Chapter 3

Virus Glycoproteins Tagged with the Human Fc Domain as Second Generation Vaccine Candidates

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Abstract  Traditional vaccines such as inactivated or live attenuated vaccines, are gradually giving way to more biochemically defined vaccines that are most often based on a recombinant antigen known to possess neutralizing epitopes. Such vaccines can offer improvements in speed, safety and manufacturing process but an inevitable consequence of their high degree of purification is that immunogenicity is reduced through the lack of the innate triggering molecules present in more complex preparations. Targeting recombinant vaccines to antigen presenting cells (APCs) such as dendritic cells however can improve immunogenicity by ensuring that antigen processing is as efficient as possible. Immune complexes, one of a number of routes of APC targeting, are mimicked by a recombinant approach, crystallizable fragment (Fc) fusion proteins, in which the target immunogen is linked directly to an antibody effector domain capable of interaction with receptors, FcR, on the APC cell surface. A number of virus Fc fusion proteins have been expressed in insect cells using the baculovirus expression system and shown to be efficiently produced and purified. Their use for immunization next to non-Fc tagged equivalents shows that they are powerfully immunogenic in the absence of added adjuvant and that immune stimulation is the result of the Fc-FcR interaction.

Keywords  Immune-complexes • Crystallizable fragment • Fc receptor • Glycoprotein tagging • Vaccine delivery
3.1 Introduction

The use of immune complexes (ICs) as vaccines has a long history. Early animal vaccines such as for *Foot and Mouth Disease Virus* were mixtures of virus antigen and immune sera and were developed followed the observation that, while passive transfer of an immune serum would provide a short window of immunity, the use of a serum mixed with live virus provided long lasting immunity (Lombard et al. 2007). Even today, the principle continues to be used in new animal vaccine development, to provide a stronger immune response to a standard dose of vaccine antigen, to overcome the infectivity that remains in an attenuated virus preparation or to overcome existing immunity, for example maternal immunity (Haddad et al. 1997; Jeurissen et al. 1998). In man too, the use of ICs has been shown to be beneficial. Treatment of chronically infected Hepatitis B patients with Hepatitis B surface antigen complexed with immunoglobulins was sufficient to boost immunity (Wen et al. 1999). Immune complexes have also been shown to overcome the problem of pre-existing immunity that limits the therapeutic use of adenovirus expression vectors, through the purposeful generation of adenovirus-antibody complexes which then target immune processing cells in preference to the normal target of Coxsackie Adenovirus Receptor (CAR) bearing cells (Leopold et al. 2006). In all these cases, the formation of an immune complex, through the incubation of antigen and antibody, effectively decorates the antigen with the crystallizable fragment (Fc) of the immunoglobulin heavy chain and thereby enables its interaction with Fc receptors (FcR) present on antigen presenting cells (APCs) such as dendritic cells (DCs). Once targeted in this way, the antigen is efficiently taken up and processed by APCs and the subsequent adaptive immune response is accelerated and intensified (Fig. 3.1). Both the major histocompatibility processing pathways, that is Major Histocompatibility Complex (MHC) class I and MHC class II, are active in APCs with the relative split between their activity associated broadly with the processing of either endogenous (e.g. virus) or exogenous (e.g. bacteria) antigens which are also recognized by various pattern recognition receptors (PRRs) within the processing cell. In general endogenous antigens processed by the MHC class I pathway activate CD8+ T cells while exogenous antigens are processed by MHC class II to activate CD4+ T cells. However, both processing pathways co-exist in APCs and some exogenous antigens can also be “cross presented” on MHC class I, especially those capable of binding FcRs on DCs (Regnault et al. 1999). Delivery of an antigen to APCs, particularly DCs, via an IC is therefore an effective “one stop shop” to efficient presentation to the immune system irrespective of the nature of the immunogen and is capable of stimulating the response by 100–10,000 fold when compared to antigen alone (Kunkl and Klaus 1981; Ma et al. 2005; Obregon et al. 2006).

3.2 Examples of ICs

Such is the value of immune targeting to APCs on the strength and longevity of the immune response obtained that various different methods have been used to mimic IC formation in addition to the direct mixing of antigen and antibody.
In influenza vaccine development for example, inactivated PR8 virus was treated with $\alpha 1,3$ galactosyl-transferase \textit{in vitro} to add $\alpha$-Gal epitopes to glycosylated influenza proteins (Abdel-Motal et al. 2007). Humans carry a natural high level of anti $\alpha$-Gal antibodies so immunization with the modified glycoproteins leads to opsonisation of the vaccine by anti-$\alpha$-Gal antibodies and increased immunogenicity following the interaction between the Fc portion and FcRs on APCs (Abdel-Motal et al. 2007). Whereas random endocytosis of uncomplexed antigens by APCs leads to poor internalization and consequent low immunogenicity, IC mediated entry targets APC cell surface receptors to effectively initiate an immune response (Abdel-Motal et al. 2009). Recombinant ICs have also been constructed. In a novel concept, Chargelegue et al., fused the sequence for a fragment of tetanus toxin to the C-terminus of the heavy chain of a monoclonal antibody specific for the same antigen. The overall construct was expressed in transgenic tobacco plants where it was found to fold and function as an antibody in which the complementarity determining regions (CDRs) of one molecule recognized the tetanus tag on another, leading to the formation of a complex which, when purified, was highly immunogenic (Chargelegue et al. 2005). The application of similar techniques to a larger number of targets has also been discussed (Paul et al. 2011). A conceptually similar vaccine has also been reported for \textit{Streptococcus pneumoniae} in which the fusion is made between pneumococcal...
surface protein A and a single chain antibody whose CDRs specifically targets human FcR (hFcγRI). The vaccine concept was tested in mice transgenic for hFcγRI where the use of the fusion protein gave higher levels of reactive immunoglobulin in both serum and bronchoalveolar lavage fluid when compared to non-fused surface protein and resulted in a higher level of protection against *S. pneumoniae* challenge (Bitsaktsis et al. 2012).

Antigen-Fc fusion proteins take the concept of a genetic fusion between antigen and the Fc effector domain a stage further by removing the requirement for CDRs completely and directly conjugating the Fc domain to the antigen (Fig. 3.2). This has a number of advantages, not least that it removes the requirement for the availability of a pre-existing antibody and provides a simple manufacturing process as the antigen-Fc complex can be purified to homogeneity by affinity chromatography on protein A or protein G matrices. The uncertainty of ICs formation and the need to purify discrete size classes of complex in order to remove excess antigen or antibody is therefore avoided. By virtue of the single disulfide bond between Fc monomers, recombinant proteins that pass through the secretory system of an expressing cell become disulfide linked dimers, offering the potential to cross link FcRs on binding even when the antigen concerned is naturally monomeric. As a result of these advantages a number of Fc fusion proteins have been expressed and characterized as candidate vaccines (Abdel-Motal et al. 2007, 2009; Chen et al. 2007; Konduru et al. 2011; Pleass 2009; Zaharatos et al. 2011).

This strategy has been also extensively investigated for the generation of cancer vaccines where the formation of antigen-antibody complexes using tumor markers leads to Fc receptor mediated uptake by APCs and stimulation of T cell responses which are able to delay tumor growth (Durrant et al. 2010; Metheringham et al. 2009).

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**Fig. 3.2** Different forms of immune complex ranging from antigen-antibody complexes *(left)*, recombinant antigen-antibody complexes *(RICS)* in which an antigen sequence is fused to an antibody heavy chain of known specificity *(center)* and direct antigen-Fc fusion proteins which by-pass the need for complementarity determining region (CDR) specificity *(right)*.
3.3 A Universal Expression Solution

To enable a generic solution to the addition of an Fc-tag to a variety of candidate antigens, rapid cloning and expression cassettes have been developed based on existing high throughput expression strategies, for example those based on baculovirus expression (Pengelley et al. 2006). The transfer vector, designed primarily for the baculovirus expression system although also capable of expression in *Escherichia coli* and mammalian cells, combines a strong expression promoter with a well expressed and cleaved signal peptide, a universal multiple cloning site based on the restriction enzyme SfiI and a C-terminal Fc-tag provided by the sequence for the human IgG1 Fc domain (Fig. 3.3). It should be noted that the glycosylation of the Fc-domain is essential for FcR binding and thus the production of functional protein requires expression in eucaryotic cells (Jefferis 2010). Almost any coding sequence can be generated by the polymerase chain reaction to include the differential SfiI sites at the 5′ and 3′ ends which, upon cloning with the necessary adjustment

Fig. 3.3 Baculovirus transfer vectors for the expression of target proteins tagged at the C-terminus with the human crystallizable fragment (Fc) domain (top) or the transmembrane (TM) domain of the G protein of *Vesicular stomatitis virus* (VSVG<sub>TM</sub>) (middle). In both cases the consequences of expression are shown schematically (extreme right). The reading frame in both vectors across the unique SfiI sites used for cloning is shown (bottom)
for reading frame into the universal vector, result in the secretion of a protein which is tagged at the C-terminus by human Fc, from baculovirus infected insect cells. In our experiments it has also been useful to produce a parallel expression vector in which the same multiple cloning region is appended, at its 3′ terminus, to the sequence encoding the transmembrane (TM) domain from the Vesicular stomatitis virus (VSV) G protein. The origin of the tag is not important but the VSV GTM is well characterized and several antibodies to it are available commercially. The same fragment cloned into this vector again results in the production of the target protein but which is, instead of secreted, displayed on the plasma membrane of the baculovirus infected cell (Fig. 3.3). The use of these vectors in combination allows for the production of a target protein as a soluble Fc-tagged molecule which can be purified from the infected cell supernatant and a non-Fc tagged equivalent attached to the infected insect cell surface. Together recombinant protein targets, expressed using this pair of vectors, allow for purification of the target to homogeneity, the generation of a target specific serum and the ability to detect seroconversion to the target antigen by flow cytometry or enzyme-linked immunosorbent assay (ELISA) on cells expressing the VSV GTM tagged form of the protein. No previous antigen specific serum is required for this methodology as the expression of the Fc-tagged and VSV GTM tagged forms of the target can both be confirmed by use of commercially sourced antibodies. To date, this strategy has been employed for a large number of viral glycoproteins and has proven to be widely applicable. A list of published and unpublished examples from the authors’ laboratories is shown in Table 3.1.

We have focused on the use of the baculovirus expression system for the production of candidate vaccine materials as a result of the advantages that have been attributed to it. These include high throughput, high expression levels for most of the proteins tested, correct post-translational modifications and lack of microbial or mammalian pyrogens or endotoxins (Hu et al. 2008). In addition, as shown recently in a direct head-to-head comparison of the same protein (Human Immunodeficiency virus, HIV, envelope protein) expressed in mammalian or insect cells, the immunogenicity of insect derived glycoproteins is somewhat higher than the equivalent material from mammalian cells as a result of insect cell glycosylation which generally appears to benefit antibody generation (Kong et al. 2010). Baculovirus expression technology is now mature with a number of products already in the marketplace.

Table 3.1 Examples of virus glycoproteins expressed and purified as Fc fusion proteins that have been shown to be strongly immunogenic following immunization

| Virus                | Target protein                        | References            |
|----------------------|---------------------------------------|-----------------------|
| HIV-1 *Influenza A virus* | Envelope gp120; outer domain          | Chen et al. (2007, 2008) |
|                      | Hemagglutinin                         | Loureiro et al. (2011), Shelton et al. (2011) |
|                      | Human H1,H1sw,H3, Avian H4,H5,H7,H8,H9,H10, H13 |                       |
| HCV                  | E2 Envelope protein                   | Fenouillet et al. (2008) |
| SARS Coronavirus     | S Spike protein                       | Mathewson et al. (2008) |

HCV Hepatitis C virus, HIV-1 Human Immunodeficiency virus 1, SARS severe acute respiratory syndrome
and many more in the pipeline (van Oers and King 2011). When the baculovirus expression system was applied to the production of influenza vaccines for example, it proved to be a very valuable facility for both seasonal and pandemic vaccines (Treanor 2009; Tripp and Tompkins 2008).

### 3.4 Application to HIV gp120

The need for an improved HIV envelope vaccine has been widely discussed (Walker and Burton 2010). In its final mature form, the envelope glycoprotein of HIV-1 is composed of two polypeptide chains: gp120 is responsible for binding to the primary and secondary receptors used by the virus for cell entry and gp41 is responsible for fusion of the viral and host cell membranes (Fenouillet et al. 2007). The initial receptor binding step, enabled by gp120, is the predominant target for the generation of neutralizing antibodies which compete for receptor binding and prevent virus entry (Pantophlet and Burton 2006). Antibodies to gp120 have been shown to be capable of neutralizing many HIV clades both in vitro (Binley et al. 2004) and in vivo (Baba et al. 2000; Kitabwalla et al. 2003) but recent work suggests that only strongly receptor blocking monoclonal antibodies (mAbs) have the ability to prevent infection in a Simian/Human immunodeficiency virus (SHIV) challenge animal model; weak or non-neutralizing mAbs were essentially ineffective (Burton et al. 2011). In polyclonal human sera, a functionally similar class of response to bivalent recombinant gp120 used as a candidate HIV-1 vaccine is either weak or not present (Pitisuttithum et al. 2006), plausibly as a consequence of the various immune evasion mechanisms used by HIV; glycan shrouding of sensitive sites (Wei et al. 2003), profuse sequence variation (Catasti et al. 1995, 1996) and structural heterogeneity (Moore et al. 2006; Yuan et al. 2006). A number of attempts have been made to design novel gp120 immunogens with the aim of eliciting a more widely neutralizing immune response than the wild-type molecule. Examples include the purposeful engineering of glycan sites (Li et al. 2008), forced immune focus on the V3 domain (Zolla-Pazner et al. 2008), the use of stabilized envelope trimers (Beddows et al. 2007; Kang et al. 2009) and forced in vitro evolution (Du et al. 2011). Some of these approaches have generated improvements in envelope immunogenicity, including improved neutralizing responses, but no single candidate has yet emerged to dominate the list of vaccine candidates that routinely induce a strong cross clade neutralizing antibody response. Part of the problem is that HIV gp120 is naturally poorly immunogenic. In addition, when antibody is produced it is often to non-neutralizing surfaces such as the inner domain. The inner domain is part of three defined gp120 structural domains, the inner domain, bridging sheet and outer domain (OD), which rearrange somewhat upon CD4 binding (Chen et al. 2005). The inner domain and bridging sheet are the source of heterogeneity within monomeric gp120 in solution (Yuan et al. 2006). Thus, generating immunity to the less mobile domain, that is the OD, could be part of an immune focusing strategy to generate stronger and more cross reactive immunity. The potential is clear as the epitope for a broad ranging,
neutralizing human mAb, 2 G12, maps to the OD (Sanders et al. 2002; Scanlan et al. 2002) and a number of lectins which potently neutralize virus infectivity \textit{in vitro} also bind to the OD (Balzarini et al. 2004, 2005). However, the OD is heavily glycosylated and immunologically silent, making immunization with the isolated domain challenging. We used the sequence of the OD of gp120 HIV-1CN54, a B/C recombinant clade originally isolated in China (Rodenburg et al. 2001; Su et al. 2000) to investigate if Fc tagging could enable an immune response. To do this, the sequence encoding the OD of gp120 \textit{CN54} (residues 251–481) was amplified and cloned into the Fc vector described (cf. Fig. 3.3). Recombinant baculoviruses were generated by established methods (Pengelley et al. 2006; Zhao et al. 2003) and OD-Fc was purified from the infected cell supernatant by a combination of chromatography on lectin (\textit{Lens culinaris})-sepharose and protein A sepharose. The former is a very useful generic way of enriching glycoproteins expressed in insect cells by virtue of the fact that they are highly mannosylated. Mammalian glycoproteins present as contaminants from the serum additions to the media are sialylated and therefore pass through the column. The protein A capture step provides essentially purification to homogeneity so that the level of contaminating insect cell protein is extremely low or not apparent. Fc-tagged full length gp120 \textit{CN54} was also expressed and purified and two further OD variations were also constructed. OD\textsubscript{DL3} is a variant that removes the immunodominant V3 loop from the gp120 sequence while OD2F5 replaces the loop with a well-known cross reactive neutralizing epitope derived from the membrane proximal region of gp41 (Law et al. 2007). Non-tagged gp120 or OD provided suitable controls. Purified OD\textsubscript{CN54}-Fc fusion proteins migrated as a single band of \(~85\) kDa by SDS-PAGE and gp120\textsubscript{CN54} -Fc migrated at \(~130\) kDa consistent with addition of the Fc domain \((~25\) kDa) to the gp120 sequences used in each case (Fig. 3.4). There was no significant cleavage of the OD in this format unlike that described previously for the HIV\textsubscript{YU2} OD (Yang et al. 2004). Gel electrophoresis using a sample buffer with non-reducing agent demonstrated that both proteins were dimers, as expected, of fusion to the Fc domain. As the antibody molecule

\textbf{Fig. 3.4} Cartoon representation of the expression cassettes used for the expression of \textit{Human immunodeficiency virus 1} (HIV-1) gp120 and the outer domain (OD) as fusion proteins with human crystallizable fragment (Fc) \textit{(left)} (SP, signal peptide from the baculovirus major surface glycoprotein gp64; C-terminal block is an additional His tag). SDS-PAGE analysis of the purified proteins from each construct prior to use for immunization showing the high degree of purity achieved \textit{(right)} (the locations of gp120-Fc and OD-Fc are indicated)
Virus Glycoproteins Tagged with the Human Fc Domain is made up of discrete domains there is little reason to suppose that fusion to the Fc domain would impact on the folding of the outer domain (or any other antigen sequence cloned into the same vector). This was formally confirmed by ELISA with antibodies that reacted with both linear and conformation epitopes showing that tagged and non-tagged forms of gp120 reacted equivalently. Indeed, we have not found any obvious effect on overall folding following Fc tagging of the molecules listed in Table 3.1 with the exception of epitopes known to be located close to the fusion junction.

To assess their potential as immunogens, tagged and non-tagged proteins were used at 10 μg per dose to immunize groups of mice. No adjuvant was used in these immunizations. The sera obtained were pooled and assayed by ELISA for titer against purified gp120<sub>CNS4</sub> HIV-1 gp120 alone in the absence of adjuvant was very poorly immunogenic even after repeated immunization. However, both gp120-Fc and OD-Fc fusion proteins elicited significant gp120 titers with gp120<sub>CNS4</sub>-Fc providing a higher titer than OD<sub>CNS4</sub>-Fc as expected of a larger antigen with more potential epitopes (Fig. 3.5). In addition, the generation of a serum to ODADL3, despite it being deleted for a major immunogenic determinant, was notable and the serum generated to OD2F5-Fc reacted with a recombinant protein containing the 2F5 epitope demonstrating that the outer domain can act as a scaffold for other epitopes. These data confirm that Fc tagging is an effective solution to overcoming poor immunogenicity and is a beneficial strategy for even extremely poor immunogens such as the OD domain. In other experiments, abrogation of the Fc-FcR interaction by mutagenesis of the contact site within the Fc domain lead to the loss of immunogenicity demonstrating that targeting to APCs via interaction with FcR is the mechanism of immune enhancement as expected (Chen et al. 2007).

**Fig. 3.5** Serum responses to immunization in the absence of adjuvant with isolated gp120 or gp120-crystallizable fragment (Fc) or outer domain (OD)-Fc fusions. Left – seroconversion was detected by ELISA using non-tagged gp120 as the immobilized antigen. Right – Western blot using the sera generated to OD<sub>V3</sub> (tracks 1 and 2) or OD2F5 (tracks 3 and 4). The samples loaded on the gels were His tagged OD (no Fc domain) (tracks 2 and 4) and a recombinant protein expressing the 2 F5 epitope (tracks 1 and 3).
As a result of the positive immune response to the OD fragment, the same immunization strategy (use of OD-Fc in the absence of adjuvant) was used to generate a panel of mAbs to this highly conserved structural domain. Six mAbs to the domain were identified by screening on OD-Fc and non-related Fc-tagged molecule. The mAbs were used as probes on a variety of HIV gp120s and revealed a fine selectivity which demonstrated serum specificity for the immunizing sequence (HIV-1 CN54). Three of these mAbs reacted very strongly with the tagged and non-tagged OD. Subsequent epitope mapping and neutralization tests showed that two of these mAbs were specific for the V3 loop and that they strongly neutralized HIV virus carrying the same envelope protein (Chen et al. 2008). Thus tagging generates a broad response to epitopes across the target sequence including to epitopes of direct relevance for vaccine design.

The use of the Fc domain in this way has not gone unnoticed as human Fc directly fused to other HIV candidate vaccine targets have also shown promising results. Qi et al., using a peptide mimetic of the gp41 hairpin fusion intermediate, induced cross-reactive antibodies following the coupling of the peptide to human Fc and the immunization of mice (Qi et al. 2010). Notably, neither the free peptide nor a peptide oligomerized by the addition of the foldon sequence derived from bacteriophage T4 fibritin alone induced substantial antibody responses. Cross-reactive neutralizing antibodies raised against gp41 were also generated in rabbits when a gp41-Fc fusion protein was used as the immunogen (Zhang et al. 2009). It was suggested from this study that the Fc domain benefited the longevity of the immunogen following immunization and that this was a facilitating factor in the responses observed. A Gag-Fc fusion protein has been shown to generate mucosal immunity when used for nasal immunization (Lu et al. 2011) with immunity being demonstrated at sites distal to the site of immunization. A study that has explored the mechanism of immune enhancement in mice in more detail compared various subclasses of Fc for their effectiveness. The Fc domain from murine IgG2a was found to be superior to the Fc domain from murine IgG1 for three different immunogens tested, two from HIV and one from Influenza virus (Zaharatos et al. 2011). Murine IgG2a is the mouse equivalent of human IgG1 as used in the studies with the OD described here and both molecules interact with the mouse Fcγ receptor (Pleass 2009). These data therefore confirm earlier observations with knockout mice that the primary mechanism for antibody mediated enhancement of immunity is through interaction with the Fcγ receptor (Wernersson et al. 1999).

3.5 Application to Influenza

Unlike the situation with HIV, where a successful vaccine has yet to be developed, influenza vaccines have been in clinical use for many years. The problem in this case is not the generation of immunity per se but rather that the rapid drift of the virus within the population requires regular vaccine updates and more rapid methods for vaccine generation. In addition, the fear of a pandemic outbreak has brought
to the fore concerns around vaccine stockpiling and antigen sparing (the use of lower amounts of immunogen to enable more doses). A rapid gene-to-vaccine approach to influenza vaccination using Fc-tagging could make a contribution to both of these concerns. The hemagglutinin (HA) is the glycoprotein responsible for Influenza virus entry into host cells via its sialic-acid receptor binding activity and, as a result, is the major target of neutralizing antibodies (Cross et al. 2001; Nicholson et al. 2003; Skehel and Wiley 2000). A successful influenza vaccine is able to elicit neutralizing antibodies that block HA and sialic acid binding and consequently prevent the spread of the virus in the population. As a first step in a feasibility study therefore, Fc tagging was investigated as a generic solution for making a range of influenza HAs immunogenic in the absence of added adjuvant. As before, the full length external glycoprotein sequence of the HA was amplified and cloned into baculovirus transfer vectors for the expression of a secreted Fc-tagged HA and a cell surface bound VSV G<sub>Tm</sub>-tagged HA. Ten different HAs have been expressed using these formats (Table 3.1) and all have resulted in efficient secretion of the Fc-tagged protein which could be purified from insect cell supernatants by the combination of lectin and protein A affinity chromatography already described. When tested by red blood cell binding or by flow cytometry the tagged proteins were shown to be functionally folded and estimates of their size in solution showed them to be oligomeric, predominantly hexamers, presumably driven by both the dimerization potential of the Fc domain and the trimerization associated with full length HA (Fig. 3.6).

Purified HA-Fc proteins, the example shown is for H5 HA, were used to immunize Balb/c mice in the absence of adjuvant as before, but in this case a dose escalation study was carried out in order to assess the minimum dose required for efficient seroconversion. The antigenicity of the candidate vaccines was evaluated in the post-immunization sera by ELISA using H5 HA-VSVG<sub>Tm</sub> fusion protein captured to the ELISA plate surface with a layer of snowdrop lectin (Galanthus nivalis lectin, GNA).

**Fig. 3.6** Bioactivity of hemagglutinin (HA)-crystallizable fragment (Fc) fusion protein shown by protein A sepharose bound with increasing concentrations of fusion protein incubated with red blood cells leading to hemadsorption (left). Cartoon of the presumed interaction of HA-Fc fusion to form the observed hexamer in solution (right)
Seroconversion to H5 was apparent at all doses tested with the titer of response correlating with the dose of immunogen (Fig. 3.7). In this experiment as little as 16 ng of HA-Fc in the absence of additional adjuvant resulted in seroconversion which can be extrapolated to doses used in man by the use of a formula based on body surface area (Reagan-Shaw et al. 2008). Based on a dose of 100 ng (which gave an appreciable titer), an adult human weight of 60 kg and mouse weight of 20 g, the human equivalent dose would be 24 mg, less than the minimum dose shown to seroconvert in trials of baculovirus expressed non-tagged HA (Treanor et al. 2006). It is evident therefore that HA-Fc fusion proteins are potent immunogens. Moreover, the strategy for H5 works equally well for a range of other HAs suggesting it is universally applicable (Loureiro et al. 2011) and the mechanism of immune enhancement has been shown, as was the case with HIV immunogen, to be via the Fc-FcR interaction (Zaharatos et al. 2011).

A successful vaccine strategy in influenza would be worthless if it generated an inappropriate antibody response but two sets of data suggest that this is not the case for the use of HA-Fc fusion proteins and that the response generated is relevant. Firstly, isotyping the serum responses to immunization has shown that the predominant profile is that expected from previous studies of soluble recombinant HA immunogens (Treanor et al. 2001). IgG1 was the main antibody isotype produced followed by some IgG2a and IgG2b antibodies (Fig. 3.8), a T helper (Th) 2 response with some Th1 contribution that is typical of the immune response to recombinant HA vaccines (Weldon et al. 2010). Use of the kappa light chain also dominated the serum responses and there was no appreciable induction of IgG3,
IgA, or IgM. The overall titer of each subclass fell with the reducing dose of immunogen and there was no evidence for a different pattern of response as the level of immunogen was reduced. Secondly, the serum generated to H5 was able to prevent HA binding to red blood cells (RBC) by the H5 serum, a receptor binding inhibition assay, as measured by flow cytometry (bottom)

**Fig. 3.8** Isotyping of the serum after immunization with the highest dose of H5 hemagglutinin (HA)-crystallizable fragment (Fc) fusion showing the polyvalent spectrum of the serum (top). Inhibition of HA binding to Guinea-pig red blood cells (RBC) by the H5 serum, a receptor binding inhibition assay, as measured by flow cytometry (bottom)
antibodies relevant to neutralization of the virus, that is to the receptor binding domain (RBD) of the HA, were generated as part of the polyvalent response. Similar responses have been generated against human H1 and H3 and avian H5, H7 and H9 serotypes and detailed epitope mapping using peptide arrays has confirmed that the predominant response is always generated to the RBD located at the top of the HA1 subunit although some antibodies are directed to the HA2 domain. In addition, expression of the HA1 domain alone as an Fc fusion protein generates high titers of serotype specific antibodies with no cross reaction on the HA2 domain. These sera are well suited to diagnostic assays aimed at resolving individual virus serotypes without recourse to reverse transcriptase-polymerase chain reaction (RT-PCR) or virus neutralization assays both of which require specialist facilities and take time. As noted, baculovirus expression has already been used extensively for the generation of candidate influenza vaccines (Cox et al. 2008; Nichol and Treanor 2006; Treanor et al. 2007; Treanor 2009; Weldon et al. 2010) so a change of HA format is all that is required to allow HA-Fc fusion proteins to effectively substitute for the non-fused molecule. These would provide rapid immune responses with smaller doses of immunogen without compromising the spectrum of the antibody response. Combinations of HA subtype and or modified forms of HA-Fc fusion proteins may be capable of generating the broadly cross reactive antibodies targeting the fusion stalk of HA that offer the possibility of long term protection against future pandemic strains (Corti et al. 2011; Ekiert et al. 2009; Sui et al. 2009).

3.6 Suitability of the Fc Domain for Human Use

A natural concern with the use of the human Fc as an immune enhancement tag is that it could break tolerance and generate a degree of autoimmunity. However, with suitable quality control at the purification stage this possibility seems very small. Firstly, intravenous Ig therapy is already used to treat a number of severe viral infections such as Junin virus (Enria et al. 2008) and has also been used for the treatment of severe H1N1 infection (Hung et al. 2011). Secondly, the Fc domain itself is widely used in clinical applications with many Fc fusion proteins undergoing clinical trial for several human diseases (Schmidt 2009). For example, Etanercept is approved to prevent tumor necrosis factor (TNF)-dependent inflammatory disease, Alefacept is used to treat psoriasis, Abatacept is approved to treat rheumatoid arthritis and Romiplostin is approved as a therapy for chronic immune thrombocytopenic purpura (Schmidt 2009). All of these drugs contain the Fc domain of human IgG1 antibody, as used here, linked to an effector protein. The use of the Fc domain in these cases is not for immune targeting but to provide a significant extension to the half-life of the proteins in plasma and an increased avidity for the ligand through Fc mediated dimerization (Schmidt 2009; Zhang et al. 2009). Many of these treatments require repeated doses and autoimmunity has not emerged as a significant issue suggesting that the Fc domain is not immunogenic in its own right when present as part of an Fc fusion protein (Schmidt 2009).
3.7 Conclusions

The expression of Fc tagged proteins has been shown to be generally applicable solution to improving immune targeting for a number of virus and bacterial vaccine candidates. The addition of Fc improves the titer of the resulting serum without compromising the spectrum of the antibody response and, based on widespread use of the Fc tag in therapeutic applications, its use appears safe. The use of Fc fusion proteins as vaccine candidates for both animal and human markets would therefore appear to offer much in terms of speed, efficiency and uniformity of manufacture.

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