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Immunity to Influenza

The Challenges of Protecting an Aging Population

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Abstract

Influenza viruses cause annual epidemics and occasional pandemics of acute respiratory disease. Improved vaccines that can overcome the decline in immune function with aging and/or can induce broader immunity to novel pandemic strains are a high priority. To design improved vaccines for the elderly, we need to better understand the effects of age on both innate and adaptive immunity. In a murine model, we have determined that defects in antigen-presenting cell (APC) expression of pattern-recognition molecules, costimulatory molecules, and cytokine production may play an important role in the reduced clonal expansion of T cells in aging. The use of immunomodulators such as adjuvants may overcome some of the defects of aging immunity and may also be useful in the development of improved vaccines for avian influenza A subtypes that pose a pandemic threat. Several novel strategies including the use of ISCOM-formulated vaccines, mucosal delivery, or DNA vaccination provided cross-subtype protection that could provide an important component of immunity in the event of a pandemic.

Key Words

Influenza virus
Immune response
Aging
Pandemics

Influenza: Epidemics and Pandemics in an Aging Population

Epidemics of acute respiratory disease caused by influenza A or B viruses result in substantial morbidity and mortality each year *(1,2)*. Although the highest attack rates of influenza infection occur among young children, the risk of hospitalizations and death from pneumonia are highest among elderly persons (>65 yr of age). The elderly account for >90% of influenza-related deaths, which

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are largely a result of pneumonia or exacerbation of cardiopulmonary or other chronic conditions. From 1990–1999, influenza resulted in an average of approx 36,000 deaths annually in the U.S. *(3),* almost double the previous estimate of 20,000 annual influenza-related deaths determined for the period from 1976 to 1990. This dramatic increase is likely, in part, owing to the aging of the U.S. population *(4).*

Vaccination is the primary strategy for reducing the morbidity and mortality associated with influenza *(5).* An inactivated trivalent influenza vaccine containing two influenza A viruses (H1N1 and H3N2) and a type B virus is recommended in the U.S. for persons in this high-risk elderly population. In healthy younger adults, the vaccine may be 70–90% effective in preventing influenza illness, if the vaccine antigen is antigenically closely matched with the circulating epidemic strain. Although the vaccine can be 80% effective in preventing death in elderly nursing home residents, it may be only 30–40% effective in preventing influenza illness *(6,7).* As the elderly become an increasing proportion of the population, the development of more effective measures to reduce the impact of influenza in this population is an urgent public health need. Our research seeks to better understand the limitations of immunity in aging and to develop improved preventive and therapeutic strategies for the elderly.

In addition to annual epidemics, influenza A viruses causes occasional pandemics of respiratory disease. Wild aquatic birds are the natural hosts and the original source of influenza A viruses that infect humans and other mammalian species. Influenza A viruses bearing each of the known 15 hemagglutinin (HA) and 9 neuraminidase (NA) serologically distinct subtypes infect avian species *(8).* Influenza A viruses possessing novel HA and one or more other genes derived from avian influenza viruses sporadically emerge in humans and have the potential to cause a pandemic of influenza if the virus is capable of spreading in a population that lacks immunity to the novel HA. Only influenza A viruses possessing H1, H2, or H3 HA genes and N1 or N2 NA genes have caused widespread respiratory illness in humans, including three pandemics in the 20th century. However, since 1997, three subtypes of avian viruses (H5N1, H7N7, and H9N2) have caused a limited number of human infections, some of them fatal *(9,10).* The direct transmission of wholly avian viruses from birds to humans has raised awareness that a virus with the potential to cause another influenza pandemic may emerge from these sporadic transmissions of avian viruses to humans. Traditional strategies for vaccination against avian H5 viruses yielded vaccines that were poorly immunogenic in healthy younger adults *(11,12).* Another pandemic could occur at any time, causing excess morbidity and mortality worldwide with a higher proportion of deaths likely to occur in adults <65 yr of age *(13).* Thus, there is an urgent need for effective pandemic influenza vaccines for all ages. A second goal of our research is to better understand the pathogenesis of avian viruses in mammalian species and to evaluate novel vaccine strategies to protect against influenza subtypes that pose a pandemic threat.

Immunity and Aging

A decline in immune function is a hallmark of aging that affects the ability to resist infectious diseases and respond to preventive and therapeutic vaccinations. Like influenza, the incidence of severe disease owing to RSV and pneumococcal pneumonia also increases in the elderly *(14).* The incidence of bacterial infections of lungs, urinary tract, skin, and soft tissues and reactivation of inactive tuberculosis and herpes zoster also increase in elderly individuals *(15–17);* infectious diseases tend

to be severe and more likely to result in death in aged individuals. Because the immune function in the elderly operates at a suboptimal level, this population is especially vulnerable and may become a target for epidemic diseases and also become a source for the spread of disease to the general population. With improved healthcare and advances in medical science, the percentage of elderly in the population is steadily increasing worldwide; however, the morbidity owing to a number of infectious diseases is still high in this segment of population when compared with that of younger individuals. Hence, our research goals are to better understand the causes of immune dysfunction in aging and develop strategies that confer improved prevention and control, not only for influenza, but also for other diseases that may be controlled by vaccination.

A number of adaptive immune functions including cytokine secretion, clonal expansion, and function of antigen-specific T and B cells, antigen-presenting cell (APC) function decline or are altered with age, leading to decreased efficacy of preventive vaccination *(18).*

Toll-Like Receptors and Innate Immunity

The physical and functional integrity of plants, lower vertebrates, and mammals, including humans, is maintained against the onslaught of multitude of microorganisms through evolutionarily conserved sets of molecules, namely Toll-like receptors (TLR). These receptors recognize conserved molecular patterns associated with pathogens and upon ligation secrete a wide range of antibacterial peptides that destroy the bacteria, yeast, fungi, and perhaps some viruses *(19).* In mammals, when the integrity of epithelial surfaces is compromised, TLR ligation results in the secretion of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin (IL-6), which initiate an inflammatory response to clear the invading organism. In addition, the inflammatory response results in the recruitment of cells of adaptive immunity to initiate clearance of the pathogens by generating a specific immune response, if the antigen dose is high. The nature of the ligands that are recognized by TLRs have only recently been elucidated and the list of known ligands continues to grow. Microbes, microbial products and pharmaceuticals that are ligands for TLR2, -3, -4, -5, -6, -7 and -9 have been identified *(20–23).* TLR1 and -2, and -2 and -6, recognize the cell wall components of Gram-positive bacteria and yeast, whereas TLR4 recognizes the predominant Gram-negative bacterial product, LPS. Recently TLR3, -5, -7, and -9 have been shown to recognize double-stranded RNA (poly I:C), bacterial flagellin, immiquimod, and CpG ODN, respectively. As the susceptibility to and severity of bacterial, mycotic, and viral infections increases with age, defects at the level of expression and function of TLRs may explain why the elderly often fail to present classical symptoms of infectious diseases and fail to elicit adequate adaptive immune responses to infection or immunization.

Expression of TLR in Aging

To address the status of innate immunity in aging, we examined the expression of all known murine TLR (TLR1 to -9) on macrophages from young and aged mice by real-time reverse transcription polymerase chain reaction (RT-PCR), because macrophages play a crucial role in the initiation of an inflammatory response and facilitate tissue-repair process. We examined whether the TLR-expression pattern varies with the anatomical location and activation state of macrophages by looking at splenic and thioglycolate-elicited peritoneal macrophages *(24).* TLR expression on both macrophage

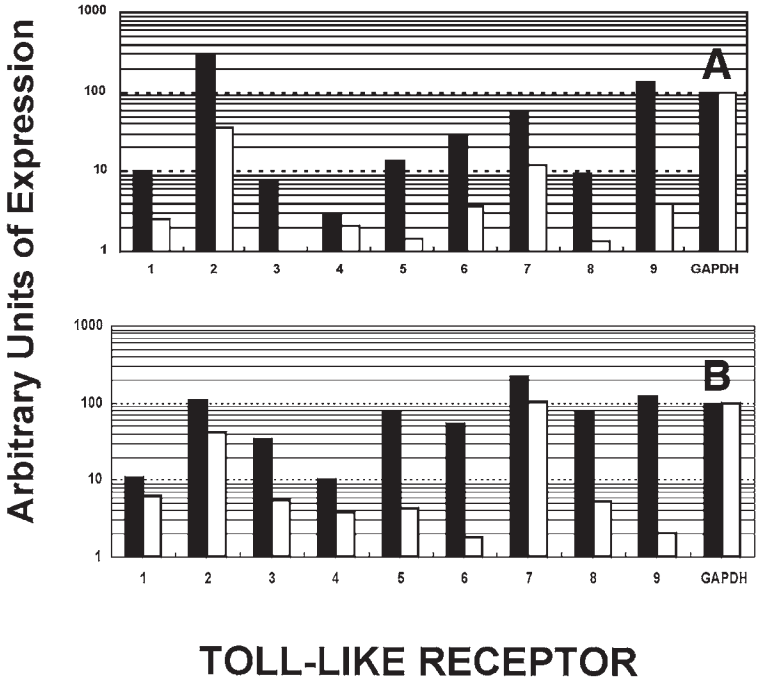

Fig. 1. TLR expression by real-time RT-PCR on splenic and thioglycollate-elicited macrophages from young and aged mice. TLR expression was quantified from splenic **(A)** and thioglycollate-elicited **(B)** macrophages from 5 to 10 young (2–3 mo) and aged (18–24 mo) C57BL/6 mice and expressed as relative units normalized to GAPDH expression. Solid and empty bars correspond to macrophages isolated from young and aged mice, respectively. The experiment was repeated three times with similar results.

populations declined with age with expression of TLR9 showing the greatest reduction followed by TLR5, -6, -3, -2, -4, and others (Fig. 1).

When TLR2, TLR3, TLR4, TLR5, and TLR9 were ligated with zymosan, poly I:C, LPS, flagellin, and ODN containing CpG, respectively, they secreted substantially lower levels of proinflammatory cytokines and chemokines, TNF-α, IL-6, α, and CCL5 (Table 1). A decline in chemokine and cytokine expression may result in a poor inflammatory response, reduced recruitment of cells of adaptive immunity at the site of infection/inflammation, a protracted repair process, and, possibly, failure to present classical disease signs. A lack of presentation of clinical signs at the onset of infection may delay suitable intervention modalities and potentially lead to an increase in severe and fatal viral and/or bacterial disease. One approach to improve immune responses to vaccine preventable diseases such as influenza in the elderly population would be to supplement vaccines with immunomodulators, which facilitate effective recruitment of professional APCs and support their differentiation, maturation, migration, and function in the presentation of antigens to specific T and B cells.

Defects in Adaptive Immunity in Aging

It has been shown that T-cell function as measured by alloresponses, proliferative responses to mitogens or antigens, cytokine

Table 1. Impaired Pro-Inflammatory Cytokine and Chemokine Secretion From Macrophages in Aging[a]

	TNF-α pg/mL		IL-6 pg/mL		CCL5 pg/mL		MIP-1α pg/mL	
Stimulus	Young	Aged	Young	Aged	Young	Aged	Young	Aged
None	120	8	69	50	<1	<1	6	<1
Zymosan	5539	746	4425	104	665	44	3113	259
Poly I:C	1404	637	1718	285	8200	987	6578	130
LPS	7586	1958	28853	2854	1055	1044	1614	1000
Flagellin	8427	865	7599	909	8604	906	344	470
CpG	406	231	1038	413	1617	906	229	93

[a] Young and aged B6 macrophages (5×10^5) were stimulated with lipopolysaccharide (LPS), poly I:C, zymosan A, stimulatory CpGODN, flagellin, or were cultured untreated for 48 h. The amounts (in pg/mL) of the indicated cytokines and chemokines secreted in response to TLR stimulation were detected by enzyme-linked immunosorbent assay (ELISA).

Table 2. Primary Immune Responses, Not Recall Responses, Are Impaired in Aged Mice

Groups	Immunogen[a]	Age when tested (mo)	HI titer[b]	IFN-γ pg/mL from T cells[c-*]
Primed at 2 and 6 mo	H1N1 virus	18	1920	10,257
Naive	H1N1 virus	18	240	605
Naive	PBS	18	≤15	<250
Primed at 2 mo	H1N1 virus	4	1920	39,295
Naive	H1N1 virus	4	960	35,316
Naive	PBS	4	≤15	<250

[a] DBA/2 mice (10 per group) were inoculated by the ip route with 200 HAU of A/Texas/91 live influenza virus.

[b] HI = Hemagglutination inhibition antibody titer was determined in serum collected 21 d after immunization.

[c] Splenic T cells were cultured for 48 h with influenza NP peptide (147–154) known to be a dominant CTL epitope and the amount of IFN-γ in culture supernatants was determined by ELISA.

secretion, and cytotoxic activity decline with aging in humans and animal models *(18,25,26).* Whether these reduced T-cell responses are owing to defects at the level of T cells themselves, or the APC, or both T cell and APC, are not clear. Although recall (memory) responses appear to be intact, the primary antibody and T-cell responses to neoantigens are severely impaired. This is shown in Table 2. Mice primed with influenza virus as young adults mount a substantial recall response in old age, a recall response similar to that achieved in younger mice. However, the primary immune response, especially the T-cell response, to influenza virus in aged mice was substantially poorer than that observed in young mice *(27).*

Because T-cell activation requires the interaction of APC to present peptide-bound MHC and provide costimulatory signals, poor T-cell responses in aging could be owing to delayed APC-T-cell contact kinetics, decreased TCR affinity, decreased adhesive forces between APC-T cell conjugates, defects in immunological synapse formation, lack of or suboptimal costimulation, defects in antigen processing, or a combination of these factors.

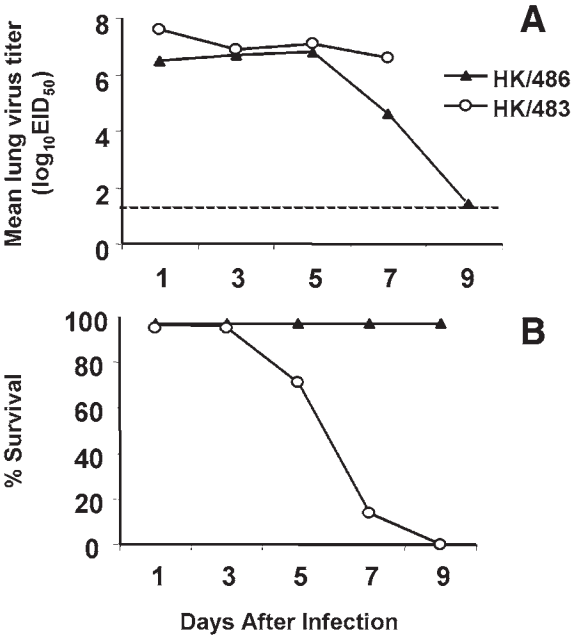

Fig. 2. Comparison of lung virus titers **(A)** and survival **(B)** of BALB/c mice infected with 100 50% mouse infectious doses of avian influenza A H5N1 A/Hong Kong/483/97 (HK/483) or A/Hong Kong/486/97 (HK/486) viruses. Mice (4–5 per group) were euthanatized at the indicated days postinfection and titers of individual lungs were determined by titration of homogenized tissues in embryonated chicken eggs. Values are expressed as $\log_{10}$ 50% egg infectious doses (EID_{50}). The limit of virus detection was $10^{1.2}$ EID_{50} (dotted line). Another seven mice in each group were observed daily for mortality for 14 d postinfection (p.i.).

Studies by others as well as our own findings provide evidence in support of some of these mechanisms. Previous studies in humans suggested that the decreased efficiency of clonal expansion of T cells from elderly individuals appears to be owing both to factors inherent in the T cells and to poor accessory cell function *(28).* Studies in mice demonstrated that the precursor frequency of memory cytotoxic T cells specific for influenza was entirely dependent on the age of the APC donor *(29).* Although a number of mechanism have been proposed, none of them can satisfactorily account completely for the extensive immune dysfunction observed in aged individuals.

Recently, we have shown that a decline in antigen-driven clonal expansion of CD8 T cells is primarily owing to defects at the level of the APC. CD8 T cells from old mice responded to the same extent as CD8 T cells from young mice when stimulated by peptide-pulsed APC from young mice. However, even CD8 T cells from young mice underwent poor clonal expansion when stimulated with peptide-pulsed APC from old mice. In addition, young T cells needed longer contact with APC from old mice to undergo similar clonal expansion when the APC are from young mice. We also determined that the expression of both CD80 and CD86 molecules on the APC declined with aging. Hence, poor expansion and delayed contact kinetics may be owing to either declined integrin expression levels and/or suboptimal costimulation as the expression of cell-adhesion molecules (CAM) on APCs declines with age (Rockwell-Plow-

den, unpublished data). Perhaps they contribute to decrease the overall avidity of APC–TCR interactions. Our data suggests that there is no decrease in TCR affinity because old T cells clonally expand to the same extent as those from young mice as long as the APC are from young mice. These in vitro findings have also been confirmed and extended in vivo. We found that adoptively transferred influenza NP-specific T-cell receptor (TCR) transgenic T cells from young mice underwent extensive clonal expansion in response to antigen when the recipients were syngeneic young mice, but underwent poor clonal expansion in aged recipient mice. Likewise, adoptively transferred transgenic T cells from old mice proliferated better in response to antigen in younger recipients than in aged recipients. These findings indicate that the APC function is primarily responsible for the observed defects in T-cell expansion in aging. Whether defects in antigen-processing capacity contribute to the observed defects in APC function remains to be determined. Our studies indicate that strategies that enhance APC function in terms of cytokine and chemokine secretion and/or upregulate costimulatory molecules is crucial for effective and successful preventive and therapeutic immune intervention strategies for the elderly.

Efficacy of Adjuvanted Vaccines in Aged Mice

The use of adjuvants and other immunomodulating agents is a logical approach to increasing the efficacy of traditional inactivated influenza vaccines. Unfortunately, adjuvants have largely failed to provide the needed enhancement of influenza vaccine immunogenicity in humans *(30).* One exception may be MF-59-supplemented influenza vaccines, which induced higher antibody titers than nonadjuvanted influenza vaccine in elderly persons, although reactogenicity was also increased *(31,32).* Although an MF-59 adjuvanted vaccine is now licensed in Europe, it is not available worldwide and thus, a safe adjuvant for use with inactivated and subunit influenza vaccines that stimulates immunity, particularly in elderly persons or against a novel pandemic vaccine, remains a significant need. We have explored two strategies for the use of adjuvants with a traditional inactivated influenza vaccine. The first involved the use of a nonionic block copolymer adjuvant CRL 1005 for subcutaneous delivery of inactivated whole influenza-virus vaccine. In young adult naïve mice, this copolymer adjuvant significantly enhanced virus-specific IgG and hemagglutination-inhibition (HI) antibody responses and augmented the production of IL-2 following vaccination *(33).* Influenza vaccine formulated with 2.5 mg of CRL 1005 adjuvant significantly enhanced the protective efficacy of the inactivated vaccine in either the upper- or lower-respiratory tract of mice (Table 3). In aged mice, the copolymer adjuvant substantially enhanced the serum HI antibody response to influenza vaccine and significantly reduced lung-virus titers following challenge with live virus compared with lung titers in mice that received vaccine without the adjuvant. A second dose of vaccine plus adjuvant further boosted this protective effect (Table 2).

The Pandemic Potential of Avian Influenza Viruses and the Need for Vaccines

In the event of another pandemic, new influenza vaccines will be required for individuals of all ages. The likelihood of another pandemic is highlighted by the events of recent years in which three distinct avian influenza-virus subtypes have crossed the species barrier to infect and cause disease in humans. In 1997, highly pathogenic avian

Table 3. Protective Efficacy of Influenza Vaccination With CRL1005 Adjuvant in Young and Aged Mice[a]

Expt no.	Age of mice	Vaccine group	No. doses	*n*	Prechallenge serum HI antibody titer	Lung virus titer ($\log_{10}$ $EID_{50} \times SD$)[b]
1	Young	CRL1005 alone	1	7	10	6.6×0.5
		Vaccine alone	1	7	20	4.6×1.9
		Vaccine + CRL1005	1	8	160	< 1.5[c]
2	Aged	CRL1005 alone	2	9	5	6.6×0.8
		Vaccine alone	1	7	5	6.4×0.8
		Vaccine + CRL1005	1	7	10	4.6×2.0[d]
		Vaccine alone	2	8	40	4.1×1.8
		Vaccine + CRL1005	2	10	160	1.7×1.1[e]

[a] Mice received 1.5 μg of influenza vaccine alone, vaccine supplemented with 2.5 mg of copolymer adjuvant, or copolymer adjuvant alone.

[b] Mice were challenged with 1000 50% mouse infectious doses of X-31 virus and lung virus titers were determined 4 d later.

[c] $p < 0.01$ compared with group receiving vaccine alone.

[d] $p < 0.05$ compared with aged group receiving one dose of vaccine alone.

[e] $p < 0.01$ compared with aged group receiving two doses of vaccine alone.

H5N1 influenza viruses circulated among poultry on farms and in retail markets in Hong Kong *(34)*. The H5N1 viruses were transmitted from infected poultry to humans, causing 18 documented cases of respiratory disease, including six deaths *(9,35,36)*. In 2003, H5N1 viruses were again isolated from two cases in Hong Kong, one of them fatal *(10)*. Human respiratory infection with avian H9N2 viruses in Hong Kong and southern China have also been documented *(37,38)*. In early 2003, a third subtype of avian influenza virus caused disease in humans. Highly pathogenic avian H7N7 viruses caused outbreaks in poultry farms in the Netherlands and more than 80 human culture-confirmed H7N7 infection were documented among individuals involved in the control of the outbreak *(10)*. The majority of these cases had conjunctivitis, although a few respiratory illnesses were also reported, including one fatality from acute respiratory-distress syndrome. These cross-species transmissions were limited by the inefficient person-to-person spread of these wholly avian viruses. However, as human infection with avian strains become more prevalent, the generation of an avian-human reassortant virus that can transmit efficiently among humans, and thus create a pandemic, becomes more likely. Thus, there is an urgent need to better understand the pathogenesis of avian viruses in humans and to develop vaccines that would be effective among all age groups in the event of a pandemic.

Pathogenesis of Avian Viruses in Mammalian Species

Avian influenza viruses have caused a spectrum of human illness from mild respiratory or conjunctival infections to severe and fatal disease. However, limited information on the biologic, immunologic, and histopathologic properties of these viruses in humans has necessitated studies in animal models to better understand the ability of avian influenza viruses to infect and cause disease in mammals. The biological and molecular factors that are important for avian virus to infect and cause disease in humans remain

unknown. We have established two mammalian models with which the biologic mechanisms and molecular basis of virulence of avian influenza viruses can be explored.

In mice, avian H5N1 viruses isolated from humans could be separated into two distinct phenotypes of pathogenicity when administered by the intranasal route. Replication of viruses of low pathogenicity (lethality) was restricted to the respiratory tract and virus was cleared from the lungs by day 9 p.i., causing only transient weight loss and minimal lethality at very high doses. In contrast, the highly pathogenic viruses spread systemically; infected multiple organs, including the brain; and resulted in the death of mice by 6–9 d p.i. *(39,40).* Viral antigen was detected in both glial cells and neurons shortly before the mice succumbed to infection *(40).* The H5N1 viruses that were highly lethal and neurotropic for mice also caused depletion of lymphocytes in blood, lung, and lymphoid tissue, and diminished production of pro-inflammatory cytokines such as IL-1β and interferon-γ (IFN) *(41),* but not TNF-α in the respiratory tract. In contrast, replication in the brain by these viruses was associated with production of all three cytokines in brain tissue. Because other investigators reported that the H5N1 viruses isolated from humans in 1997 were potent inducers of pro-inflammatory cytokines including TNF-α *(42)* or resistant to the effects of TNF-α in vitro *(43),* we investigated the role of TNFα in the pathogenesis of H5N1 viruses in the mouse model. Mice deficient in TNF-α receptor exhibited reduced morbidity but similar lung-viral titer and only slightly delayed mortality compared with wild-type mice (Gangappa, unpublished data).

It was noteworthy that both HK/483 and HK/486 viruses, which exhibited dichotomous pathogenicity phenotypes in mice, appeared equally pathogenic in the outbred ferret model. In comparison to current human H3N2 viruses, which cause only modest lethargy, fever, and no significant weight loss, the disease caused by infection with the human H5N1 strains was extremely severe and lethal in 20–30% of ferrets infected with a range of doses (*44;* and unpublished data). Virus was isolated from the upper- and lower-respiratory tract, brain, spleen, and intestine. Histological findings in H5N1 virus-infected ferrets included extensive bronchiolar inflammation; necrosis of bronchial epithelium and supportive exudates in bronchial lumen; and presence of glial nodules, neuronophagia, and perivascular inflammation in brain tissue. The latter histopathology in brain tissue was consistent with observations of neurological symptoms in ferrets during the second week of infection *(44).*

The final elucidation of the molecular, virologic, and immunologic properties that confer avian influenza virus virulence in mammalian species will require the use of both in vivo and in vitro systems. It is likely that no one animal model or cell type will reflect all components of the complex phenotypes and genotypes of virulence, because virulence depends both on viral determinants and their interaction with host cell-gene products.

Pandemic Influenza Vaccines

Another pandemic of influenza could occur at any time and with little warning. However, current influenza vaccines, which are based on the induction of strain-specific neutralizing antibodies, take a minimum of 6 mo to produce *(45)* and offer optimal protection only when the vaccine antigen is closely matched with the circulating strain. Therefore new approaches that stimulate broader cross-protection against multiple influenza subtypes may provide an approach for vaccination against pandemic strains and may be an important first line of defense allowing time for the development of

an optimal strain-specific vaccine. Animal studies have demonstrated that infection with one influenza A subtype can provide partial protection, termed heterosubtypic immunity (Het-I), against subsequent infection with a different influenza A subtype *(46,47).* The mechanisms of Het-I are not completely understood but the available evidence suggests that $CD8^+$ T cells, $CD4^+$ T cells and non-neutralizing antibody-recognizing conserved viral epitopes may all play a role.

Although Het-I has been reported in humans, its duration and effectiveness is unknown *(48).* We have investigated several strategies for the development of vaccines that induce Het-I against avian viruses with pandemic potential. A traditional inactivated H1 subtype vaccine formulated with immunostimulating complexes (ISCOM) induced broad cross-protection in mice against challenge with human H2N2 and H3N2 viruses or avian H5N1 and H9N2 viruses *(49).* Parenteral or intranasal delivery of influenza-vaccine antigen as ISCOM induced class I-restricted $CD8^+$ cytotoxic T lymphocytes (CTL) and non-neutralizing subtype cross-reactive antibodies. The effector activity of the CTL was dependent on perforin and not on IFN-γ production suggesting that functional lytic activity was required. The subtype cross-reactive antibodies were able to facilitate viral-titer reduction by macrophages in influenza virus-infected canine kidney cells.

Intranasal delivery of inactivated whole-virus vaccine with a modified *Escherichia coli* heat-labile enterotoxin mucosal adjuvant [LT(R192G)] was also shown to be an effective strategy for the development of subtype cross-reactive protective immunity *(49).* Mice vaccinated intranasally with inactivated H3N2 vaccine were protected against challenge with a human H1N1 or highly lethal H5N1 virus. Although $CD8^+$ T cells contributed to viral clearance, they were not essential for the protective effect afforded by this vaccination strategy. In contrast, B cells were essential for Het-I and the protective effect was associated with the presence of non-neutralizing IgA and IgG antibodies that recognized subtype cross-reactive epitopes on HA.

A third vaccine strategy involved the use of DNA vaccine rather than traditional inactivated influenza protein-based vaccine. Such vaccines overcome the need for growth and purification of the virus in vaccine production and thus could be rapidly prepared in the event of a pandemic without the need for higher laboratory containment required by highly pathogenic strains. Intramuscular inoculation of DNA vaccines encoding the conserved nucleoprotein (NP) and matrix (M1 and M2) proteins of influenza A H1N1 virus protected mice from lethal challenge with an avian H5N1 virus of moderate virulence, but was unable to protect animals from a highly virulent strain that attained near-peak titers as early as 24 h postinfection *(50).* Because the influenza A/NP+ A/M DNA vaccine elicited IFN-γ producing CD8 T cells with lytic activity, the protective effect was likely owing to subtype cross-reactive CTL that contributed to pulmonary viral clearance and survival. Taken together, these three vaccine approaches demonstrate that multiple immune components contribute to Het-I and that modifying vaccine components, route of delivery, and/or use of adjuvant may all provide enhanced subtype cross-protection, which would be highly desirable in the event of a pandemic.

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