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Why is Co-infection with Influenza Virus and Bacteria so Difficult to Control?

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Why is co-infection with influenza virus and bacteria so difficult to control?

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Abstract

Influenza viruses are genetically labile pathogens which avoid immune detection by constantly changing their coat proteins. Most human infections are caused by mildly pathogenic viruses which rarely cause life-threatening disease in healthy people, but some individuals with a weakened immune system can experience severe complications. Widespread infections with highly pathogenic strains of influenza virus are less common, but have the potential to cause enormous death tolls among healthy adults if infection rates reach pandemic proportions. Increased virulence has been attributed to a variety of factors, including enhanced susceptibility to co-infection with common strains of bacteria. The mechanisms that facilitate dual infection are a major focus of current research, as preventative measures are needed to avert future pandemics

The death rates that are associated with influenza virus infections follow a cyclic pattern which fluctuates with seasonal changes in humidity. Occasional spikes in the numbers of fatalities mark random changes in pathogenicity, which occur when new mutations are introduced by the error-prone viral polymerase. More pronounced changes in pathogenicity mostly occur when two viruses exchange RNA-segments, thus producing a hybrid (reassorted) strain with virulent properties, or when a zoonotic virus first starts replicating in human populations (Greenbaum et al., 2012). Some avian viruses, including variants of the H5N1 and H7N9 strains that recently began causing sporadic infections in humans, are highly pathogenic to people without pre-existing immunity and have the potential to exact substantial death tolls in all age groups including healthy adults (Watanabe et al., 2012). Infections with novel avian viruses have become more frequent during recent years, fueling speculation that another devastating pandemic could soon develop.

The “Spanish flu” of 1918 was the most severe influenza pandemic on record, when 95% of the mortality was attributed to bacterial co-infection (Morens et al., 2008). Several common strains of bacteria were found in sputum samples from cadavers, including *Streptococcus pneumoniae* (Morens et al., 2008). Other pandemics occurred in 1957 and 1968 but the death tolls were less severe and coinfections with *Staphylococcus Aureus* were more prevalent, which may reflect increased use of antibiotics and the emergence of drug-resistant bacteria (ROBERTSON et al., 1958; McCullers, 2006). Preventing infections with

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methicillin-resistant Staphylococcus aureus (MRSA) has become an integral part of hospital care, underscoring the need for systematic control of anti-bacterial medication and providing an impetus in the search for alternative approaches. The mortality rates that were recorded during prior pandemics were so severe that stockpiling antibiotics and vaccines for pneumococcal infections has been touted as a major priority in preparation for future pandemics (Klugman and Madhi, 2007).

Influenza viruses became a major focus of research efforts after the ‘Spanish Flu’ caused an estimated 50 million deaths around the globe (Loo and Gale, Jr., 2007; Luk et al., 2001; Shanks, 2014). Influenza virus-related deaths were not a novel occurrence at that time, but the monumental loss of human life was unprecedented and garnered enormous public support for research efforts which ultimately led to the implementation of government-sponsored vaccination programs in many developed countries, including the US. Understanding why secondary infections with common strains of bacteria often emerge during influenza virus infection is an important frontier for current research efforts. While some progress has been made, much remains to be learned about the way that the virus interferes with immune regulation and undermines protective T cell responses. Susceptibility to bacterial co-infection increases with age and the most severe complications usually arise in people greater than 65 years old. Age-related changes in immunity also reduce the impact of vaccination due to weak B cell responses and declining antibody production (Duraisingham et al., 2013; Kogut et al., 2012).

Bacterial replication and adherence

The human body is host for a diverse constellation of bacterial species, collectively referred to as the microbiota (Hooper et al., 2012). Most of these bacteria are commensal strains which are maintained in mutually beneficial symbiosis with the host and provide many useful functions such as the provision of essential nutrients and immune protection through competition with more pathogenic strains. Sometimes small quantities of harmful bacteria are maintained within the microbiota without severe consequences. Many pathogenic microbes (or pathobionts) colonize exposed tissues such as the skin, where they will be well-positioned to invade the body should the opportunity arise. People that are genetically predisposed to subclinical infections can become ‘carriers’ of pathogenic bacteria, including MRSA. Carriers may be particularly vulnerable to co-infection with bacteria and influenza virus, since disruption of the mucosal barrier is sometimes sufficient for outgrowth of a previously harmless microbe. It is likely that viral infections also attract immune cells to new regions of the body and thus distract resident cell populations from the task of controlling bacterial growth.

Influenza viruses enter the body from the oral or nasal cavities and attach to the membranes of host epithelial cells. These tissues are also frequently sites of bacterial colonization. A pseudo-stratified epithelial layer includes specialized cells which produce mucus and surfactant. The primary function of the mucus is to trap noxious organisms before they adhere to the walls of the airways. Some particulate matter becomes trapped in the mucus and is expelled from the airways by ciliated epithelial cells. Influenza viruses with highly glycosylated coat proteins are more readily expelled from the lungs by the mucociliary

escalator, than poorly glycosylated variants which are more likely to cause deep lung infections and facilitate bacterial entry into the mucosa (McCullers, 2006). Some bacteria use mucinases to degrade the host proteins, while others take advantage of virus-induced damage in the lungs. Neuraminidase is a viral coat protein with enzymatic activity which is required for viral replication (Nayak et al., 2009). Bacterial colonization in the lungs increases when the viral neuraminidase disrupts a layer of sialylated mucins on the host cells, which act as decoy receptors for invading microbes, thus exposing cryptic sites for bacterial adherence (Peltola et al., 2005). Large quantities of sloughed host cells and increased mucus production in the airways also provide nutrients for bacterial growth (Siegel et al., 2014). Damage to the epithelial layer uncovers the basement membrane and reveals extracellular matrix proteins which provide additional sites for bacterial attachment (McCullers, 2006).

Cytokines cause immune damage and promote wound healing in the lungs

Enhanced pathogenesis during bacterial superinfection is the result of complex interactions between immune cells which elicit synergistic inflammatory responses and disrupt the integrity of the epithelial barrier. Current data, mostly derived from animal-models, point to multifactorial mechanisms of co-pathogenesis including strain-specific virulence factors of the virus. Some variations in disease severity have been linked to viral products such as the cytotoxin encoded by PB1-F2 (Alymova et al., 2014; McAuley et al., 2007), which causes host cell death and contributes to a ‘cytokine storm’ during infection with some pandemic and avian strains (de Jong et al., 2006; Conenello et al., 2007). Cytokines belong to large families of soluble molecules with pro- or anti-inflammatory properties, including interleukins (IL) which are primarily released by white blood cells and orchestrate the response to infection. In the lungs a cytokine storm can lead to irreparable tissue destruction as proinflammatory cytokines damage the alveoli. Defects in immune activation have also been attributed to the virally-encoded non-structural protein 1 (NS1) (Fernandez-Sesma, 2007), which interferes with signaling molecules and nuclear translocation (Jia et al., 2010).

Human deaths occur in two waves during infections with highly pathogenic strains of influenza virus. The ‘cytokine storm’ is responsible for many early deaths, resulting from fever, anorexia and severe lung damage leading to hypoxia. Loss of barrier function in the lower respiratory tract can facilitate acute-respiratory distress syndrome (ARDS). Neutrophils and macrophages are scavenger cells that engulf and destroy noxious organisms, including extracellular bacteria and debris from infected cells. During respiratory virus infection, severe damage to the lungs is accentuated by early and excessive infiltration of myeloid cells, including mononuclear phagocytes which kill alveolar epithelial cells by releasing a lytic molecule called TRAIL (Brincks et al., 2008). Macrophages play a key role in mediating inflammation by releasing large quantities of pro-inflammatory cytokines after they encounter necrotic cells and other debris. The proinflammatory cytokines include interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) are required for immune protection, but can become toxic when released in large amounts. Neutrophils are recruited into the lungs by IL-8 and may be important for controlling bacterial replication, however, an excessive neutrophil response is associated with a poor prognosis for ARDS susceptible patients (Tate et al., 2011). Symptoms of ARDS include diffuse

alveolar damage with accumulations of dead cells in protein-rich fluid which inhibits oxygen exchange.

The bacteria that are most commonly found in human sputum during influenza virus infection include strains that colonize the nasal passages of many healthy people (Ballinger and Standiford, 2010; Iverson et al., 2011) including *Staph. Aureus* (Metersky et al., 2012; McCullers, 2006) and group A streptococcus (GAS), which is a gram-positive bacteria that can also colonize human tonsils. Symptoms of infection with *Strep. pneumoniae* in children often include *otitis media*, while data from animal models suggest that influenza virus infection assists bacterial migration from the nasopharynx to the middle ear (Wren et al., 2014). Bacterial co-infection often becomes evident as the clinical symptoms of viral infection begin to improve and some patients experience recrudescence of fever, dyspnea and cough with milky sputum.

A variety of factors contribute to the synergy that is observed during viral and bacterial coinfection, including suppression of innate immune cells even during a robust cytokine response. Interferons are a family of proinflammatory cytokines with antiviral properties (Bonjardim et al., 2009). In animal models increased susceptibility to *Strep. pneumoniae* was linked to high concentrations of type I Interferon (IFNαβ) and negative-regulation of γδ T cells, which are an important source of interleukin-17 (IL-17) and recruit neutrophils into the lungs (Li et al., 2012). Reduced numbers of neutrophils are often found in the lungs during the recovery from influenza virus infection, partly due to prolonged desensitization of Toll-like receptors (TLR) which are involved in recognition of bacteria (Didierlaurent et al., 2008). Some data from animal models indicate that neutrophils do not play a major role in immunity during early bacterial infection, but may become more important as the infection progresses (Sun and Metzger, 2008).

Cells of the adaptive immune system help control subclinical bacterial infections in healthy people, including specialized CD4+ T cells which make IL-17 (called Th17 cells) (Cohen et al., 2011). Development of Th17 cells requires exposure to transforming growth factor-β (TGF-β) and IL-6 (Yang et al., 2008; Bettelli et al., 2006), which are expressed in the lungs during influenza virus infection (Schultz-Cherry and Hinshaw, 1996; Dienz et al., 2012). These cytokines induce mildly pathogenic Th17 cells which make suppressive cytokines (i.e. IL-10 and TGF-β) (McGeachy et al., 2007), as well as interleukin-21 (IL-21) which promotes auto-proliferation. The pathogenicity of the Th17 response increases when IL-1 and IL-23 are present, leading to reduced IL-10 and IL-21 expression (Cua et al., 2003). Although IL-6 is important for immunity to influenza virus infection (Dienz et al., 2012) excessive quantities can prove detrimental by reducing the numbers of regulatory T cells which synthesize suppressive cytokines such as IL-10 and TGFβ (Rincon, 2012).

As the viral titers decline, an anti-inflammatory state is induced by suppressive factors such as IL-10, which is required to restore homeostasis but may also increase susceptibility to bacterial co-infection (Sun et al., 2009). Macrophages change their cytokine response after consuming host cells that are undergoing controlled cell death (apoptosis) by producing anti-inflammatory cytokines (i.e. TGFβ and IL-10). Phagocytosis must occur quickly to prevent the dying cells from undergoing secondary necrosis, when intracellular components are

released and expose danger associated molecular patterns (DAMPS) which rekindle inflammation. During the recovery from influenza virus infection, macrophage activity is suppressed through inhibitory receptors such as CD200 (Snelgrove et al., 2008) and insensitivity to bacterial products which are recognized by Toll-like receptors (Didierlaurent et al., 2008). Influenza virus infection induces high concentrations of T cell-derived interferon (i.e. IFN-γ), which has been linked to slow clearance of microbial infections by alveolar macrophages due to reduced expression of scavenger receptors that are used to engulf particulate matter including extracellular bacteria and dying cells (Sun and Metzger, 2008). Other cytokines promote wound healing, including interleukin-22 (IL-22) which reduced lung damage during co-infection with *Strep. pneumoniae* (Ivanov et al., 2013). Some injured host cells express unusual surface proteins during wound healing (Puchelle et al., 2006), thus exposing additional cryptic sites that can be used by bacteria for adherence to the walls of the airways where they can resist the action of the mucociliary escalator (McCullers, 2006).

TGF-β is released from the host cells as a latent complex which is normally activated by extracellular proteases (Jenkins, 2008), but can also be activated by the viral neuraminidase (Schultz-Cherry and Hinshaw, 1996), making some seasonal strains of influenza virus less pathogenic than newer avian strains (Carlson et al., 2010). New data suggest that viral neuraminidase may promote bacterial growth in the lungs by altering the concentrations of TGFβ. Some bacteria use integrins which are regulated by TGFβ for adherence (Li et al., 2015). In addition TGFβ is an important regulator of the adaptive immune response, which reduces the numbers of activated CTLs in the lungs and induces regulatory CD4 T cells (Treg) that make IL-10 (Berod et al., 2012; Brincks et al., 2013). In animal models, reduced numbers of Th17 cells were linked to severe co-infection with *Strep. Pneumoniae* by a mechanism that involved IL-10 (van der Sluijs et al., 2004; McKinstry et al., 2009; Sun et al., 2009). In contrast, studies of human cells suggest that antigen-specific Tregs utilize CTLA-4 and PDL-1 (i.e. surface receptors involved in negative regulation of T cells) to suppress the Th17 response during control of commensal *Strep. pneumoniae* (Pido-Lopez et al., 2011). Similarly *Klebsiella pneumoniae* is a gram-negative bacteria that can be found in the lungs during influenza virus infection and is controlled by Th17 cells (Chen et al., 2011) suggesting that related mechanisms may be involved in susceptibility to co-infections with a variety of bacteria.

Interleukin-1 (IL-1) is a pyrogenic cytokine which is expressed in the host cells during influenza virus infection. Activation requires multiprotein complexes known as ‘inflammasomes’ which are an important trigger of the immune response (Ichinohe et al., 2009). Influenza virus infected mice became highly susceptible to infection with *Staph. Aureus* when Th17 responses were suppressed by a mechanism involving reduced IL-1β expression (Robinson et al., 2013; Robinson et al., 2014). Suppressed Th17 responses also promoted colonization with *Staph. Aureus* by reducing the concentrations of an antimicrobial peptide (i.e. neutrophil gelatinase-associated lipocalin) that limits bacterial growth (Robinson et al., 2014). Other studies linked enhanced susceptibility to co infection with *Staph. Aureus* with an impaired response by natural killer (NK) cells and weak antibody-dependent cellular cytotoxicity (ADCC) (Small et al., 2010).

The impact of dual infection is not limited to enhanced bacterial growth, but also impacts immunity to the virus. Animal models have consistently shown increased viral titers during bacterial superinfections and delayed clearance. Studies suggest that *Staph. Aureus* produces proteases which cleave hemagglutinin, thus producing fusion-competent virus particles with enhanced infectivity (Tashiro et al., 1987). Symptoms of viral replication were also exacerbated during coinfection with methicillin-resistant *Staph. Aureus* (MRSA), when IL-13 suppressed the interferon response (Rynda-Apple et al., 2014). Reduced numbers of NK cells, which have lytic activity, could also delay viral control during Infection with *Staph. Aureus*. Similarly suppressed anti-viral CTL responses and cytokine expression were observed in the lungs during co-infection with *Strep. pneumoniae* (Blevins et al., 2014). Further defects in immune activation occur when dendritic cells become infected with influenza virus, leading to suboptimal maturation and increased apoptosis (Bender et al., 1998; Fernandez-Sesma et al., 2006). As ‘mature’ dendritic cells are essential for T cell priming, suboptimal activation may suppress immunity to both bacterial and viral infections thereby permitting outgrowth of pathogens that would normally be suppressed. Collectively these concepts are presented in Figure 1.

Preparations for a new pandemic – a case for stockpiling anti-viral medications and antibiotics

The drugs that are currently approved by the Food and Drug Administration (FDA) for treating influenza virus infections in humans target two viral genes. The most widely used medications are neuraminidase inhibitors (e.g. *Oseltamivir* and *Zanamivir*) which promote early recovery by reducing the amount of virus that is released into the lungs (Nayak et al., 2009). Other drugs, such as such as *Amantadine* and *Rimantadine*, block M2 proton channels which are required for virus to enter the cytoplasm of the host cells (Moorthy et al., 2014). The available drugs are becoming less efficacious as resistant viruses emerge (van der Vries et al., 2011; Thorlund et al., 2011) and the search for new pharmaceuticals must be a top priority in preparations for managing future influenza pandemics. New strategies that are under consideration include inhibitors of the viral polymerase (Furuta et al., 2013), as well as enzymes which impede infection by cleaving sialic acid residues from the surface of the host epithelial cells (Marjuki et al., 2014). Data from animal models suggest that treatment with Doxycycline (an inhibitor of matrix metalloproteinases (MMPs) can reduce lung injury, although viral replication and weight loss were not changed (Ng et al., 2012). Similarly, ARDS patients experienced reduced lung injury during treatment with a neutrophil elastase inhibitor (Iwata et al., 2010). As drug-resistant bacteria become more prevalent the new treatments must target ubiquitous strains such as MRSA. Some progress has been made as recent studies suggest that synthetic antibiotics (Pasberg-Gauhl, 2014), or plant extracts such as flavonoids (Wang et al., 2014) can augment bacterial clearance when used in combination with conventional remedies. Similar responses were observed when Chevalone E (an extract from marine animals) was used in combination with antibiotics (Prompanya et al., 2014).

Concluding remarks

For many years vaccines have been produced from inactivated components of ubiquitous viruses which are used to induce high concentrations of serum antibodies (Baez et al., 1980; Cox et al., 2004). These vaccines are not optimal because they do not include pandemic strains and offer little protection against viruses with novel coat proteins (Luke and Subbarao, 2006; Vardavas et al., 2010). Increasingly frequent of infections with new strains of avian influenza virus (including H5N1 and H7N9) have been reported during recent years (Watanabe et al., 2012; Belser et al., 2011) highlighting the possibility that another pandemic could occur in the not too distant future. Ideally preparations for such an event will include the development of new classes of vaccines for both viral and pneumococcal infections, which target drug-resistant strains (Tripp and Tompkins, 2014; Hoft et al., 2011; Klugman and Madhi, 2007; Chung and Huh, 2015).

The advent of new medications and inventions that improve hygiene are likely to reduce the severity of a future influenza pandemic. Though such improvements are cause for optimism, other aspects of modern life are cause for concern as widespread access to high speed transportation and high density animal husbandry can facilitate the transmission of highly contagious pathogens. Drug-resistant viruses and bacteria are becoming increasingly commonplace with widespread use (and abuse) of antibiotics or anti-viral medications, including neuraminidase inhibitors (Thorlund et al., 2011). Complacency could have dire consequences, since relatively low death rates (1–2%) could be sufficient to cause massive numbers of casualties on a global scale. Animal models faithfully recapitulate many salient features of bacteria co-infections, thus revealing important insights into the mechanisms that promote synergy between different organisms, but much more remains to be learned before effective treatment and prevention strategies will be ready to provide immunity on a global scale. Promising research initiatives include high-throughput RNA-sequencing and computer modeling to analyze cytokine signatures in patients with community-acquired infections.

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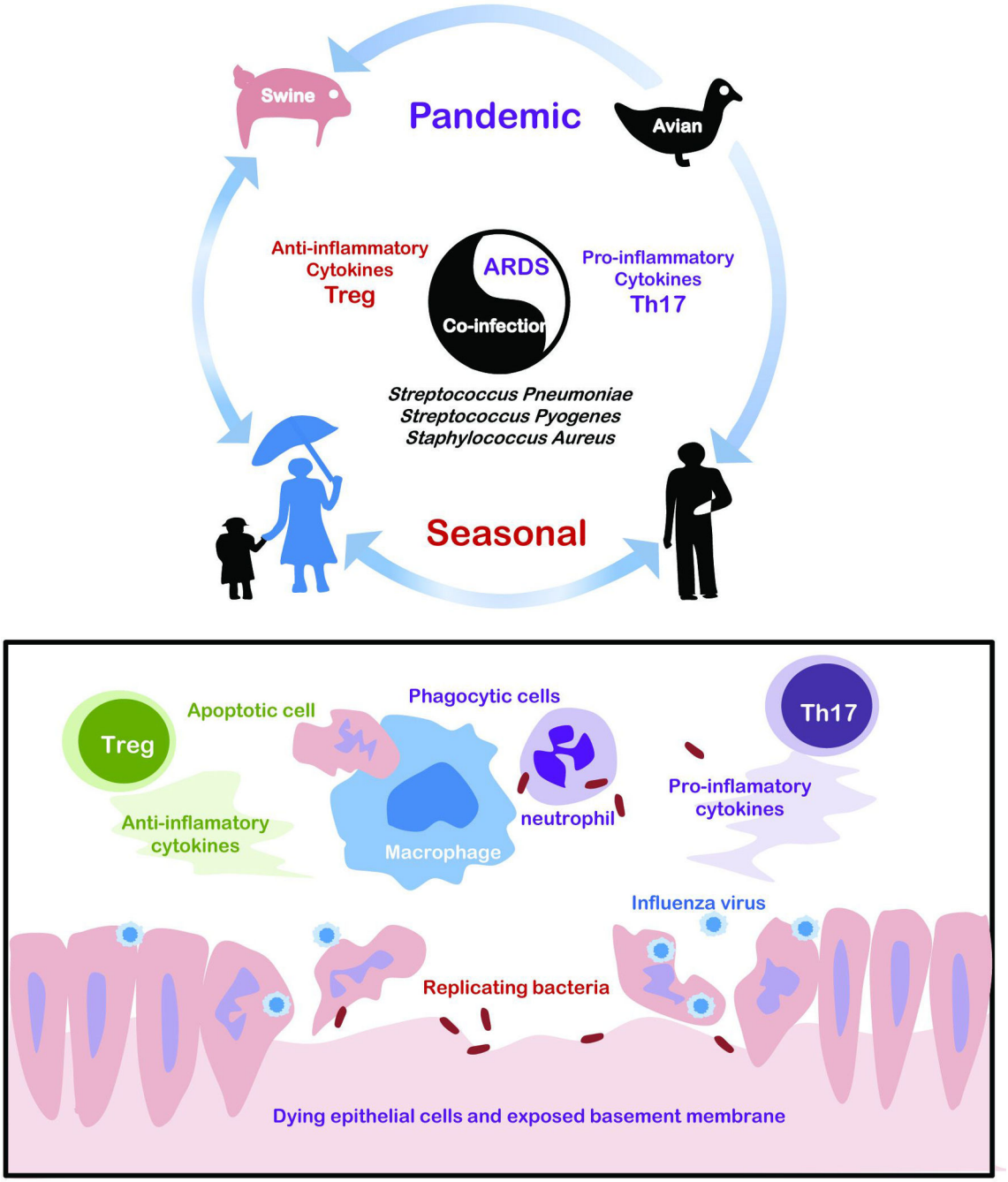

Figure 1.

Influenza virus infection damages the lungs and promotes bacterial growth by changing the cytokine response. Severe damage to the alveoli usually occurs during infection with highly pathogenic strains of influenza virus, including reassorted strains that are adapted for replication in birds or swine. Bacterial growth often begins when the virus disrupts the epithelial barrier and induces cytokines which reduce the numbers of Th17 cells in the lungs. Immune protection is further compromised by inefficient phagocytosis by macrophages and suppressive cytokines which are released by regulatory T cells. Many current antibiotics and

anti-viral medications are not sufficient to protect against drug-resistant strains of influenza virus or bacteria such as MRSA.