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Heterosubtypic Immunity To Influenza A Virus: Where Do We Stand?

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Abstract

Influenza A virus (IAV) strains are denoted by the subtype of their hemagglutinin (HA) and neuraminidase (NA) virion surface proteins. Major changes in HA subtype among strains circulating in humans are referred to as “antigenic shift”. Antigenic shift can occur by two means; direct transmission of a zoonotic strain to humans or through reshuffling of the segmented genome in cells co-infected with animal and human strains. The lack of circulating anti-HA antibodies in human populations to a novel IAV results in extremely high frequency of illness and the potential for severe morbidity and mortality on a world-wide basis; the dreaded pandemic. Such pandemics could be partially controlled by developing a vaccine that generates effective heterosubtypic immunity (HSI) based on immune recognition of IAV antigens conserved across all viral strains. While it has long been known that T cells exhibit such broad cross-reactive specificity that could provide effective HSI, recent animal studies suggest a potential role for antibodies as well. Here we review current knowledge of the mechanisms contributing to HSI to influenza and speculate on the potential for this approach to contribute to public health.

Keywords

Influenza A Virus; heterosubtypic immunity; antibodies; T-cells

Introduction

It is estimated that annually influenza virus infects over 24 million Americans, causing ~40,000 deaths, and costing upwards of 87 billion dollars for health care and diminished productivity [1]. Due to the constant antigenic evolution of influenza viruses, influenza vaccines must be reformulated each year. The potential emergence of a pandemic, particularly from introduction of highly lethal avian influenza A virus (IAV) strains into the human population, combined with vaccine supply shortages, have made improving influenza vaccination a public health priority. The idea of creating a universal flu vaccine has long been a goal of influenza researchers. Vaccines have been shown to elicit cross-protective immunity against widely divergent IAV strains in animals, but their potential in humans has not been tested.

IAV possesses a negative strand RNA genome made up of eight gene segments that encode 11 defined gene products: HA, NA, nucleoprotein (NP), two matrix protiens (M1 and M2),

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three polymerases (PB1, PB2, PB1), and three non-structural proteins (NS1, NS2, PB1-F2). All of the non-glycoprotein genes (with the exception of PB1-F2) are highly conserved between IAV strains, and elicit cross-reactive antibody and T cell responses. The glycoproteins are highly variable. There are 16 known subytpes of HA and 9 known subtypes of NA. For the most part, glycoprotein subtypes are non-crossreactive serologically and display limited cross-reactivity for T cell recognition.

Strains circulating in humans change each year, mostly by point mutations in genes encoding HA and NA. These changes are believed to be principally driven by antibody mediated selection of neutralization resistant mutants. This process is termed “antigenic drift”, and ultimately necessitates vaccine reformulation. Antigenic shift, by contrast, reflects the introduction of IAV strains with hemagglutinin genes that the population has not previously experienced. Such viruses have the potential to be transmitted to nearly 100% of the human population with dire consequences. Direct transmission of highly lethal H5N1 avian strains to humans clearly occurs, but as yet, such strains have not demonstrated the capacity for efficient transmission in humans.

Heterosubtypic immunity (HSI) is defined as immunity generated by a given IAV subtype or its antigens that protects against challenge with other IAV subtypes (e.g. immunity to H1N1 protecting against an infection with H3N2). The idea of HSI to influenza has tantalized the scientific community for over 50 years. Schulman and Kilbourne first demonstrated that infecting mice with one IAV strain reduced pathology and decreased viral pulmonary titers upon challenge with a heterosubtypic IAV [2]. This phenomenon was specific for IAV, as infection with an influenza B virus (which is distantly related to IAV) did not afford the same protection.

These findings demonstrated the existence of limited cross-protective immunity between IAV subtypes. While the HSI did not provide sterilizing immunity (i.e. prevent infection) it clearly reduced pathogenesis and mortality. Under conditions that may be more representative of the naturally occurring human to human transmission, HSI may appear even more robust. When mice are challenged with the A/Puerto Rico/8/34 (H1N1) (PR8) virus via a route that limits initial infection to the upper nasal passages, the infection can spread to the trachea and lungs but is not lethal. Mice pre-immunized with A/Port Chalmers/1/73 (H3N2) virus, challenged in this intranasal model, prevent dissemination of infection from the upper respiratory tract [3]. These experiments demonstrate that pre-existing HSI immunity to IAV can ameliorate illness following challenge with IAV infection. HSI has also been demonstrated in other animal models such as ferrets, chickens, pigs and cotton rats [4-8].

Here, we describe the components of the adaptive immune response that are responsible for providing effective HSI and discuss the ramifications for designing a cross-protective vaccine for humans.

Heterosubtypic T-Cell Responses

IAV specific T-cells recognize viral peptides associated with major histocompatibility complex class I and II molecules. Immunogenic peptides are derived from all of the viral gene products. The majority of T cells specificities, including most of the abundant specificities (i.e. the immunodominant responses) recognize peptides derived from the conserved cytoplasmic/nuclear viral proteins. This is primarily because they represent the majority of the coding sequences translated by infected cells. Since the internal proteins are highly conserved between IAV subtypes, T cells are considered a prime effector mechanism for the generation of HSI. Although pre-existing IAV-specific T cell responses cannot prevent infection, they can participate in a more rapid response that enhances virus clearance and reduces pathology. The role of such “cross-reactive” T cells in humans has not been clearly established. In humans,

CTL activity has been shown to correlate with reduced virus shedding in the absence of antibodies to HA and NA, though not with reduced symptoms [9]. Some studies have shown evidence of protection afforded by prior exposure to influenza [10-12]. Human T cells specific for human IAV have been shown to respond to conserved determinants from swine and avian influenza strains [13], providing a basis for human protection to zoonotic subtypes.

The vast majority of information regarding T cell responses to IAV has been obtained from experiments in mice. This is due to the defined genetic background of inbred laboratory strains, the reagents available to characterize mouse immune responses and the power afforded by the many strains of "knockout mice" available. Unfortunately, the main reason that these strains are "susceptible" to influenza infection is due to deletions or mutations in one particular interferon inducible gene, *Mx* [14]. The gene product of *Mx*, Mx1, resides in the nucleus of influenza infected cells and inhibits transcription, and therefore replication, of orthomyxoviruses [15]. The Mx1 gene has been shown to have at least a hundred fold affect on susceptibility to virus [16]. The human protein MxA has a similar role but functions slightly differently. Human MxA is a cytoplasmic protein and is thought to block replication at a step subsequent to transcription [15]. However, humans are still infected with flu indicating that Mx alone is not able to control IAV infection. Only recently have groups begun to study influenza infection in congenic inbred mice that express a functional Mx protein [17,18]. It will be interesting to examine the effect of the expression of Mx in mice on the generation and magnitude of the anti-IAV T cell responses and therefore the generation of HSI.

The subtype cross-reactivity of T-cells was first demonstrated by the Doherty laboratory [19] and quickly confirmed by the Askonas [20] and Braciale [21] laboratories. These studies demonstrated that influenza A specific cytotoxic T lymphocytes (CTL) generated from infected mice could lyse target cells infected with a heterologous strain. Yap and Ada soon demonstrated that T cells could mediate protective HSI. Transfer of splenic T cells reduced virus lung titers and increased survival of mice following heterosubtypic challenge [22]. Protective immunity correlated with the cytotoxic activity of transferred cells [23]. Shortly thereafter culturing of $CD8^+$ T cell clones in vitro was developed and studies were performed which demonstrated protection from the transfer of cross-reactive (HSI) $CD8^+$ T cells [24,25]. It was also discovered that the influenza nucleoprotein (NP) was a major target of T cell-mediated cross-reactivity in BALB/c mice [26]. Askonas's laboratory demonstrated that the adoptive transfer of NP-specific cytotoxic T cells could provide enhanced virus clearance and increased survival to both homologous or heterosubtypic virus challenge [27]. It was also shown in the chicken model that T cells could mediate HSI [6].

Vaccination against conserved proteins or infection with attenuated influenza virus has been successful in providing HSI in mouse models. Cold adapted viruses are selected based on their limited ability to replicate at temperatures above 38°C to 39°C. Consequently, their replication is limited to the upper respiratory tract. Since cold adapted viruses are now in use in humans as live attenuated vaccines, their ability to induce effective HSI is a critical issue. Early studies performed using cold-adapted influenza viruses clearly demonstrated HSI [28,29]. A recent report has confirmed that cold-adapted viruses induce HSI and showed that $CD8^+$ T cells play a key role in the phenomenon [30].

In addition to attenuated viruses, other immunization strategies have produced HSI. Immunization of both inbred and outbred mice with a recombinant chimeric protein consisting of NS_1 and HA_2 (referred to as D2 protein) has also been shown to induce cross-reactive CTL and confer HSI [31-33]. DNA immunization demonstrated that anti-NP $CD8^+$ T cell responses could be generated that correlate with protective immunity against IAV challenge [34-36]. More recently, the DNA vaccine prime, adenovirus boost studies of Epstein and colleagues [37] have also demonstrated protective HSI to NP. Recent studies have shown that an

experimental prime-boost vaccination induced more potent HSI than cold-adapted virus in a simultaneous head-to-head comparison [38].

The role of $CD8^+$ T cells in HSI has been examined by using knockout mice, antibody depletion of specific cell populations, and transgenic T cell receptor (TCR) mice as a source of defined T cells in adoptive transfer experiments. We referred to several of these reports above. A key study showed that depletion of $CD8^+$ cells abrogated HSI partially in the nose and lungs of virus-primed mice [39]. Other studies that have demonstrated additional roles for $CD8^+$ T cells in HSI that deserve mention include: studies that demonstrate the important role of perforin and Fas-dependent pathways in $CD8^+$ T cell clearance of influenza [40]; a study which defines the role of $CD8^+$ T cell homing to the lungs in protection [41]; and protection of mice following adoptive transfer of TCR transgenic cells (24). It is critical to note that while $CD8^+$ T cells appear to play an important role in HSI, other immune effector mechanisms also participate. HSI is not abrogated by depletion of $CD8^+$ T cells via antibody treatment or through use of $\beta 2m^{-/-}$ mice [42], though not for challenge of highly pathogenic H5N1 [43].

The role of $CD4^+$ T cells in HSI has been studied less extensively than $CD8^+$ T cells. $CD4^+$ T cells are important for class switching and somatic mutation of B cells and therefore the development of highest affinity anti-IAV antibody responses [44]. In mice depleted of $CD4^+$ cells, neutralizing antibody titers are greatly reduced [45]. However, depleting $CD4^+$ cells prior to influenza infection results in only a slight delay in viral clearance [45,46]. This may be due to the fact that the anti-IAV $CD8^+$ T cell response is not detectably modified by the absence of $CD4^+$ T cells [46].

Whether $CD4^+$ HSI immunity is beneficial remains controversial. Adoptive transfer of $CD4^+$ T cells into nude mice accelerates neutralizing antibody responses compared to wild-type mice [44], indicating that $CD4^+$ T cell memory could potentially accelerate antibody levels during a heterosubtypic infection. Depletion of $CD4^+$ T cells prior to a subsequent challenge with a heterosubtypic virus partially abrogated HSI in the nose [39]. However, in a study of HSI protection against lethal challenge, CD4 depletion had little effect on survival of normal mice, but some impact was still observed in $\beta 2m^{-/-}$ mice [42]. It has been reported that cytolytic $CD4^+$ T cells could play a role during influenza infection [47] but perhaps only in the absence of cytolytic $CD8^+$ T cells is their role in HSI observed.

Other elements of the cellular immune system could potentially contribute to HSI. NKT cells and γδ T cells are abundant cells that typically lack CD4 and CD8 surface markers. $CD1^{-/-}$ mice exhibit HSI indicating that NKT cells, which are absent in these mice, are not essential for HSI [48]. Likewise, mice deficient in γδ T cells also generate a cross-protective immune response. Interestingly, unlike wild type mice, γδ T cell deficient mice depleted of both $CD4^+$ and $CD8^+$ T cells lose HSI, indicating that γδ T cells can contribute to HSI [48].

It is clearly established in the mouse and avian [6] IAV models that T cells can mediate protective HSI, and should be considered for vaccination strategies. There are a number of important limitations. First, the relevance to human influenza remains to be established. Second, even in mice truly sterilizing immunity is not possible with T cells since they require a substantial amount of viral replication to respond following heterosubtypic infection. Third, T cells are potentially a double edged sword since they can greatly enhance immunopathology [49,50]. Balancing protective vs. destructive effects of cellular immunity is one of the challenges of rational vaccine design.

Role of Antibodies in HSI

Neutralizing antibodies, mainly against HA, are clearly the major contributing factor for homosubtypic protection and are the historic target of influenza vaccination. NA-specific

antibodies have also been shown to reduce viral titers [51-54], morbidity, and virus shedding [55,56]. While HA and NA can possess conserved epitopes between subtypes, their role in HSI is generally thought to be minimal or non-existent. However, crossreactive antibodies to any conserved IAV epitopes may contribute to HSI. Indirect support for the concept of antibody mediated HSI is provided by studies of mice lacking terminal deoxyribonucleotidyltransferase (TdT), which adds nucleotides during V-D-J rearrangement to increase antibody and TCR diversity. $TdT^{-/-}$ mice exhibit a defect in HSI, despite having similar levels of CTL activity [57]. This is consistent with a role for antibody diversity in HSI, but further studies are required to eliminate a role for $CD4^{+}$ T cell diversity and to more rigorously demonstrate the ability of antibodies to mediate HSI via passive transfer experiments.

One study has demonstrated that mice unable to generate antibodies exhibit partial HSI [48], suggesting that antibodies, like T cells, can contribute to HSI, but are not essential. However, another study failed to see protection against highly pathogenic strains of IAV [43]). Antibody dependent HSI does not require IgA or IgA transcytosis into the lung mucosa [42,48], despite the correlation between mucosal anti-viral IgA levels and protective homotypic immunity.

More direct support has been demonstrated by studies in neonatal mice that vaccination of the mothers and even foster-nursing protected the pups from a heterosubtypic challenge [58], indicating that maternal antibodies can provide cross-protective immunity. In another study, administration of vaccines by a mucosal but not a systemic route induced IgG and IgA antibodies in both serum and lungs that cross-reacted with a heterosubtypic virus [43].

Logic dictates the most promising HSI vaccine targets are highly conserved viral gene products. During IAV infection, antibodies are probably routinely generated to all of the conserved gene products. Although NP is highly immunogenic and is expressed on infected cell surfaces in reasonable quantities [59], there is no solid evidence that antibodies specific for NP (or nuclear/cytoplasmic viral proteins) provide significant HSI.

M2, on the other hand, is an extremely promising HSI serological target. M2, a highly conserved protein expressed on the surface of infected cells and to a limited extent, on virions. M2 is highly conserved, and is abundantly expressed on the infected cell surface. M2's ectodomain is short, but highly conserved and antigenic. While early studies of antibody responses to M2 demonstrated mixed protective effects [51,60-63], treatment with anti-M2 antibodies and vaccination to generate anti-M2 antibodies can provide effective HSI [64-69]. The in vivo emergence of escape mutants clearly demonstrates the anti-viral potency of anti-M2 antibodies [70]. ADCC (antibody-dependent cell-mediated cytotoxicity) has also been suggested as a mechanism contributing to HSI by antibodies to the conserved antigen M2 [71]. If M2 is such an active target of HSI, why is effective HSI difficult to clearly demonstrate in humans? Importantly, the ectodomain of M2 appears to be weakly immunogenic during natural human IAV infection [72]. Thus, induction of anti-M2 ectodomain antibodies is a hopeful HSI vaccine strategy.

Route of Infection and Duration of HSI

Fazekas de St Groth demonstrated that respiratory tract infection was superior to i.p. or subcutaneous infection in generating protective anti-influenza immunity against homotypic infection [73]. This appears to be true for HSI as well. A study by Nguyen et al. has demonstrated that mice immunized via the intranasal route under full anesthesia (total respiratory tract infection) generated long lasting cross-protective immunity against an HSI lethal challenge. Mice primed by other routes (including i.v. and i.p.) were not as well protected despite robust CTL generation detectable in the spleen [74] and the fact that memory cytotoxic T lymphocytes (CTL) generated by i.p. inoculation can travel to the mediastinal lymph node and lung during a second, heterosubtypic challenge [75]. Mucosal administration using

adjuvant has also been shown to provide heterosubtypic protection [43]. Altogether, these data indicate that the route of initial infection can greatly affect the generation of protective HSI.

When designing an effective vaccine strategy the length of time the individual is protected is critical. In animal models the duration of protection provided by HSI depends upon where and how you look. The Gerhard group demonstrated that HSI in the nose began to disappear at about 4-5 months post infection, but it remained in the lung for over 7 months. Mice immunized with a DNA prime/adenovirus boot regimen to create anti-NP HSI could be protected from highly pathogenic H5N1 lethal challenge 5 months post vaccination [37]. Also in the ferret model, which is considered more similar to human than mice, HSI was detected 18 months post infection [8].

Epidemiologic studies of HSI in humans examined the impact of cases of flu infection preceding the 1957 pandemic, and suggest a period of protection of at least a few months [11] to perhaps several years [10]. Only the Slepushkin study covered a large enough population to begin to look for a correlation between the length of time in between exposures and protection. Prior infection in the spring had a significant apparent protective effect in both the summer and the fall pandemic waves. There is a tremendous need to update these studies on a large scale using cohorts of patients with well defined infection histories.

Concluding Remarks

The ever constant evolution of influenza viruses poses an ever changing challenge to the human host defense. The potential of pandemic influenza outbreaks and the emergence of highly pathogenic strains have enhanced interest in the ongoing investigations of HSI. Studies to dissect the individual components of immunity have revealed that multiple immune components may be required for maximal HSI. The immune system is incredibly plastic and it makes sense that the host defense has developed redundant features to ensure that pathogens cannot escape. Perhaps the key to developing an effective HSI vaccine is not concentrating on one effector mechanism but developing a vaccine to optimally stimulate them all against the conserved components of the virus.

To date, studies of HSI have been performed in mice, ferrets, pigs, chickens, and cotton rats. It is critical to extend HSI studies from the cageside to the bedside. Cold adapted live attenuated IAV vaccines have great potential for induction of HSI due to their abilities to induce local immunity at the point of viral transmission and generate $CD8^+$ T cell responses. In addition there are promising DNA/adenovirus regimens that also generate highly effective HSI in animal models. Though ultimately it would be optimal to develop a vaccine that generates life-long HSI, the ability of vaccines to induce HSI that persists for even a few weeks could still save millions of lives until matched vaccines are available during a pandemic.

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