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Respiratory Viral Vaccines

Chapter 51

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The respiratory mucosa is vulnerable to invasion by a number of important human pathogens, including a variety of viruses. Proficient mucosal, antigen-specific immune responses are essential to prevent the disruption of critical functions that may be substantially impaired in the course of respiratory virus infection. Unfortunately, the most severe respiratory complications tend to occur on first exposure in very young children and among the elderly in whom immunocompetence has begun to erode. As such, vaccines that effectively target those populations are needed to provide protection where it is most urgently needed. Passive transfer of specific immunoglobulin is generally not protective against respiratory virus infections. Only two vaccines for respiratory viruses, influenza and adenovirus, have been licensed for use; neither vaccine is highly effective and neither is recommended for use in children less than 2 years of age. The difficulty in designing beneficial mucosal vaccines is compounded by the fact that the mucosal immune system appears to be prone to polarizing toward either T helper (Th) type 1 (Th1) or Th2 dominance, the latter of which can actually lead to immune-mediated airway obstructive disease. A more complete understanding of the mechanisms underlying this polarization could well lead to superior strategies for priming the mucosal immune system against respiratory pathogens. The importance for doing so is manifest, since these viral agents are a leading cause of morbidity among adults and of mortality among the very young and the elderly. Notably, more persons in the 20th century perished in the 1918 influenza pandemic than perished in both world wars.

What follows is a summary of the salient features of the pathogenesis of respiratory viruses responsible for the greatest public health impact, together with current and anticipated progress in vaccine development, including the use of adjuvants and other biological modifiers to enhance vaccine performance. The pathogens causing the largest burden of respiratory disease in humans include influenza (flu) virus, respiratory syncytial virus (RSV), parainfluenza viruses (PIVs), rhinoviruses, coronaviruses, and adenoviruses.

INFLUENZA VIRUS

Viral pathogenesis

Influenza is a single-stranded negative-sense RNA virus belonging to the Orthomyxovirus family and is classified as influenza viruses A, B, and C. The influenza virus genome is divided into seven (influenza C) or eight (influenza A and B) segments that encode a variable number of gene products. All influenza viruses share the property of binding to mucus (to sialic acid–bearing cell surface receptors), and the three types are distinguished on the basis of antigenic differences in the nucleocapsid and matrix proteins (Wright and Webster, 1996). Influenza A viruses are most commonly responsible for severe respiratory illness in humans, followed by influenza B. Influenza C is only rarely responsible for lower respiratory disease in humans. Influenza A viruses are distinguished by their rapid antigen variation, which is accomplished through two mechanisms, antigenic shift and antigenic drift. Antigenic shift results from recombination in the hemagglutinin (HA) and neuraminidase (NA) genes between parent strains, a process believed to involve intermediate animal hosts, particularly migratory waterfowl and pigs. Fifteen HA and nine NA subtypes have been identified. Antigenic drift results from continuous mutational changes in the HA and NA proteins. Shift variants emerge constantly against which previously induced antibodies have reduced avidity, and these are responsible for annual epidemics of influenza. The periodic emergence of shift variants, for which large numbers of people in the population have no history of exposure, can lead to influenza pandemics. Both types of variation pose considerable problems for influenza vaccination strategy and currently make annual vaccination with

updated vaccine formulations essential for the control of influenza disease.

Influenza viruses replicate rapidly, are highly infectious, and spread principally by aerosol. Influenza is an infection of primarily the upper respiratory tract. During the course of infection the virus spreads to the lower respiratory tract, where it can cause viral pneumonia or enhance susceptibility to bacterial infections. Virus replication peaks within 48 hours after exposure and then slowly declines for approximately 1 week (Wright and Webster, 2001). Upon recovery, the patient is permanently immune to reinfection to the identical strain but may be fully susceptible to shift variants. Protection from symptomatic infection with drift variants usually persists for several years (Frank *et al.*, 1987).

Influenza infection is a major cause of morbidity and mortality in most areas of the world, resulting in at least 20,000 deaths and 114,000 hospitalizations annually in the United States (Centers for Disease Control and Prevention [CDC], 2000). Infections occur most frequently in children and adults, but the elderly are regarded as the highest risk group for life-threatening disease (Webster, 2000). Very young children and persons of all ages with chronic lung disorders are also at high risk for serious complications and death from influenza infection (Betts and Treanor, 2000).

The relative importance of innate, humoral, cellular, and mucosal immunity for the control of and protection from influenza virus infection remains poorly understood. High levels of proinflammatory cytokines such as IL-6 and interferon (IFN)-α are induced by influenza infection, usually peaking by day 2 (Hayden *et al.*, 1998; Kaiser *et al.*, 2001). These cytokines are believed to play a direct role in impairing viral infection and in driving the immune response toward a $CD4^+$ Th1 cytokine pattern. A transient infiltration of natural killer (NK) cells has also been observed during influenza infection and has been suggested as an important early defense mechanism (Skoner *et al.*, 1996). Neutrophils also play an important role in clearance of virus from the lung. Mice irradiated to reduce the number of peripheral polymorphonuclear leukocytes have increased viral titers after influenza infection of the lung (Wright and Webster, 2001). In addition, deficiency in the C3 component of complement, which has well-described opsonin activities, also results in impaired ability to clear influenza (Wright and Webster, 2001). However, enthusiasm for nonspecific defenses must be tempered by the observation that they function poorly against shift variants, particularly in the populations at greatest risk. Thus, innate defenses would seem inadequate alone to prevent and control influenza infections. Moreover, influenza virus directly impairs neutrophil function, which appears to be responsible for a reduced capacity to clear bacterial infections in complicating pneumonias (Ruben and Cate, 1987; LeVine *et al.*, 2001)

The presence of antibodies specific for HA and NA are an absolute requirement for prevention of influenza infection (Webster, 2000). Mucosal IgA antibody in nasal and bronchial secretions appears particularly effective for the neutralization of influenza virus and may also play an important role in viral clearance (Tamura *et al.*, 1998). Humoral IgG antibodies to HA and NA are also produced in response to influenza, and these enhance resistance to influenza virus infection in both humans and animal models (Wright and Webster, 2001).

Neutralizing antibodies against influenza are directed to the surface glycoproteins HA and NA and thus against the specific influenza subtype (i.e., they are homosubtypic). T cell responses, in contrast, are predominantly directed to epitopes on invariant proteins such as NP and matrix protein and are thus influenza type–specific (heterosubtypic). The results of studies comparing mucosal and systemic immunization suggest that the former induces an effective memory response, while the latter does not (Gorse and Belshe, 1990; Clover *et al.*, 1991; Brühl *et al.*, 2001).

The role of T cells in prevention of influenza infection, especially in humans, is not well defined. However, since $CD8^+$ T cell responses are directed mainly against the shared proteins among A strains, protection would be expected to be highly cross-reactive. In fact, in humans, cross-protection occurs only among drift variants and is more effective when levels of cross-reactivity detected by antibodies are high (Wright and Webster, 2001). The relevance of cross-reactive $CD8^+$ T cell immunity to influenza has been studied in mice in circumstances where antibody-mediated cross-reactivity to HA or NA epitopes is avoided. In such studies, effective cross-protection occurs for a month or so and appears to principally involve "effector memory" $CD8^+$ cytotoxic T lymphocytes (CTLs) present in alveolar spaces and interstitial tissues of the respiratory tract (Woodland, 2003; Hogan *et al.*, 2001). Although similar cells may be present in the draining lymphoid tissues, these cells seem not to participate in the protective response. After a few months post–primary infection, the mucosal effector memory $CD8^+$ T cells have largely disappeared and no longer participate directly in immunity to reinfection. In these circumstances, if they lack cross-reactive antibody, the $CD8^+$ T cell–primed animals become susceptible to clinical disease upon challenge, although the syndrome may be shorter and of milder severity than occurs in challenged naïve animals (Woodland, 2003). Accordingly, $CD8^+$ T cells would seem to play at best a minor role in effecting resistance to secondary infection, except in the immediate post–primary infection period.

Less is known about the function of $CD4^+$ T cells in mediating protection against reinfection. However, it is also believed that $CD4^+$ T cells (in mice at least) play little or no role in mediating protection to secondary infection. Nevertheless, the $CD4^+$ T cell response is required for optimal antibody responses to influenza proteins and may also be needed for robust $CD8^+$ T cell responses (Epstein *et al.*, 1998).

The function of T cells in immunity is mainly required to effect recovery from infection. In primary infections, this is chiefly a function of $CD8^+$ T cells and may largely involve both cytotoxicity and cytokine production (Cerwenka *et al.*, 1999; Sarawar *et al.*, 1994). In the mouse, the $CD8^+$ T cell response becomes evident around 7 days after primary

infection and peaks at day 10, a time that corresponds with viral clearance (Eichelberger *et al.,* 1991; Flynn *et al.,* 1998; Riberdy *et al.,* 2000). The induction of $CD8^+$ T cell responses occurs both in the nasal associated lymphoreticular tissue (NALT) and in draining lymph nodes. However, the effector $CD8^+$ T cells responsible for viral clearance from the lung likely derive largely from the mucosal lymphoid site.

The crucial outcome of primary infection is the establishment of immunological memory. In the mouse, T cell memory cells are found in both nonlymphoid and lymphoid sites. The memory in nonlymphoid sites, which includes the respiratory tract itself, is of shorter duration than lymphoid memory (Hogan *et al.,* 2002). Recall from both sites takes at least 4–5 days, a circumstance explaining why T cells are not readily available to contain infection in the early stages of infection. However, once the T cells are recruited and activated into effectors, both $CD8^+$ and $CD4^+$ T cells participate in viral clearance (Doherty *et al.,* 1992). $CD4^+$ T cells function by producing type 1 cytokines, particularly IFN-γ.

The recent understanding of immunity to murine influenza may have implications with regard to vaccine design. Thus, it would seem that mucosal memory is an important goal for vaccines against influenza. Moreover, it is suspected that different types of vaccines may variably stimulate long-term mucosal and systemic memory. The immune response to influenza is thus complex, and crafting effective vaccination strategies requires accounting for a variety of factors, including route of inoculation, vaccine composition and type, and strain variation.

Vaccine development

The inactivated influenza vaccine in current use in the United States comprises three split influenza strains; these strains are selected on the basis of the previous year's surveillance data on the most prevalent subtypes, and vaccine composition may vary from year to year, particularly the influenza A component. In contrast to natural infection, intramuscular inoculation of inactivated trivalent influenza vaccine induces the production of serum antibodies but is not effective at inducing mucosal or cell-mediated immune responses. Some influenza-specific systemic antibody transudates to the lung and is credited with this vaccine formulation's observed reduction of severe lower respiratory tract disease. Nonetheless, the immunity elicited by inactivated, parenterally administered vaccine is strain-specific and of short duration, making annual revaccination necessary. In addition, the protection rate varies by age group and is especially low in the populations most susceptible to disease complications and death: the elderly, infants, and persons with chronic pulmonary conditions (Wright and Webster, 2001). The basis for the reduced efficacy of vaccine in the elderly is not well understood; studies have variously implicated heterogeneity in the response to the component strains and in the type of adaptive immune response that is generated (Webster, 2000; Remarque, 1999; Treanor and Falsey, 1999; Bernstein *et al.*, 1999).

Since respiratory infection by influenza induces both humoral and mucosal antibody as well as cross-reactive cell-mediated immunity (CMI), it is widely believed, although not proven, that vaccine efficacy will be improved through nasal administration. A number of nasal vaccine strategies are under investigation, including the use of live attenuated strains (Bradshaw and Wright, 2002; Belshe, 1999; Gruber *et al.,* 1996; Murphy, 1993); recombinant (Berglund *et al.,* 1999; Ferko *et al.,* 1998; Watanabe *et al.,* 2002), virosomal (Cusi *et al.,* 2000), DNA (Ljungberg *et al.,* 2002; Ban *et al.,* 1997), peptide (Matsuki *et al.,* 1999; Yedidia *et al.,* 1998; Jeon and Arnon, 2002), and purified subunit vaccines (Barchfield *et al.,* 1999; Asanuma *et al.,* 2001; Saurwein-Teissl *et al.,* 1998); and immune-stimulating complexes (ISCOMs) (Sjölander *et al.,* 2001; Sjölander *et al.,* 1997).

Nasally administered, live, attenuated, cold-adapted, trivalent influenza-virus vaccine (ca vaccine) may represent a convenient and effective approach to the prevention of influenza in children. The segmented genome characteristic of influenza facilitates reassortment between two strains dually infecting individual cells. By exploiting this phenomenon, the vaccine antigens can be updated annually, substituting genes encoding the HA and NA antigens from contemporary influenza A and B viruses for those present in established master attenuated strains (Bradshaw and Wright, 2002). Ca vaccine will be administered with a spray device that delivers an aerosol of large particles to the upper respiratory tract.

The vaccine has been extensively field-tested in subjects ranging from 6 months to 65 years of age and is well tolerated, immunogenic, and protective, particularly in young children (Belshe *et al.*, 1998; Belshe *et al.*, 2000; Edwards *et al.*, 1994; Treanor *et al.,* 2000; Boyce *et al.,* 2000; Gruber *et al.*, 1996; Murphy, 1993). In one field trial of seronegative children, the individual viruses present in the vaccine induced fourfold or greater increases in titer in 61% to 96% of recipients (Belshe *et al.*, 1998). A clinical trial involving 4000 participants of all ages revealed that, while the inactivated vaccine induced higher levels of serum antibody than the live vaccine, the latter induced much higher levels of mucosal immunity (Edwards *et al.*, 1994). The vaccine has been shown to induce strain-specific mucosal IgA in the majority of children after two doses (Boyce *et al.*, 2000). In a challenge study of 103 adult volunteers, the protective efficacy of ca vaccine was estimated at 85%, compared with 71% for the currently available inactivated vaccine (Treanor *et al.*, 2000). In a separate study of children aged 1 to 6, the vaccine was determined to be 92% effective at preventing culture-confirmed influenza A and B infection (Belshe *et al.*, 2000). One early study of influenza vaccine in children suggests that risk for acquiring influenza infections in all age groups could be reduced substantially if community-wide coverage levels of 70% were achieved in young children (Monto and Kioumehr, 1975). This is considered an achievable goal with ca vaccine, in large part because the nasal route of administration is more readily accepted than subcutaneous or intramuscular injection, particularly for children.

Ca vaccine has been associated with some side effects, generally mild. Recipients have been found to be at slightly elevated risk for rhinorrhea and low-grade fever (Belshe *et al.*, 1998). Elevated fever is also observed in a comparable fraction of inactivated influenza vaccine recipients, and ca vaccine has not been associated with Guillain-Barré syndrome, which is observed at very low incidence in inactivated-vaccine recipients (Lasky *et al.*, 1998).

While the live, attenuated vaccine appears to be less efficacious in the elderly, one study suggested that conventional inactivated trivalent vaccine given in combination with nasal ca vaccine may enhance protection in elderly recipients (Treanor and Betts, 1998). The live, attenuated intranasal vaccine was licensed for use in late 2003. However, the Advisory Committee on Immunization Practices has recommended its use only in persons between the ages of 5 and 49. Thus, the vaccine is not yet available for the age groups at highest risk for life-threatening disease (Harper *et al.,* 2004).

A nasal virosomal vaccine consisting of purified HA and NA encapsulated in lecithin has also been evaluated in clinical trials in Europe. Study populations included children (6–12 years old), adults, and the elderly (>60 years old); the vaccine induced both influenza-specific systemic IgG and mucosal IgA and was estimated to be 85% efficacious in all adults and had 89% efficacy in children. The highest immunogenicity was seen when the vaccine was coadministered with the adjuvant LT (heat-labile toxin of *Escherichia coli*) (Cusi *et al.,* 2000). Unfortunately, this vaccine was associated with an increased incidence of Bell's Palsy or facial paralysis, and it has been taken off the market. The Semliki Forest virus (SFV) recombinant vaccines expressing HA and NA have been shown to induce protective, strain-specific secretory IgA (S-IgA) antibody responses in mice, and SFV recombinant vaccine expressing influenza NP elicited antigen-specific CTLs that protected mice from infectious challenge (Berglund *et al.,* 1999). ISCOMs, including influenza HA and NA, administered nasally induced protective immunity in mice, with measurable antigen-specific mucosal and systemic antibody and CMI (T helper and cytotoxic activity) (Sjölander *et al.,* 1997). Both the cytokine profile and localization of the T cell response induced by ISCOMs was shown in a separate study to depend upon the type of adjuvant used (Sjölander *et al.,* 2001).

It has been suggested that plasmid DNA vaccines have an advantage over recombinant viral vectors such as SFV because no immune response is generated against the vector itself. As such, it should be possible to revaccinate indefinitely without a reduction in the expression of the target proteins. A nasal plasmid DNA vaccine expressing influenza HA has been evaluated in mice but, disappointingly, failed to elicit detectable antibody in the respiratory tract (Ban *et al.,* 1997). A similar vaccine given parenterally was able to induce a CMI response (Ljungberg *et al.,* 2002). However, DNA vaccines in general are more effective in mice than in humans. In fact, one must conclude that currently there is little enthusiasm for a DNA vaccine against human influenza.

Other strategies are also under investigation for vaccination against influenza. For example, there is interest in exploiting the common mucosal immune system to induce respiratory immunity by delivering vaccines at other mucosal sites. Trials with an oral vaccine thought to engage the intestinal Peyer's patches by means of particle formations of inert microspheres containing protein antigens represent one such approach under investigation (Clancy *et al.,* 1995). Another approach that is under study is the use of epidermal powder immunization, in which trivalent split vaccine is administered to mice with a compressed-air injector. An initial study demonstrated both systemic and mucosal antibody, as well as enhanced protection from infectious challenge (Chen *et al.,* 2001). Coadministration of almost every candidate vaccine with various antigens is also being actively explored, as is the tactic of using both intranasal and parenteral vaccine to induce maximal immunity.

Given the intensive level of activity in this field, it seems very likely that new alternatives to the currently used vaccine will be available within a relatively short period.

RESPIRATORY SYNCYTIAL VIRUS

Viral pathogenesis

Respiratory syncytial virus (RSV) is the most important cause of serious lower respiratory tract illness among infants and children in the United States, particularly before the age of 2 years. In the United States alone, RSV infections account for between 85,000 and 144,000 hospitalizations and thousands of deaths each year, and health care costs have been estimated at several million dollars annually (Shay *et al.,* 1999; Institute of Medicine, 1985). Together with parainfluenza virus 3, these two viruses are responsible for nearly one-third of all cases of respiratory tract disease necessitating hospitalization of children. Most hospitalizations resulting from RSV infection are of newborn infants with no prior exposure to RSV and, in general, hospitalized infants carry no recognized risk factors for severe disease in comparison with other infants of the same age. In developed countries throughout the world, RSV is the leading cause of morbidity and mortality among infants and young children (Crowe *et al.,* 1997). Respiratory virus infections are estimated to cause about 4 million deaths each year in children less than 5 years of age, and RSV is the leading contributor to those deaths. RSV is also a significant cause of severe respiratory tract illness in elderly and immunocompromised persons (Nicholson *et al.,* 1997; Walsh *et al.,* 1999). Infants born prematurely and persons with underlying heart or lung disease (notably, bronchopulmonary dysplasia) are at heightened risk for developing severe RSV disease (Hall *et al.,* 1986; MacDonald *et al.,* 1982; Groothius *et al.,* 1988). RSV disease displays seasonality, with most community outbreaks occurring during the winter months. There is currently no effective treatment for RSV disease, although prophylactic administration of RSV antibody is approved for high-risk infants and appears to offer protection against severe disease.

RSV (as with most other respiratory viruses) has been implicated as a predisposing factor for the development of otitis media, leading to speculation that an effective vaccine could also reduce the morbidity associated with that disease (Anderson, 2001). RSV spreads very efficiently among infants and young children; most have primary infection by age 2 years.

RSV is a nonsegmented negative-strand RNA virus, a member of Paramyxoviridae. The virus codes for 11 proteins, including three transmembrane surface proteins (G, F, and SH), the virion matrix protein (M), the nucleocapsid and polymerase proteins (N, P, M2-1, and L), the putative transcription-replication regulatory factor (M2-2), and two nonstructural proteins (NS1 and NS2). The genome consists of 15,222 nucleotides. Two antigenically distinct subgroups of RSV have been characterized on the basis of both antigenic and sequence variability: RSV A and RSV B. Subgroup A has been most frequently isolated in community outbreaks and appears to be associated more frequently with severe disease. The attachment glycoprotein (G) contains most of the observed variability between subgroups. Strains of RSV within a subgroup display a high level of antigenic similarity, but the antigenic diversity between subgroup A and B strains can be as high as 95% for G and as high as 50% for the fusion protein (F) (Collins *et al.,* 2001).

RSV establishes infection in the upper respiratory tract, replicating initially in the nasopharynx. Symptoms manifest after 4 to 5 days. If virus spreads to the lower tract, which often happens in the most susceptible 2- to 7-month-old age group, severe bronchiolitis and pneumonia develop within 1–2 days. Symptoms persist for 2–3 weeks, with continual viral shedding. Except in immunocompromised individuals, virus rarely spreads beyond the superficial epithelium. Lung lesions in severe disease are likely a consequence of direct virus-induced tissue damage along with an immunopathological reaction (Collins *et al.,* 2001).

Animal models have played an important role in acquiring information about the pathogenesis of RSV (Brandenburg *et al.,* 2001). The mouse model has been especially useful, permitting robust studies, for example, aimed at defining the underlying mechanisms responsible for the failure of formalin-inactivated RSV vaccine. Other animals, such as the cotton rat and nonhuman primates such as the African green monkey, bonnet monkey, and chimpanzee have also provided useful insights into RSV disease. Such models, as for all infectious agents, have serious limitations, however. Mice, for instance, are not fully permissive for RSV infection, and challenge doses do not cause death. Cotton rats are more susceptible to infection, but the range of immunological reagents available for mouse studies is not available for the rat, and the high body temperature of this animal complicates the assessment of the attenuation of temperature-sensitive strains developed as vaccine candidates. Nonhuman primate models have permitted essential preclinical studies of vaccine tolerance and virulence, but these animals are still less permissive than humans for RSV infection. As such, the critical assessments of candidate RSV vaccines will need to be performed in human subjects and ultimately will involve assessment in the youngest, most vulnerable population, to whom an RSV vaccine will need to be targeted.

The immunological response to RSV is complex and dependent on multiple factors, including the age of the patient, level of immunological maturity, and presence of maternal, passively transferred antibodies. The antigenic diversity of RSV allows both subgroups to circulate in a community at the same time. While exposure to an RSV of one subgroup confers short-lived resistance to disease by subsequent infection with the alternative subgroup, protection against the homotypic subgroup is superior. Since antibodies to G and F are considered to be principally responsible for resistance to reinfection, and since the intertypic variation is greatest in these same proteins, there is the possibility that a bivalent RSV vaccine will be required to achieve optimal effectiveness.

The humoral response to RSV is directed almost exclusively against the F and G surface glycoproteins, and these proteins are the major neutralization targets for mucosal and serum antibody. While several components of the immune system have been implicated in the control and resolution of RSV infection, the increased resistance to reinfection observed in persons previously infected seems to be mediated primarily by RSV-specific secretory and serum antibodies (Mills *et al.,* 1971; Prince *et al.,* 1985). Serum IgG antibodies protect the lower but not the upper respiratory tract; the role of protecting the latter is largely the province of S-IgA antibodies. Although antibodies confer protection against severe disease, the protection is incomplete, and multiple reinfection, even by the same strain, can take place despite high circulating titers of neutralizing antibody. A recent study of antibodies cloned from natural responses to RSV revealed that the virus engineers an evasive tactic by presenting the F antigen in multiple forms during an immune response, skewing the antibody response toward the production of nonneutralizing antibodies (Sakurai *et al.,* 1999). The study also revealed that the neutralizing power of an antibody was more important than its isotype, showing in a mouse model that an IgG monoclonal antibody (mAb) with high neutralizing titer was more protective than an IgA antibody with lower neutralizing titer.

RSV-specific CMI can lead to both advantageous and immunopathologic endpoints during acute infection. Resolving acute infection appears to require the presence of CTLs, the peak activation levels of which coincide with virus clearance (Collins *et al.,* 2001). The period of RSV shedding is prolonged in animals or patients that lack functional T cells (Cannon *et al.,* 1987; Bangham *et al.,* 1986). $CD8^+$ CTLs are present in the circulation of persons who have had a known primary exposure, and both $CD4^+$ and $CD8^+$ T cells can eliminate RSV from infected animals. While CTLs play an important role in viral clearance, they are not believed to contribute substantially to the prevention of reinfection, largely because the response is very short-lived.

There are two curious aspects of immunity to RSV. First, natural infection fails to result in resistance to reinfection. In fact, repeated infections can occur throughout life (Hall *et al.,*

1991). Second, pulmonary disease following infection appears to result mainly from a host immune inflammatory response (Varga and Braciale, 2002). This situation was exaggerated following the use of a formalin-inactivated vaccine in children. Many vaccine recipients developed severe disease, several of which were lethal following natural infection with RSV (Kapikian *et al.,* 1969). The explanation for the ineffective and untoward immune response to RSV remains obscure. Thus, infection induces neutralizing antibody production as well as $CD4^+$ and $CD8^+$ T-cell responses to peptides from most viral proteins. For unknown reasons, infection can occur in the presence of neutralizing antibody and T-cell immunity. The latter form of immunity, however, is of brief duration, especially in the respiratory tract (Varga and Braciale, 2002). Solutions to these immune mysteries are likely to emerge from ongoing studies in animal model systems.

As mentioned previously, several animal models are available to study RSV immunopathology, none of which is ideal. Most recent progress has come from studies in the BALB/c mouse strain. In such animals, it is possible to simulate vaccine-induced pulmonary disease. For example, if RSV G protein–specific Th2 $CD4^+$ T cells are transferred into mice that were subsequently RSV-infected, then a pulmonary disease reminiscent of that in vaccinated children occurred (Alwan *et al.,* 1994). Other approaches have since confirmed that memory $CD4^+$ T cell responses to the G protein are responsible for pulmonary immunopathology. Surprisingly, in BALB/c mice a single peptide of the G protein is the recognized epitope, and the memory T cells involved are highly oligoclonal (Varga *et al.,* 2000). The cells that recognize the peptide can be of either Th1 or Th2 effector phenotype, but the Th2 cells, although the minor population, are responsible for lesions (Varga *et al.,* 2000).

$CD8^+$ T cells also play a role in immunopathology since they serve to influence the phenotype of the G protein–specific $CD4^+$ T cells involved. Thus, in the adoptive transfer model described previously, the cotransfer of M2 protein–specific $CD8^+$ T cells along with G-specific $CD4^+$ T cells resulted in diminished pathology (Alwan *et al.,* 1994). Moreover, incorporation of the M2 MHC class I–restricted epitope into G protein constructs used to prime for injury-provoking memory $CD4^+$ T cells reduced pulmonary disease expression (Srikiatkhachorn and Braciale, 1997). Thus the $CD8^+$ T-cell response is protective, a lesson for future vaccine design. Unfortunately, the environment of the lung, or perhaps exposure to one or more RSV proteins, appears to impair the function of $CD8^+$ T cells. In addition, such cells persist only briefly in the lungs. Residence of $CD8^+$ T cells in the lung impairs their antigen-induced cytokine production, compromising their protective efficacy (Chang and Braciale, 2002). The exact mechanism of $CD8^+$ T cell functional damage is not understood, but its mechanistic resolution could lead to the design of effective vaccines against RSV.

In recent years, evidence has mounted that both infectious RSV and individual RSV proteins are capable of modulating the innate and antigen-specific host immune responses. The F protein has been implicated in the contact-mediated impairment of peripheral blood lymphocyte (PBL) proliferation (Schlender *et al.,* 2002). PBLs exposed to RSV-infected cells or to cells expressing F protein in the absence of infectious RSV were arrested in G_0G_1 phase. Activation marker could still be induced on the surface of these cells with use of T-cell mitogens. The RSV nonstructural proteins NS1 and NS2 were shown in a separate study to coordinately antagonize the antiviral effect of type 1 IFN (Schlender *et al.,* 2000). Both of these effects were observed for both human and bovine RSV and to function most effectively when cells of the homologous species were used. The F protein was also shown to augment innate immunity through the receptors CD14 and Toll-like receptor 4 (TLR4) by inducing the proinflammatory cytokine IL-6 (Kurt-Jones *et al.,* 2000). Recently, the CX3C chemokine receptor 1 (CX3CR1) has been shown to facilitate RSV infection. The G glycoprotein binds to CX3CR1, and a short region of G has been shown to have moderate amino acid homology to fractaline, the only CX3C chemokine identified thus far (Tripp *et al.,* 2001). More recent studies indicate that glycoprotein G has CX3C chemokine activity that can be blocked by anti-G antibody and that the G glycoprotein is responsible for generating the enhanced disease caused by inactivated RSV (Tripp, personal communication). Finally, a study of $CD8^+$ T cells infiltrating the lung parenchyma in BALB/c mice showed they are impaired in both cytolytic activity and cytokine secretion (Chang and Brachiale, 2002). This impairment appears to be attributable to a T-cell-receptor signaling defect that is in turn dependent upon pulmonary infection by RSV. Thus, it seems clear that RSV has developed a number of mechanisms for altering or curtailing the host immune response.

Vaccine development

The preceding section documents some of the difficulties associated with developing safe and effective vaccines for RSV. One- to 2-month-old infants are the population most likely to develop RSV infections requiring hospitalization, and as such they constitute a critical target group for RSV vaccine. Live virus vaccines tend to be more immunogenic than other types of vaccine at this age, when maternal antibody and immunologic maturity can render the immune system refractory to antigenic stimulation (Crowe, 2001). Unfortunately, live virus vaccines also pose a potential risk, since an insufficiently attenuated virus may actually cause disease in young infants. In addition, candidate RSV vaccines must induce a robust circulating titer of neutralizing antibody, since they have been shown to play an instrumental role in protection against reinfection. Finally, any candidate vaccine for RSV will need to be carefully evaluated to ensure that it does not cause the disease enhancement on primary exposure to wild-type virus that was associated with formalin-inactivated vaccine (Kapikian *et al.,* 1969). Although there is no vaccine currently licensed for RSV, a variety of vaccine development strategies have been assessed, including those involving peptide (Jiang *et al.,* 2002), subunit (Simoes *et al.,* 2002; Goetsch *et al.,* 2001; Power *et al.,* 2001; Prince *et al.,*

2000), virus vector (Dollenmaier *et al.,* 2001; Schmidt *et al.,* 2002), plasmid DNA/RNA (Andersson *et al.,* 2000; Fleeton *et al.,* 2001), and live attenuated vaccines (Collins and Murphy, 2002; Crowe *et al.,* 1999; Wright *et al.,* 2000).

While subunit and peptide vaccines provide a higher level of safety than vectored or live virus vaccines, they generally have the disadvantage of failing to induce protective levels of neutralizing antibody. This is primarily due to the fact that most of the neutralizing antibodies to RSV are directed against the mature, membrane-bound/virion-associated form of F, and subunit and peptide vaccines present the immature form of F. At least two subunit/peptide RSV vaccines are being actively pursued. One is a purified preparation of the F glycoprotein and the other is a glycoprotein G specific peptide conjugated to the albumin-binding site of the streptococcus G protein, which serves a carrier function. Neither appears to be sufficiently immunogenic in young infants, and both are being targeted for use in older children and the elderly. Efforts are being made to address the need to induce adequate levels of neutralizing antibody by expressing F glycoprotein in a suitable vector. In one study, for example, the immunogenicity of a human rhinovirus replicon expressing the RSV F protein was evaluated in mice; while the majority of the F protein produced was of the immature form, a fraction was expressed as the mature form, and titers of neutralizing antibody were readily measurable in immunized animals (Dollenmaier *et al.,* 2001). Similar observations have been made for RSV glycoproteins expressed in vaccinia and bovine parainfluenza virus (Crowe 2002; Schmidt *et al.,* 2001). Plasmid DNA vaccines are transfected into cells on administration, leading to a transient expression of the immunogenic antigens. The approach has been demonstrated to be effective in mice with use of recombinant constructs containing either RSV F or G protein in an SFV expression system (Fleeton *et al.,* 2001). However, there is scant evidence that such an approach will prove sufficiently immunogenic in humans. The pursuit of live attenuated RSV vaccines has begun to yield promising results. One potential advantage to a live attenuated RSV vaccine (also possibly applicable for plasmid DNA or vectored vaccines) is the possibility of intranasal administration. This immunization strategy has been demonstrated to mimic a natural RSV infection, inducing a neutralizing antibody response in both the nasopharyngeal mucosum and in serum, as well as cell-mediated immunity (Crowe, 2002).

Initially, live candidate RSV vaccines were derived in the conventional fashion, by passaging the virus repeatedly in culture at low temperature, leading to a strain of RSV that was attenuated in chimps as well as seropositive adults and children (Crowe, 2002). This virus was further mutagenized chemically to produce a series of temperature-sensitive mutants that, while clearly more attenuated, remained capable of causing transient respiratory illness in infants when administered intranasally. Furthermore, some of these mutations have been shown capable of reverting during replication in vivo (McIntosh *et al.,* 1974). However, extensive sequencing data have provided a catalog of specific temperature-sensitive mutations for RSV, and the next generation of candidate RSV vaccines is being created with use of site-directed mutation (Collins and Murphy, 2002). With use of the best current generation of attenuated strains as a starting point, a cDNA copy of the virus is produced, and various characterized mutations are introduced, whereupon the cDNA copy can be transfected into cells to produce infectious virions of the new vaccine strain. By this approach, multiple attenuating mutations (point mutations, gene insertions, deletions) can be introduced into RSV and evaluated independently and in combination for immunogenicity, pathogenicity, and stability in test animals and humans. The possibility of introducing immunomodulatory genes, such as IL-2 or IL-12, into an RSV live virus vaccine is also being explored as a means for driving the anti-RSV toward the Th1 cytokine profile and, concomitantly, to reduce the risk of causing vaccine-enhanced illness.

PARAINFLUENZA

Viral pathogenesis

Parainfluenza viruses (PIVs) are single-stranded, negative-sense RNA viruses belonging to the family Paramyxoviridae. The envelopes of these viruses display two glycoproteins, hemagglutinin-neuraminidase (HN) and fusion (F) proteins, against which the major antibody response is generated. Four distinct serological types have been identified, termed PIV-1 through PIV-4. Human PIVs are a major cause of acute respiratory infections, particularly in infants and young children (Chanock *et al.,* 2001). PIV-1 is responsible for the majority of cases of croup, and PIV-3 is the second most frequent etiologic agent, after RSV, in pediatric lower respiratory disease. More recently, PIVs, predominantly PIV-3, have been shown to cause acute pneumonia, persistent infection, and death in immunocompromised patients. It is estimated that in the United States, PIV-1, -2, and -3 are responsible for about 20% of cases of pediatric respiratory disease requiring hospitalization (Murphy *et al.,* 1988).

PIV subtypes exhibit clear differences in epidemiology. PIV-1, -2, and to a lesser extent PIV-4 occur most commonly in the fall and winter months, whereas PIV-3 infections occur year-round, with peak infection in the spring and summer (Laurichess *et al.,* 1999). PIV-1 and PIV-2 are effectively controlled by maternal antibody in infants, which delays the onset of disease caused by these subtypes until the preschool years. PIV-3 infection is unimpaired by maternal antibody, so that half of all children seroconvert to this subtype during the first year of life (Glezen *et al.,* 1984).

In common with influenza and RSV, reinfection by the homologous subtype occurs, even in the presence of virus-specific antibody. In general, reinfection is restricted to the upper respiratory tract, probably because serum IgG, which is present in the lungs, endures longer than secretory IgA in the upper respiratory tract. The predominant humoral immune response is directed against HN and F, which are involved in virus attachment and fusion, respectively

(Chanock *et al.,* 2001). Both serum IgG and S-IgA antibodies are produced in response to PIV infection, but their contribution to viral clearance is uncertain. T-cell immunity is thought to play an important role in recovery from PIV infection, since disseminated disease, including spread to the brain, can occur in T-cell–immunosuppressed patients (Fishout *et al.,* 1980). Virus shedding also tends to be more prolonged in persons with impaired cell-mediated immune responses (Rabella *et al.,* 1999). Additional support for the participation of cell-mediated immunity in PIV recovery comes from studies in mice, where the participation of $CD8^+$ CTLs can effect virus clearance in the absence of other cell types (Hou *et al.,* 1992). Conversely, patients with PIV-3-induced bronchiolitis display elevated levels of PIV-3-specific lymphocyte transformation, suggesting that immunopathogenic responses to PIV-3 may occur (Chanock *et al.,* 2001). Maternally acquired anti-PIV antibody appears to protect infants from severe PIV-3-associated lower respiratory tract disease in infants, suggesting an important role for humoral immunity. However, nasal wash IgA produced by infants during primary infection with PIV-1 or PIV-2 is usually unable to neutralize virus (Chanock *et al.,* 2001).

In adults, PIV illnesses spread only in rare instances to the lower respiratory tract. Nonetheless, PIV has been increasingly identified as the causative agent in serious respiratory illness in elderly care facilities. Rates of pneumonia in this population have been reported to be as high as 29%, and deaths due to PIV have also been confirmed. PIVs have also been shown to exacerbate chronic obstructive pulmonary disease in the elderly (Chanock *et al.,* 2001).

PIV vaccine development

Given the complex and variable pathology of PIVs, compounded by uncertainties about the relative contribution of various facets of immunity in controlling infection, vaccine development for these viruses is somewhat problematic. No vaccine has been licensed to date; nonetheless, several approaches to PIV vaccine development are currently under investigation. Early candidate vaccines were formalin-inactivated but failed to provide any protection against PIV infection or disease. Fortunately, in contrast to the experience with inactivated RSV vaccine, enhanced PIV disease in infants was not a consequence of vaccination with formalin-inactivated PIV vaccine (Chin *et al.*, 1969). Two PIV-3 vaccine candidates, a live chimeric vaccine (HN and F of human PIV-3 recombined into bovine PIV-3) (Schmidt *et al.*, 2001b) and a cold-adapted vaccine strain (cp45) (Karron *et al.*, 1995), have undergone clinical trials and were determined to be both safe and immunogenic in seronegative infants ≥6 months of age.

A number of attempts have been made to develop a live-attenuated intranasal vaccine with temperature-sensitive, cold-adapted PIV. However, attenuated PIV strains were discovered to retain the capacity to infect central neurons in the nasal cavity, and as such they had the potential for causing adverse events in the central nervous system (Mori *et al.*, 1996). Thus, the strategy of oral immunization with this vaccine was investigated in mice as an alternative approach to establishing anti-PIV mucosal immunity, and this strategy was found to provide protection. This has not yet been attempted in primates or human subjects. In addition, purified HN and F and viral vectors expressing these proteins are immunogenic in various animal models but have yet to be evaluated in primates and humans (Chanock *et al.*, 2001).

Hamsters, cotton rats, mice, and ferrets have been useful small animal models for the evaluation of candidate PIV vaccines. Chimpanzees have also been employed, and recent evidence suggests that African green monkeys provide a superior animal model for the assessment of live PIV vaccines (Durbin *et al.,* 2000).

It seems that we are on the brink of developing acceptable PIV vaccines. Recent efforts to apply the technique of reverse genetics with PIV3 could lead to the development of vaccine strains that lack neurotropism and would thus be suitable for nasal administration. The payoff from such powerful new techniques has yet to materialize, but the prospects appear promising.

ADENOVIRUSES

Viral pathogenesis and vaccine development

The human adenoviruses are a large family of double-stranded DNA viruses comprising more than 50 serotypes, divided into six subgroups (A–F). A small number of serotypes in subgroups B and C are estimated to cause between 5% and 15% of all respiratory diseases in children and about 3% of respiratory illnesses in adults. In children, adenovirus infections can cause severe interstitial pneumonia that results rarely in death. Symptomatic respiratory infections are usually febrile and are often accompanied by conjunctivitis. Under conditions in which persons are housed in close quarters, such as military barracks, dormitories, and long-term-care facilities, adenoviruses can cause large-scale epidemics of acute respiratory disease. Subgroup A viruses such as Ad31 have also been associated with pneumonia in immunocompromised patients. Neutralizing antibodies directed against the capsid proteins (hexon and fiber proteins) are considered to be primarily responsible for the prevention of reinfection by adenovirus. While these viruses cause both upper and lower respiratory disease, only serum antibodies appear to have a role in protective immunity (Horwitz, 2001). A highly effective live virus vaccine against serotypes 4 and 7 was administered to military recruits until 1998. This vaccine was administered orally in enteric coated capsules; the virus, which was not attenuated in the nasopharyngeal cavity, could replicate in the gastrointestinal tract without causing disease, generating a protective response in both the upper and lower respiratory tract (Howell *et al.*, 1998). The vaccine went out of production in 1996, and supplies have now unfortunately been exhausted. The loss of this vaccine has already precipitated respiratory disease epidemics among military recruits, and adenovirus has now reemerged as the leading cause of febrile respiratory disease

in this population. A similarly constructed quadrivalent vaccine for serotypes 1, 2, 3, and 5 should be developed and assessed for use in children, since those serotypes cause more than 80% of adenovirus-associated respiratory disease in young children (Schmidt *et al.*, 2001a).

Since recombinant adenoviruses efficiently transfer foreign genes into host cells in vivo, a great deal of attention has been focused on the use of these viruses as vectors to express recombinant genes for gene therapy (Carroll *et al.*, 2001). This is achieved by deleting the E1 region from the wild-type viral genome. The adenoviruses used for gene therapy have extensive gene deletions and thus cannot replicate in normal cells. The primary interest in adenovirus vectors is for use in correcting gene defects such as cystic fibrosis and to make certain cancers more immunogenic. Similarly, the well-characterized immune modulatory effects of these viruses are being explored as a means for controlling autoimmune disease and transplant rejection.

RHINOVIRUSES

The human rhinoviruses (HRVs) are nonenveloped, positive-strand RNA viruses of the family Picornaviridae. More than 100 antigenically distinct serotypes have been identified. HRVs are the etiologic agents responsible for more than 50% of common colds. HRVs are highly infectious and replicate rapidly in the epithelium and adjacent lymphoid tissues of the upper respiratory tract, causing symptoms in 1–4 days. Usually lesions are confined to the upper respiratory tract, mostly the nose, and only very rarely does pneumonia occur. Recovery occurs quickly and appears to depend mainly on innate defense mechanisms, especially IFNs. Characteristically, nasal and serum antibodies appear late after infection and can be delayed until 21 days in primary disease. Titers may continue to rise until 4–5 weeks after infection, and immunity, once developed, is stable and long-lasting (2–4 years). However, it remains uncertain whether serum IgG transudate, nasal IgA, or both defend against reinfection (Couch, 2001). Because so many antigenically distinct serotypes occur in nature and no infection is homotypic, vaccine development for the prevention of HRV infection is theoretically possible but probably not practical.

CORONAVIRUSES

First isolated in 1965, human coronaviruses are enveloped, positive-strand, RNA viruses with a large (30,000-nucleotide) genome. The viruses are classified as two serotypes (designated simply 1 and 2), represented respectively by the strains 229E and OC43. Coronaviruses are estimated to cause between 15% and 30% of common colds. The disease has a longer incubation and shorter course than disease due to rhinoviruses. Occasionally coronaviruses cause severe lower respiratory tract disease in infants and small children. Recovery from infection leaves patients immune, but the duration of immunity is shorter than that for rhinoviruses (Holmes, 2001). The mechanism of immunity is not understood, and no attempts have been made to develop a vaccine against human coronavirus infection.

This situation may be about to change. By April 2003, the world had suddenly become aware of coronaviruses as human pathogens. Around November 2002, new coronavirus strains appeared in Southern China that represent major respiratory pathogens. The syndrome has been termed *SARS*, for severe acute respiratory syndrome (Ksiazek *et al.*, 2003; Drosten *et al.*, 2003). Unlike previously described coronavirus strains, which usually cause mild upper respiratory tract lesions, the SARS strains affect the lower tract, markedly damage alveolar epithelial cells, and may become viremic. Furthermore, infection results in death, and in some communities the mortality rate may be as high as 5%. Modern travel is rapidly disseminating the virus around the world, and given the high mortality and the lack of effective antiviral therapy or vaccines, this is causing considerable alarm. The origin of the new virus remains unknown, but its genomic structure suggests it is a zoonosis, or a recombination of animal and human strains. The National Institutes of Health (NIH) and the governments of other countries are in the process of significantly funding coronavirus research to find improved means of diagnosis, treatment, and control. As mentioned above, vaccines are not available for human coronavirus, but some animal coronaviruses are controlled by vaccines, the efficacy of which is not well established (Ladman *et al.*, 2002). It appears likely that vaccines against the SARS strains will represent a high priority issue for the future.

THE MUCOSAL IMMUNE SYSTEM AND VIRAL VACCINES

Obstacles and possible strategies

The example of formalin-inactivated RSV vaccine illustrates the potential hazards of immunizing humans with the aim of inducing protective immunity at a mucosal surface. Because mucosal surfaces are continuously visited with a large variety of foreign, albeit mostly harmless antigens, the local immune system is predisposed to be hyporesponsive to most of these materials. The perturbations caused by invading microbes, such as bacteria and viruses, are thought to arm the mucosal surface to a state of responsiveness. Consequently, nasally administered vaccine viruses, which have been carefully designed to exhibit limited virulence, may insufficiently disturb the mucosal surface to generate an effective immune response. In addition, even when preparations are sufficiently immunogenic, resident antigen-presenting cells in concert with T lymphocytes can direct individual responses toward Th1 or Th2 dominance (Neurath *et al.,* 2002). The cytokine profile secreted by activated Th1 cells promotes the development of antigen-specific cytotoxic T lymphocytes, and Th2 cells promote the activation of B cells to produce specific antibodies, including S-IgA antibodies. Both responses make important contributions to the resolution of

respiratory infections, but overproduction of STAT6 and GATA-3 can polarize the immune response toward Th2 cell hyperactivity, leading to allergic responses such as asthma (Neurath *et al.,* 2002). Similarly, activation of the transcription factor T-bet can lead to Th1 hyperresponsiveness, leading to immunopathologic responses such as that seen with Crohn's disease (Ouyang *et al.,* 2000). Thus, respiratory viral vaccines (including materials coadministered to enhance immunogenicity) must be carefully evaluated to ensure that they lack the capacity to induce harmful immune responses in recipients.

A variety of experimental adjuvants have been evaluated for their ability to improve the immunogenicity of mucosal vaccines. The two most widely investigated adjuvants are the heat-labile enterotoxin of *Escherichia coli* (LT) and cholera toxin (CT) (Pizza *et al.,* 2001). Normal LT has been shown to have too much toxicity to be used as an adjuvant; however, studies of nasal immunization in mice have demonstrated that the mutated LT (which lacks the toxic ADP-ribosylation activity) provides an adjuvant effect comparable to that of the intact heterodimer. Likewise, CT has been shown to cause both inflammation and enhanced production of IgE antibody, which will likely preclude its use as an adjuvant in humans, but selected mutations of CT have resulted in reduced toxicity variants that retain adjuvancy. Another adjuvant that shows promise for nasal immunization is CpG oligodeoxynucleotide (ODN). Unmethylated CpG oligonucleotide motifs occur commonly in bacterial DNA and trigger immune responsiveness in mammals. Studies of nasal immunization using CpG ODN as an adjuvant showed that the material enhanced both humoral and cell-mediated responses and was well tolerated (McKluskie and Davis, 2001; Moldoveanu *et al.,* 1998).

Another pair of experimental adjuvants under investigation for nasal administration, proteosomes and emulsomes, were shown in animal studies to polarize immunity toward a Th1- or Th2/3-type cytokine profile. Proteosomes, a complex of neisserial membrane proteins with lipopolysaccharide from *Shigella flexneri* or *Plesiomonas shigelloides*, induced Th1-type cytokines in mice in response to nasally coadministered influenza HA (Jones *et al.,* 2002). Emulsomes are lipoidal particulate vehicles comprising a hydrophobic solid fat core surrounded and stabilized by one or more phospholipid bilayers. Emulsomes have been shown in mice to enhance nasal immunogenicity, polarizing the response toward the Th2/3 cytokine profile (Lowell *et al.,* 1997).

The coadministration of nasal influenza vaccine with the cytokine IL-12 has also been evaluated in mice and was found to substantially enhance protective antibody responses over those obtained with vaccine alone (Arulanandam *et al.,* 1999).

One significant drawback to vaccination strategies for viruses such as influenza that induce homotypic and usually transient immunity is that continual revaccination is required. T-cell immunity, which is primarily directed against nonvariant internal antigens of these viruses (heterotypic immunity), has the potential to provide cross-protection against recurrent disease. Unfortunately, $CD8^+$ CMI responses to respiratory viruses appear to decline rather rapidly (Woodland, 2003). Recent studies in mice have revealed that long-term $CD8^+$ CMI responses can be generated, particularly with use of DNA vaccination. These studies, while still relatively early, may eventually lead to vaccine strategies that target the induction of long-term, cross-reactive T-cell immunity that could reduce the requirement for revaccination. The complexities of the mucosal immune system thus present daunting challenges for vaccinologists. Many of the strategies under study have enormous promise, but they must be examined with great care to guard against the equally great capacity for inadvertent harm.

SUMMARY AND CONCLUSIONS

Respiratory virus infections are a major cause of human disease. None is satisfactorily controlled by vaccines, and for most infections licensed vaccines do not exist. The reasons for this situation are likely multiple. They include the fact that some are antigenically mobile or exist as multiple serotypes. Moreover, none persists beyond a usually brief infection/disease episode. Hence, there is no continual source of antigen, which some investigators maintain is necessary to sustain functional long-term memory (Ahmed and Gray, 1996). In addition, it has been evident since the pioneering work of Tomasi and Ogra that the biology of immunity at mucosal surfaces differs from central systemic immunity (Tomasi *et al.,* 1965; Ogra and Karzon, 1969). Initially, the focus was on the types of antibodies involved. Thus, S-IgA is usually the principal Ig isotype at mucosal sites, and this is mainly produced by lymphoid tissue associated with mucosal surfaces (Tomasi *et al.,* 1965). It seems that the half-life for IgA is less than that for IgG which dominates systemic immunity. The long-term presence of Ig requires that cells continue to produce it. Some have argued that IgG-producing plasma cells can exist for long periods at least in the bone marrow (Slifka and Ahmed, 1996). It is not clear if similar long-term IgA-producing plasma cells also exist in mucosa-associated lymphoreticular tissue (MALT). Should they be absent, this could account for the loss of protective IgA at mucosal surfaces.

Contemporary research has focused on T-cell immunity, especially immunological memory, the basis for protective vaccines. As discussed previously, T cells, especially $CD8^+$ T cells, do participate in immunity to respiratory virus infections. However, unlike neutralizing antibody, they do not function to impede infection. Instead T cells serve to effect recovery from infection, a process that involves cytotoxicity and cytokine production. Following infection, effector-memory cells are induced in lung tissues, and these are responsible mainly for the control of infection. The cells exert direct protective effects and maintain this function in nonlymphoid parenchymal tissues of the lung for some time. However, after a few months, effector memory cell activity is barely detectible. At this stage, self-renewing memory cells are present in central lymphoid tissue, and these may persist for

years, seemingly without the need for antigen restimulation to sustain them (Sprent and Tough, 2001; Wherry *et al.,* 2003). This issue, however, remains debatable (Hogan *et al.,* 2002). In addition, it is not clear if mucosal lymphoid and nonlymphoid sites similarly act as repositories of memory cells. However, some have suggested that mucosal memory is of shorter duration than central memory (Hogan *et al.,* 2001). Wherever noneffector memory cells exist, it takes 4–5 days for their recruitment as effector memory cells in lung tissues. Meanwhile, a rapidly replicating respiratory virus could be well established and be in the process of causing lesions.

Another unresolved issue is the location of cells that are recruited and activated following reinfection to become lung effector memory cells. Conceivably, it cannot be mucosal lymphoid sites if such cells do indeed disappear quickly after antigen exposure. Experiments have shown that the central lymphoid organs can provide a source of protective T cells long after initial infection (Wherry *et al.,* 2003). It could be that some forms of antigen reexposure might preferentially recruit this source of cells. Additionally, it is conceivable that certain forms of vaccination may induce a readily recruitable form of memory cells from lymphoid tissues. Novel adjustments might serve to influence such events. It is anticipated that ongoing fundamental research on mucosal and central memory should reveal clues that are exploitable to improve the efficacy of respiratory viral vaccines. We hope that any future rewrite of this review will be able to report positive progress on these issues.

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