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Immunity to avian influenza A viruses

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Summary
While the basic principles of immunity to the influenza A viruses are probably similar for all vertebrates, detailed understanding is based largely on experiments in laboratory mice. Elements of the innate response limit early virus replication, although high pathogenicity strains can trigger effusive cytokine/chemokine production and lethal shock. Virus clearance is normally mediated via $CD8^+$ effector T cells but, in their absence, the class-switched antibody response can ultimately achieve the same goal. Protection against re-infection is optimally provided by antibody (IgG and IgA) specific for the homologous viral haemagglutinin, and priming against the neuraminidase and the low abundance, conserved M2 protein can also have an effect. Influenza virus-specific plasma cells and $CD8^+$ T cells persist in the long term and the recall of the $CD8^+$ T cell response can lead to earlier virus clearance. The characteristics of the aging immune system and possible, novel vaccine strategies are also considered.

Keywords
Antibody – Cytokine shock – Cytotoxicity – Memory – Protection – Secondary response – T-cell.

Introduction

Our understanding of immunity to the influenza A viruses (IAVs) comes from the natural challenge of immunologically naïve, vaccinated and previously infected humans and other vertebrates, and from experiments in a variety of animal models. Most detailed information on its mechanism is derived from studies in laboratory mice, as we are able to make full use of the enormous spectrum of immune and molecular probes available in this species (25). The limitation is, however, that while mice are readily infected by aerosol droplets or intranasal (i.n.) exposure to both virulent and relatively non-pathogenic IAVs and can develop severe, and even fatal pneumonia, the mouse is not a natural host for influenza. Furthermore, the pathological process in the murine lung is less like the human disease than is that seen following respiratory challenge of ferrets, the species used to isolate the first human IAV in 1933. Virologists and those evaluating vaccines are generally of the opinion that the ferret is the best available mammalian model for studying influenza (32), but its value for detailed immunological analysis is severely constrained by the relative lack of reagents (64, 77). That situation is better for domestic chickens but, as discussed elsewhere in this volume (67), the pathogenesis of the infectious process in birds and mammals is somewhat different (82).

Even so, it seems reasonable to think that both the basic nature and the limitations of IAV immunity are in most senses comparable for vertebrates as diverse as birds and humans. The focus of preventive approaches (41) may, however, be quite dissimilar depending on the relative importance of financial and ethical constraints. With poultry, we are interested principally in relatively short-term solutions, and also have the possibility of taking draconian control measures in the face of an outbreak (82).

Long-term protection is much more important for people and for species like horses, where the individual animal may be of great monetary or emotional value.

Economic, technological, organisational and regulatory factors limit our capacity to get a vaccine against a novel IAV distributed rapidly and in sufficient volume to protect the human population. Consequently, there is currently increasing interest in developing more cross-reactive immunogens (24, 29, 53, 62) that could give at least some protection against seasonal variants and novel IAVs invading from avian reservoirs. The idea would be to manufacture and stockpile such a product before specific knowledge of the particular IAV is available. Even if priming in the face of a pandemic meant that those who were subsequently infected developed mild rather than severe disease, the consequent decrease in morbidity would greatly decrease the social and economic costs.

As with many questions concerning vaccination against rapidly changing pathogens (like the human immunodeficiency virus [HIV] or hepatitis C virus) it is, in fact, reasonable to think that we will need to adopt innovative products and approaches if we are to improve on the current situation. These may be used in addition to the presently available vaccines, but it is obvious that the strategies available at present could not protect more than a small proportion of the global human population in the face of a pandemic that mimicked the 1918-1919 tragedy in scope and mortality. The combination of rapid air travel and the fact that there are more than three times as many people on the planet as there were 90 years ago gives us good cause for great concern, and for moving forward with all possible dispatch.

There are papers on vaccines elsewhere in this volume, so the authors will confine this discussion to questions of immune mechanisms rather than the practical issues. For that reason, the focus is on the detailed mouse studies that provide a conceptual framework for understanding what is happening with species of real interest, including people. Some issues require little probing. There is, for instance, no doubt that the best protection against IAV challenge in all species from birds to humans, is conferred by pre-existing, high titre neutralising antibody against the homologous haemagglutinin (HA) glycoprotein, the primary target of all current vaccination strategies. Beyond that, however, the situation is less clear.

Characteristics of IAV-specific immunity

Mice primed with purified HA, or by intraperitoneal injection of the same live or inactivated IAV, are effectively protected against intranasal infection with the homologous virus. Following low dose respiratory challenge, there may be little boosting of the level of host immunity once the input inoculum is neutralised. If, however, the HAs of the priming and challenge virus are partially mismatched, there can be a level of virus growth in the respiratory epithelium (compared to naïve controls) that leads to increases in the levels of both the antibody and cell-mediated immune (CMI) responses (74). Whether or not the mouse has previously encountered a variant IAV, the subsequent events are the same, although duration and magnitude will be influenced by antigen load and will differ depending on the overall extent of virus replication.

Priming of the immune response commences with the interaction of virus with dendritic cells (DCs), particularly those that line the upper respiratory tract. These key antigen presenting cells (APCs) then migrate via afferent lymph to the regional cervical and mediastinal lymph nodes (RLNs) where the primary (naïve) or secondary (partially immune) host response develops. Viral protein accesses both the cytoplasmic and lysosomal processing pathways to provide the peptides bound to major histocompatibility complex class and class II glycoproteins (PMHCI and PMHCII) that are recognised by the $CD8^+$ cytotoxic T lymphocytes (CTLs) and $CD4^+$ helper (T_H) cells respectively. Infection of DCs by IAVs does not normally result in either cell death (13) or the release of new virus, but highly pathogenic (HP) H5N1 strains can both cause productive infection and kill certain human DC subsets (85), an effect that could obviously be immunosuppressive.

The RLNs act as a 'nursery' for the developing immune response, bringing together the APCs with those precursor (p) B cells, $CD4^+$ T_H cells, and $CD8^+$ CTLs bearing surface receptors (BCRs and TCRs) with the capacity to bind (in the main) conformed tertiary structures on viral proteins (B cells) or viral peptides presented by DC MHCI or MHCII glycoproteins (T cells). The DCs are 'activated' (18) by antigen-specific T_H cells (4) to be efficient APCs, with particular subsets in the DC population being more effective in this regard (31, 60). The various lymphocyte populations then start to divide (clonal expansion), progressively acquiring functional activity. Differentiation is cell-cycle dependent (40) and asynchronous division is thought to lead to different fates (73), such as development into CTL effectors that exit (after about 5-7 days) and migrate to the infected lung, or maintenance as less activated memory CTLp that survive long term and are available for further restimulation on subsequent viral challenge. The IAVs themselves may activate (or 'license') DCs (75) and effector CTL populations can be generated in the absence of a concurrent $CD4^+$ T cell response, but it does seem that T_H involvement is normally required for the development of high quality CTL memory (3).

Both $CD62L^{hi}$ central memory (T_{CM}) and $CD62L^{lo}$ effector memory (T_{EM}) CTLps recirculate from blood to tissue to lymph and are widely distributed throughout the host. However, only the TT_{CM}s can access the RLNs via high endothelial venules while the T_{EM}s enter via afferent lymph subsequent to migration through somatic tissues. This CD62L gating mechanism does not operate in the spleen. Lymphatic tissues are also found in birds but, while ducks have structures that resemble mammalian lymph nodes, domestic chickens lack anatomically distinct nodes, although they do have diffuse mucosal lymphoid aggregates and Peyer's patches [reviewed in (82)]. A combination of splenectomy and shutting down naïve T cell trafficking to mouse lymph nodes by treatment with the Mel-14 monoclonal antibody (mAb) to CD62L still allowed a primary immune IAV-specific response to proceed (90), indicating that although the LN may provide an ideal milieu for the developing host response, even mammals can use alternative anatomical niches.

Memory CTLps are then maintained at detectable frequency for the life of a laboratory mouse (44) and (at very low frequency) for as long as 50 years or more in humans (21), although the capacity for effective recall may be greatly diminished after 3 years (57). The T_{CM}s become more prominent in the longer term reflecting that there can be reciprocal transition between the $CD62L^{hi}$ and $CD62L^{lo}$ phenotypes. Following virus challenge, at least a proportion of the T_{CM} set will be reactivated to become $CD62L^{lo}$ CTL effectors and the resident T_{EM} set in the lung may also play an important part (20, 27, 66). It is also of note that while the T_{CM} pool is generally considered to be the 'optimal' population for recall, it does contain many minimal clonotypes expressing what are probably 'poor fit' TCRs (45).

The B cells also go through a differentiation process that leads to the development of either memory B cells or protein-secreting plasma cells that tend to localise in sites like the mucosa-associated lymphoid tissue (61) and the bone marrow (99). Both memory B cells and IgA and IgG-secreting plasma cells are readily detected in the lungs of mice that have recovered from IAV infection (43). Plasma cells no longer express surface BCRs and can be very long-lived (101). The BCRs are the precursors of secreted immunoglobulin (Ig) molecules and the spectrum available for virus recognition is modified during antigen-driven B cell differentiation by somatic diversification, affinity maturation and T_H-dependent class switching in germinal centres. Some T_H-independent IAV-specific IgA may be generated early in the course of the response (80), but class-switching to IgG1, IgG2A and other isotypes is generally $CD4^+$ T_H-dependant (10). The chicken also has organised germinal centers and plasma cell accumulations (particularly IgA secretors) in the lung (72), the paranasal Harderian gland and in intestinal sites like Meckel's diverticulum (82), while the human nasal mucosa and tonsil contain substantial populations of antibody-secreting cells (16). The nasal associated lymphoid tissue of the mouse is very limited in extent and does not seem to play an essential role in the IAV-specific response (98).

Virus clearance from the infected lung is mediated most efficiently by the $CD8^+$ CTLs which, while they can secrete effector cytokines such as γ interferon (IFN-γ) and tumor necrosis factor α (TNF-α) (51), are thought to operate primarily via perforin and granzyme mediated cytotoxicity (42, 86, 88). In the absence of $CD8^+$ T cells, the infection is controlled more slowly by the T_H-dependent antibody response. Transferring large numbers of *in vitro* activated $CD4^+$ T cells can also reduce IAV lung titres (9), although it is not obvious that IFN-γ and TNF-α-secreting $CD4^+$ T_{H1} effectors acting alone can terminate this infectious process under normal, physiological conditions. The same relationship between virus-specific $CD8^+$ and $CD4^+$ T cell-mediated effector function may also apply in chickens (65).

Once infectious IAV is eliminated from the respiratory epithelium, evidence of further, rapid CTLp division is soon lost (30) and many of the CTL effectors are thought to die, although increased numbers of antigen specific CTLps may remain in the lung for a considerable period (38). There is published evidence that IAV antigen can persist long after virus clearance (102). However, this is not an invariant finding: in a very detailed set of as yet unpublished experiments that replicated and extended this study (102), the authors have been unable to detect antigen for more than a few days after the termination of the infectious process (J. Mintern, P.C. Doherty and S.J. Turner, in preparation). Our current understanding of memory is that the T_{CM} and T_{EM} subsets are maintained by physiological processes (involving cytokines such as IL-7 and IL-15) in the absence of further TCR/PMHCI ligation (7, 71), although IAV boosting will expand CTLp numbers. Less is known about the consequences of priming $CD4^+$ T cell memory, although there is some evidence for T_H-mediated protection (17).

Innate immunity and cytokine shock in highly pathogenic influenza A virus infections

The role that innate immunity (46) plays in IAV infections can be addressed from four, related aspects. Firstly, there are the protective effects mediated by rapidly produced cytokines such as IFN-α, a topic that is reviewed elsewhere in this volume in the context of Mx gene function (see Haller *et al.* in this issue) (79). Second, there is the role of various 'immediate' effector cell populations, like the

natural killer (NK) cells and NKT cells (36) that produce cytokines such as TNF-α and IFN-γ, and can mediate antigen non-specific cytotoxicity. Then there is the possibility of using molecular reagents derived (or identified) from the analysis of the innate response as adjuvants to promote vaccine efficacy (11, 39, 47). Finally, there is the cytokine shock effect resulting from infection with HP IAVs such as the 1918-1919 H1N1 virus and the currently circulating avian H5N1 strains (59).

Blocking the effects of TNF-α and IFN-γ by using mice in which the genes for the cytokine or its receptor(s) have been 'knocked out' (-/-) by gene targeting can modify the nature of the IAV-specific response, although the consequences for virus control are generally not very obvious (33, 78, 94). Interestingly, disrupting the IFN-γ-inducible nitric oxide synthase NOS2 allows the emergence of an IFN-γ-mediated IAV clearance mechanism that is not normally apparent, illustrating the inherent complexity of the networks of cytokines, chemokines and free radicals that can potentially operate in any infectious process (68). When TNFR-1$^{-/-}$ mice were infected with HP H5N1 viruses, morbidity was reduced, but there was no effect on either virus replication and spread or the ultimate disease outcome (83).

Cytokine shock was first recognised as an important cause of HP IAV morbidity and mortality for human subjects infected with an avian H5N1 strain (14). Looking back, it seems apparent that some of the atypical aspects of the 1918-1919 pandemic, including the high acute death rates in fit, young adults, can be attributed to this cytokine 'storm' effect that has now been reproduced by infecting mice and primates with the reconstructed 1918 virus (48). Since then, there have been extensive studies showing that HP IAVs induce massive cytokine and chemokine production (54, 91) in cell types as diverse as respiratory epithelial cells, neutrophils and lung monocyte/macrophages (69). This suggests various therapeutic possibilities, including the early use of anti-inflammatory drugs (12, 28).

Cross-reactive immunity and protection

Evidence of cross-clade protection within the H5N1 viruses has given some cause for optimism (2, 37). Otherwise, apart from the exploitation of similarities between closely related viral HAs and sharing of the neuraminidase (NA) (8, 15), the discussion of cross-reactive, protective immunity has focused on two main strategies. The first strategy is to prime against the membrane exposed (e) component of the conserved, but low abundance, M2 ion channel protein found on the surface of the virion. Using a variety of vaccination protocols to stimulate an M2e specific antibody response, a reasonable level of protection can be demonstrated in both mice and domestic chickens (5, 22, 52, 82, 87). A number of companies are developing possible M2e vaccines for use in humans, and a commentary published in the *New Scientist* (63) indicates that they are safe and promote an antibody response. However, there is also a report that M2-priming exacerbated the severity of the disease in pigs following live virus challenge (35), so it would be wise to proceed with caution.

The second approach is to use the fact that most of the CD8$^+$ CTL response is directed at peptides derived from relatively conserved, internal IAV proteins (86). These are presented to the CD8$^+$ T cell receptor as PMHCI complexes, or epitopes, so any attempt to make a vaccine that primes or boosts CD8$^+$ T cell immunity must take account of the spectrum of MHCI alleles (encoded at the HLA A, B and C loci in humans) present in the target population. Each individual MHCI glycoprotein will probably bind a different peptide and, although some MHCI types may be very common in, say, Caucasians, that may not necessarily be the case for Africans. Differences between groups and the great polymorphism of MHCI types both create difficulties for any approach that seeks to prime with peptides (23), either as individual entities or in some linked form (polytopes). The same is true for CD4$^+$ T cell memory, which may be directed at peptides derived for both surface (HA) and internal (NP) virus proteins and can, at least for some epitopes, be associated with some degree of viral load reduction on subsequent virus challenge (17).

'Cold adapted' live virus vaccines are expected to mimic the normal IAV-specific response, though at a lower level due to the reduced antigen dose. There is the danger with any live IAV vaccine that the input virus may be neutralised by pre-existing, HA-specific Ig, with the consequence that there is no boosting effect (1, 6). At this stage we have only a limited understanding of the extent of CTLp expansion induced by cold adapted IAVs in humans (34). Alternative prime/boost strategies might use DNA that encodes whole proteins, or some form of engineered virus (adenovirus, poxvirus, alphavirus, etc.) or bacterial (tuberculosis, *Listeria*) vector systems which deliver the IAV protein to the cytoplasmic processing pathway (89, 97).

Heterologous subtype priming (e.g. H3N2 and H1N1) and vaccination experiments with live IAVs (including cold adapted strains) in mice show very clearly that, while having what might be regarded as physiological numbers of CD8$^+$ memory T cells cannot prevent infection or even limit virus growth in the lung over the first 24 to 48 hours or so, the more rapid expansion of memory (versus naïve) CTL precursors leads to enhanced virus clearance and

survival (30, 70). This protective effect can be shown with quite virulent viruses, providing memory T cell numbers are high (86).

Similar protection has been inferred from experiment in chickens, although the mechanistic analysis has been much less comprehensive (81). Early studies also indicated (57, 58) that established T cell memory can limit the severity of the disease in humans, and the issue is now being re-examined in the light of concerns about a possible H5N1 pandemic. A recent, very detailed analysis showed, for example, that people who would not have been exposed to an H5N1 virus had cross-reactive memory CTLps and $T_H$ps that can potentially recognise H5N1-infected target cells (50, 53). Perhaps the reason that healthy adults in their 20s to 50s do not normally succumb to seasonal IAV infection is that they are protected by memory T cells.

Although $CD8^+$ T cell memory can be very long-lived, there is also evidence that the protective effect is greater if CTLp numbers are high. Boosting this response in the face of a spreading pandemic might thus be one way of limiting the extent of severe morbidity and the duration of virus shedding, although it will not stop people from becoming infected. Taking such an approach will probably require a change of mindset for both regulatory authorities and vaccine manufacturers. As it stands, there is no evidence that prior exposure to seasonal H1N1 and H3N2 viruses protects against the extremely pathogenic H5N1 strains. If, however, an H5N1 IAV does develop the ability to spread in humans, it will more likely escape rapid control if the mortality rate is around the 2% to 5% range (as in 1918-1919) rather than the current 60% or so that (like an Ebola outbreak) will trigger an immediate 'red alert'.

Another cross-protective strategy that is being considered in the HIV field focuses on generating antibodies specific for shared carbohydrate moieties on viral surface glycoproteins (96). Given that most inactivated IAV vaccines have been produced in embryonated hen's eggs, any consequent antibody responses would be directed at avian rather than human glycosylation patterns. Live, 'cold adapted' IAV immunogens will, of course, acquire the appropriate 'species-specific' carbohydrates following replication in the infected host. However, if antibodies to carbohydrates expressed on viral HA or NA proteins are associated with any protective effect, most who work in this field would expect that to be minimal.

In the final analysis, a possible cross-reactive vaccine that might be produced and stockpiled for emergency use in the face of a severe pandemic could use a spectrum of different approaches, each of which gives partial protection. Unlike HIV, where the virus integrates into the host genome and the game is lost once it evades the initial immune control, the task with the IAVs is to reduce the severity of the acute disease and to minimise any immunosuppressive effect until the normal processes of adaptive immunity can eliminate the pathogen. Perhaps that is achievable.

The aging immune system

The incidence of morbidity (26) associated with seasonal influenza pandemics is increased in the very young (who have no pre-existing immunity) and in the elderly (55). Immunosenescence (84) is a normal feature of aging and it is generally considered that the capacity to respond to novel pathogens is diminished. Analysis of toll-like receptor (TLR 3 and TLR4) function in older adults has, for example, shown that monocytes and DCs respond less well than expected to viruses and bacterial products, indicating that there could be a general defect in stimulatory capacity (49, 95). With respect to influenza, while levels of HA-specific Ig may be reduced (19), repeated IAV challenge over the years can lead to the maintenance of substantial $CD8^+$ and $CD4^+$ memory T cell populations. In fact, T cell responses measured following *in vitro* stimulation may give a better correlate of protection than antibody levels alone (56).

Mouse studies indicate that IAV-specific $CD8^+$ memory CTLps generated early persist and give good recall responses in the very long term. However, older mice that are exposed to IAVs for the first time have substantial defects in their CTL TCR repertoires that can cause various problems, the most common being diminished responses to heterologous virus challenge (100). This loss of repertoire was most apparent for a prominent nucleoprotein epitope ($D^bNP_{366-374}$) that presents a relatively 'bland' PMHCI interface to the variable TCR CDR3β region (93) and, as a consequence, tends to select a very limited spectrum of responding TCRs. The effect was much less for the more structurally prominent $D^bPA_{224-236}$ PMHCI that recruits a more diverse TCR response profile (92).

Given that the incidence of mutation in the key sites of immunogenic peptides tends to be low for the IAVs (76), the influenza specific CTLp populations in elderly humans are likely to be the progeny of memory cells generated many years previously and restimulated as a consequence of periodic exposure to infectious virus. Analysing the long-term persistence of IAV-specific CTLp and T_H clonotypes in humans would clearly be of interest, particularly in the context of further antigenic challenge, either as a result of natural infection by seasonal IAVs or following exposure to an appropriate vaccine. Such 'at risk' populations may provide a useful target group for testing whether boosting T cell numbers has some protective value.

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L'immunité contre les virus de l'influenza aviaire de type A

P.C. Doherty, L.E. Brown, A. Kelso & P.G. Thomas

Résumé

Si les principes généraux de l'immunité contre les virus de l'influenza de type A sont probablement les mêmes chez tous les vertébrés, la connaissance précise que nous en avons repose fondamentalement sur l'expérimentation conduite sur des modèles murins. Certains éléments de la réponse innée parviennent à empêcher la multiplication précoce du virus, mais les souches très pathogènes du virus sont capables d'induire une libération de cytokines/chémokines et de causer un choc létal. L'élimination du virus se fait généralement par des cellules T $CD8^+$ effectrices ; à défaut, la réponse anticorps mise en jeu par des immunoglobulines de différentes classes atteint le même résultat. La meilleure protection contre une réinfection est assurée par les anticorps (IgG et IgA) spécifiques de l'hémagglutinine virale homologue ; l'amorçage de la réponse immune contre la neuraminidase et la protéine M2, faiblement abondante mais bien conservée au plan antigénique, peut également avoir un effet protecteur. Étant donné que les plasmocytes et les lymphocytes T $CD8^+$ spécifiques du virus de l'influenza persistent longtemps, un rappel de la réponse des lymphocytes T $CD8^+$ peut conduire à une élimination plus rapide du virus. Les auteurs examinent également les caractéristiques du système immunitaire des sujets plus âgés ainsi que les dernières innovations dans le domaine des stratégies vaccinales.

Mots-clés

Anticorps – Cellule T – Choc létal – Cytotoxicité – Mémoire – Protection – Réponse anticorps secondaire.

Inmunidad contra los virus de la influenza aviar de tipo A

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Resumen

Si bien es probable que los principios básicos de la inmunidad contra los virus de la influenza aviar de tipo A sean similares en todos los vertebrados, la mayoría de los conocimientos proceden de experimentaciones con ratones. Distintos factores de la respuesta innata limitan la replicación precoz del virus, pero las cepas de alta patogenicidad pueden inducir una producción anormal de citocinas/quimiocinas y un choque mortal. Habitualmente, las células efectoras

T CD8+ median la eliminación del virus pero, de faltar, la reacción de los anticuerpos producida por distintas inmunoglobulinas también puede lograrlo. Los anticuerpos (IgG e IgA) específicos contra la hemaglutinina viral homóloga procuran la mejor protección contra una reinfección, y la sensibilización del sistema immune contra la neuraminidasa y la proteína M2, poco presente pero bien conservada, también puede producir un efecto protector. Los plasmocitos y las células T CD8+ específicas del virus de la influenza aviar persisten largo tiempo y la memoria de la reacción de estas últimas puede acelerar la eliminación del virus. Los autores también exponen las características del sistema inmunitario de sujetos de mayor edad y nuevas estrategias de vacunación.

Palabras clave
Anticuerpo – Célula T – Citotoxicidad – Choque por citocinas – Memoria – Protección – Respuesta secundaria.

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