out to a 1/1000 dilution (Fig. 2, top panel). IVIG-Ab binding to intact H5N1 was also evident for viruses immobilized to nitrocellulose (Fig. 2, lower panel-with nitrocellulose loading equalized with the human viruses), but at titres 100-1000 fold less than for the equivalent load of H1N1 or H3N2 viruses. Collectively our findings of anti-H5N1 binding and neutralizing antibodies in human ‘herd’ IVIG preparations suggest that some individuals within the normal population have antibody activities (neutralization) directed to envelope H5N1 and to several denatured surface and internal viral proteins.

![IVIG Dot Blots of Intact Influenzas](image)

**Fig. (2). Detection of human antibodies in Australian IVIG that bind native influenza antigen of whole virions.** For examination of IVIG-Antibody binding to the envelope proteins of the intact viruses 2 μl samples of whole virus and titrations thereof were spotted onto nitrocellulose. H5N1, H3N2 and H1N1 viral loadings were equalized for total protein and matched against control samples of human albumin. Ab binding was revealed by secondary HRP-Ab treatment and chemiluminescence [7].

**Human Ab Binding to H5N1 is Proportional to Influenza Exposure**

If human Ab binding to H5N1 is the result of non-specific interactions, we would predict that serum Abs from a population minimally exposed to influenza, such as children, would bind to H5N1 virus equivalent to the serum Abs of adults. Therefore we compared the Abs in an adult serum pool (10 sera: ages 31 to 40 years) or adult (age 31 to 40 years) pooled sera. For identification of anti-influenza antibodies in adult and child sera dot blotting was performed as for Fig. (2) applying whole purified H5N1 (A/chicken/Vietnam/8/2004), H1N1 (A/human/Texas/36/1991; A/human/Johannesberg/82/96) and H3N2 (A/human/Beijing/32/1992 and A/human/Sydney/5/92) viruses and using 1/500 dilutions of a pools of child (x10) or adult (x10) sera. The top panel examines binding to H5N1 and the bottom panels, comparative antibody binding to equalized titrations of the human H1N1 and H3N2 influenza isolates. Note: The dot blots were all performed in parallel within the same blotting experiment, and reveal adult serum antibodies that bind to the envelope proteins of intact H5N1 virus.

![Adult vs Child Serum Dot Blots of Intact Influenzas](image)

**Fig. (3). Comparisons of anti-H5N1 antibodies in child (age 1 to 4 years) or adult (age 31 to 40 years) pooled sera.** For identification of anti-influenza antibodies in adult and child sera dot blotting was performed as for Fig. (2) applying whole purified H5N1 (A/chicken/Vietnam/8/2004), H1N1 (A/human/Texas/36/1991; A/human/Johannesberg/82/96) and H3N2 (A/human/Beijing/32/1992 and A/human/Sydney/5/92) viruses and using 1/500 dilutions of a pools of child (x10) or adult (x10) sera. The top panel examines binding to H5N1 and the bottom panels, comparative antibody binding to equalized titrations of the human H1N1 and H3N2 influenza isolates. Note: The dot blots were all performed in parallel within the same blotting experiment, and reveal adult serum antibodies that bind to the envelope proteins of intact H5N1 virus.

**Proportional Hetero-Subtypic Depletion of Anti-Human Influenza and Anti-Avian H5N1 Abs Following Prior-Exposure to Whole Human H3N2 Virus**

We have performed viral-Ab clearance studies to determine the inter-virus specificity of the observed anti-H5N1 activity. As shown in Fig. (5) pre-exposure of an adult serum pool to human H3N2 influenza selectively and proportionally removes both anti-human influenza and anti-avian binding Abs. This indicates firstly that these Abs are specific for influenza, and secondly that the anti-H5N1 human Abs have arisen not from the unlikely event of sub-clinical exposure to avian H5N1 virus but from repeated exposure to heterogeneous influenza challenge per se. From these findings and from other data not shown, we have found that depletion of human Abs in IVIGs or serum can be performed irrespective of whether we use the same H5N1 strain; such as a Viet/8 (i.e. same subtype); or a subtype different H5N1 virus, such as with pre-adsorption of Abs-to Viet/8 using a Viet/1203 virus (i.e. different subtype); or as we have as indicated in Fig. (5) pre-adsorbed with an unrelated human-H3N2 influenza strain (i.e. different strain) (Fig. 5). When antibody quantitation was assessed by spot titration of the total immunoglobulin in the samples pre- and post adsorption there was no
quantitative loss of serum antibodies concurrent with depletion of the cross-reactive anti-H5N1 antibodies, thus showing the specificity of the latter.

**Summary of Our Findings that Show Ab Specificity for H5N1 from Unexposed Humans**

Our findings that show anti-H5N1 human Abs in exposure naïve Australian blood have been qualified by a number of different and complementary means, namely by: 1) cell culture neutralization, 2) solid-phase immuno-assay of Ab binding to whole virus and 3) western blotting assays, all of which were confirmatory and clearly identify low level human Abs that bind to H5N1 and protect against cell infection by multiple avian H5N1 isolates in cell culture. In these studies establishment of the specificity of H5N1 interactions were satisfied by demonstration of: i) titratable Ab-binding interactions; ii) complementary binding of either normal adult human serum Abs or of highly purified IVIG-Abs; iii) Ab binding to highly purified preparations of H5N1, iv) and binding to both denatured and native H5N1 protein preparations; v) with minimal binding of children’s serum Abs to denatured or intact H5N1 virus (Figs. 1-4); vi) a lack of neutralization of an avian H7N7 isolate studied in parallel with H5N1 neutralization, and vii) the specific depletion of binding activities of IVIGs or normal serum Abs with whole virus or denatured viral proteins by Ab pre-clearing treatments (Fig. 5). In addition, in serological tests using a commercial ELISA anti-H5 mAb Hemagglutinin competition assay we have also detected viii) anti-H5 HA binding in the serum of some individuals and this activity was removed on adsorption with H5N1 viruses (S. Stelzer, B. Wong, W. Rawlinson, G. Lynch unpublished observations).

**DISCUSSION**

We provide direct evidence for *bona fide* specific and protective cross-reactive anti-H5N1 antibodies in a previously unexposed population. This activity clearly differs from the more marked Ab-activities observed in H5N1 vaccine responses [1] and from convalescent H5N1 infected individuals [10] and the modest *in vitro* activities we detect may often be masked within the low and perhaps equivocal value ranges of some assay. We have validated our observations using multiple different assays and our findings gain support from recent reports of anti-NA antibodies in normal unexposed serum [11] and of enhanced anti-H5N1 HA neutralization in healthy donors after boosting with unrelated human influenza H1N1/ H3N2 seasonal vaccines [12]. Less direct evidence has also been gained from indication of H5N1 immune protection in aged individuals [13], and from animal studies, as summarized in the bioinformatics evaluations of Bui et al. [5], that show the conservation of numerous linear and conformational epitopes (some neutralizing) across H1N1, H3N2 and H5N1 strains. The low levels of human antibodies we find bind to H5N1 viruses, orders of magnitude less than for human strains. It is important therefore to determine whether these cross-reactive antibodies could contribute to the observed age-related survival of the ~40% individuals that have been infected with H5N1 [4], perhaps through mechanisms that subdue viral infection and expansion thereby protecting those individuals against clinical disease.

Our findings and those identified in [5] suggest that functional epitope-specific Ab-targets can be maintained and conserved across viral species. Indicated is a need to focus on viral epitope structural interactions. To assist in understanding influenza immune capture and escape there are new methods for analyzing protein structures independent of primary sequence bias and these offer new possibilities for the interpretation of conserved structural epitopes that are targeted by neutralizing antibodies and which are not explained from primary sequences alone [14].

Failure of the human Abs to neutralize the H7N7 avian strain is notable, since human H7N7 infections have been documented in 1959, 1977, 1981, 1996, and in the Netherlands in 2003 with 89 confirmed infections. Interestingly H7 subtypes have been found to have irregular ocular tropisms and in general humans are not considered to be at high infection risk, and Ab responses infrequently detected [15, 16]. Hence between different avian strains, there appears to be a gradation of human antibodies for conserved inter-species protein epitopes and further study of other avian strains may confirm this.

There is need to validate our findings in models of *in vivo* infection and to determine whether there is an age-related antibody protection of H5N1 infection and pathogenicity, as has been suggested by Smallman-Raynor [13] and for which our unpublished preliminary findings are supportive. Also
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required and as highlighted in the recent Jordan report [1] is the need for resolution of the epitopes, pathways and mechanisms involved in the human antibody neutralization of H5N1 infections whether through targeting HA and NA [11] and possibly M2 [17]. It would be possible to do this by testing another virus containing a non-human neuraminidase gene, or to generate reassortant viruses containing the avian H5 and a non-human neuraminidase. It is notable that with few exceptions (i.e. one human antibody study) to date all of the antibody epitopes of influenza have been identified from animal experiments. It is unknown whether there are conserved, cross-reactive and human-specific antibody epitopes for influenza.

Our on-going studies aim to profile those individuals with the highest titres so that preparations of enriched anti-H5N1 Abs might be possible. From these candidates for quick response vaccine boosting may also be identified. A possibility bolstered by the report of enhanced anti-H5 activities of healthy donors after human influenza vaccine boosting [12]. The protective value of using human neutralizing anti-H5N1 Abs, generated from EBV treated B cells of a convalescent individual who had survived H5N1 infection, has also been shown in murine studies, along with evidence for H5N1 inter-clade cross-reactive antibody protection [10]. Of interest one of the monoclonal antibodies examined in the latter study despite being non-neutralizing in vitro gave significant protection from lethal injection in vivo [10]. Other studies have also shown that passive immune transfer of antibodies from H5 vaccine boosted mice were able to protect naive mice against homo-typic lethal challenge [18]. These findings and those from retrospective meta-analyses of convalescent blood product use in treating victims of the 1918 Spanish flu pandemic [19], indicate the potential value of passive anti-H5N1 Ab supplementation in the event of a H5N1 pandemic. While support for effective cross-reactive antibodies in nature can be gleaned from the findings of the Cleveland Family Study on the susceptibility to infection through the 1957 H2N2 pandemic, and suggests that preventative hetero-typic immunity may be accrued over the lifetimes of many individuals [20].
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