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REVIEW

Principles of antidote pharmacology: an update on prophylaxis, post-exposure treatment recommendations and research initiatives for biological agents

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The use of biological agents has generally been confined to military-led conflicts. However, there has been an increase in non-state-based terrorism, including the use of asymmetric warfare, such as biological agents in the past few decades. Thus, it is becoming increasingly important to consider strategies for preventing and preparing for attacks by insurgents, such as the development of pre- and post-exposure medical countermeasures. There are a wide range of prophylactics and treatments being investigated to combat the effects of biological agents. These include antibiotics (for both conventional and unconventional use), antibodies, anti-virals, immunomodulators, nucleic acids (analogues, antisense, ribozymes and DNAzymes), bacteriophage therapy and micro-encapsulation. While vaccines are commercially available for the prevention of anthrax, cholera, plague, Q fever and smallpox, there are no licensed vaccines available for use in the case of botulinum toxins, viral encephalitis, melioidosis or ricin. Antibiotics are still recommended as the mainstay treatment following exposure to anthrax, plague, Q fever and melioidosis. Anti-toxin therapy and anti-virals may be used in the case of botulinum toxins or smallpox respectively. However, supportive care is the only, or mainstay, post-exposure treatment for cholera, viral encephalitis and ricin – a recommendation that has not changed in decades. Indeed, with the difficulty that antibiotic resistance poses, the development and further evaluation of techniques and atypical pharmaceuticals are fundamental to the development of prophylaxis and post-exposure treatment options. The aim of this review is to present an update on prophylaxis and post-exposure treatment recommendations and research initiatives for biological agents in the open literature from 2007 to 2009.

Abbreviations

AGP, amino-alkyl glucosaminide 4-phosphate; BoNT/A–E, botulinum neurotoxins A–E; CapD, capsule depolymerase; CBR, chemical biological radiological; CDC, Centre for Disease Control (USA); CDHS, California Department of Health Services; CDV, cidofovir; ChiSys, chitosan mucoadhesive agent; CpG, unmethylated sequences of DNA; CMRI, phase I chloroform-methanol residue; ctx, cholera toxin; ctxB, subunit B of the cholera toxin; Dstl, Defence Science and Technology Laboratories (UK); DSTO, Defence Science and Technology Organisation (Australia); EEEV, Eastern equine encephalitis virus; EEV, equine encephalitis virus; EF, oedema factor; F1, fraction 1 capsular antigen; FDA, Food and Drug Administration (US); flaA–E, flagellin proteins A–E; G-CSF, granulocyte colony-stimulating factor; Had5, human adenovirus serotype 5; HC, heavy chain; HE-BAT, heptavalent botulinum antitoxin; HGS, Human Genome Sciences; IFN, interferon; INA, iodonaphthylazide; IND, investigational new drug; LC, light chain; LF, lethal factor; LT, heat-labile enterotoxin; mAbs, monoclonal antibodies; MVA, Modified vaccinia Ankara; NE, non-toxic mucosal adjuvant; ODN, oligodeoxynucleotides; PA, protective antigen; pAbs, polyclonal antibodies; PBT, pentavalent botulinum toxoid; pcDNASHc, conventional plasmid DNA vaccine; PPMO, peptide-conjugated phosphorodiamidate morpholino oligomers; pSCARSHc, plasmid DNA replicon vaccine; rhAPC, recombinant-activated protein C; rPA, recombinant protective antigen; rRV, RTA vaccine; RTA, ricin toxin A-chain; SIN, Sindbis; scFv, single-chain Fv; siRNAs, short interfering RNAs; SFV, Semliki Forest virus; TMP-SMX, trimethoprim sulphamethoxazole; USAMRIID, US Army Medical Research Institute of Infectious Diseases; VEEV, Venezuelan equine encephalitis virus; WEEV, Western equine encephalitis virus; WHO, World Health Organisation

History of biological agents

The use of biological agents to evoke fear and cause casualties and death has a long history, dating back before the middle ages. The first known use of a biological agent was in 600 BC when Helleborus root (active ingredients: protoanemonin, steroidal saponines and bufadienolides) was used to contaminate water supplies in the siege of Kirrha (Smart, 1997). In the middle ages, the Polish used projectiles filled with saliva from rabid dogs against enemies in 1650, plague-infected cadavers were deposited in Reval, Estonia by Russians in 1710 (Smart, 1997), and in 1763 smallpox-infected blankets were distributed to Native American Indians by the British (Smart, 1997).

The deliberate use of biological agents has continued in more modern times with the use of cholera and plague by the Japanese during their invasion of China (1940–1944) (Robinson and Leitenberg, 1971), and the testing of aerial bombs and cannon shells for the dissemination of anthax spores in Scotland (1941–1942) (Robinson and Leitenberg, 1971). The assassination of Georgi Markov with ricin via a modified umbrella on 7 September 1978 (Harris and Paxman, 1982) further highlights the evolution of dissemination techniques for biological agents.

More recently, biological agents have been used outside the military sphere and in the civilian domain. The use of salmonella by the Rajneeshi sect in 1984 to influence US elections (Torok *et al.*, 1997), the several failed attempts by Aum Shinrikyo to disseminate botulinum toxins and anthrax spores (1990–1995) (Olson, 1999) and the anthrax letters distributed in the USA shortly after the 9/11 attack on the World Trade Centre Towers in 2001 (Jernigan *et al.*, 2002) are evidence of non-state-based groups intentionally using biological agents in terrorist activities.

Change of the war environment

The use or potential use of biological agents has generally been confined to military-led conflicts. However, the face of war has changed significantly in the past few decades. The days of conventional, military-led war-fighting with a readily-identifiable enemy are becoming less frequent.

This change in the war environment is important for governments to consider in developing strategies for preventing and preparing for attacks by insurgents. In conventional combat, operations tend to be driven by military strategy. However, in counter-insurgency, it is intelligence and information that tend to drive operations. Therefore, the best way to combat the asymmetric nature of unconventional warfare, such as biological agents, is to develop and maintain capabilities that assist with the prevention of, and response to, biological agents that may pose a threat to societal security. These areas include personal protective equipment (Figure 1), detection technology and the development of pre- and post-exposure medical countermeasures.

Figure 1

Soldiers (A) donning chemical and biological (CB) protective suits and (B) carrying a casualty in a CB protective stretcher (photographs courtesy of the Combat Arms Training Centre).

Although the probability of an incident involving a biological agent is relatively low, the high impact risk associated with such an event necessitates that biological agents are classified as weapons of mass destruction. In contrast to conventional weapons, relatively small amounts of biological agents are capable of causing great morbidity and mortality. In addition to the burden of casualties and possible deaths on the healthcare system, an incident involving a biological agent would undoubtedly have an impact on other areas of infrastructure, including first-responder agencies, the economy, travel and tourism. Hence, one of the

major challenges with preparing for an incident involving a biological agent is not necessarily whether to have a particular capability, but the scale to which a capability should be resourced.

Current treatment of biological agents

Given the delayed onset of common symptoms that can occur following exposure to a biological agent, healthcare professionals could play a vital role in informing first responders of a potential agent. This information is imperative in preparing a response plan (including the use of personal protective equipment), preventing further casualties and/or deaths and developing and implementing appropriate decontamination arrangements.

There are a wide range of prophylactics and treatments being developed to combat the effects of biological agents. These include antibiotics (for both conventional and unconventional use), antibodies, anti-virals, immunomodulators, nucleic acids (analogues, antisense, ribozymes and DNAzymes), bacteriophage therapy and micro-encapsulation. Interestingly, despite the numerous medical advances of the past few decades, the mainstay treatment for a number of biological agents is still basic supportive care. However, given the array of research approaches available, much research is still being undertaken to combat biological agents.

Pettineo *et al*. (2009) have undertaken an extensive review of current clinical therapeutic countermeasures for biological and chemical weapons; however, they did not cover the recent research advances in potential treatments. This review aims to present an update on prophylaxis and post-exposure treatment recommendations and research initiatives for biological warfare or bioterror agents (anthrax, botulism, cholera, viral equine encephalitis, melioidosis, plague, Q fever, ricin and smallpox). It focuses on research advances and medical countermeasures of treatment of biological agents in data published from 2007 to 2009 (Table 1).

Anthrax

For a brief summary of the type, mode/mechanism of action and signs and symptoms of anthrax infection, please refer to Table 1.

Current prophylaxis for anthrax

There are two licensed anthrax vaccines available (Little, 2005; Wang and Roehrl, 2005). The US anthrax vaccine adsorbed (AVA; Emergent BioDefense Corporation; also known as BioThrax®, Emergent Biosolutions Incorporated, Rockville, MD, USA) is extracted from a cell-free culture filtrate of an unencapsulated, toxin-producing strain of *Bacillus anthracis* (V770-NP1R). The UK vaccine (Health Protection Agency) is prepared from a similar strain called Sterne 34F2. Both vaccines contain the protective antigen (PA) adsorbed to aluminium hydroxide and contain small amounts of lethal factor (LF) and oedema factor (EF). The vaccines are both effective against anthrax infection when administered prophylactically, although the vaccination protocols differ (Little, 2005; Wang and Roehrl, 2005; Scorpio *et al*., 2006). The US vaccine is administered in a six-dose primary series at 0, 2 and 4 weeks and 6, 12 and 18 months with an annual booster, while the UK vaccine requires four single injections: three injections 3 weeks apart, followed by a 6 month dose, with an annual booster.

For post-exposure prophylaxis against inhalation anthrax the Centre for Disease Control and Prevention (CDC) recommends that the vaccine AVA be used at 0, 2 and 4 weeks in combination with selected oral antibiotics. The combined use of AVA and antibiotics has been shown to prevent inhalation anthrax (Schneemann and Manchester, 2009) and may also shorten the required period of antibiotic therapy (Bossi *et al*., 2004a). However, this regime has not been approved by the United States Food and Drug Administration (FDA). Caution should be taken with children as the PA component of the vaccine may associate directly with the toxin components produced by the invading bacterium thereby potentially augmenting intoxication (Aulinger *et al*., 2005).

Although current human anthrax vaccines are effective against anthrax, they still suffer from batch-to-batch variation in composition, require multiple doses and yearly booster injections and have been associated with occasional adverse reactions (reactogenicity) (Pittman *et al*., 2001; Pittman *et al*., 2004). These limitations have prompted the development of novel vaccines that are less reactogenic, but equally efficacious with fewer doses. Research efforts focus on: (i) development of subunit vaccines targeting PA (and to a lesser extent EF and LF); (ii) evaluation of alternative vaccine delivery routes (e.g. i.m. and mucosal administration); and (iii) identification of new vaccine targets (e.g. spore and capsule antigens). Excellent reviews have been published on a number of the major achievements (Brey, 2005; Little, 2005; Wang and Roehrl, 2005; Scorpio *et al*., 2006).

Mucosal vaccination has proven to be a practical, non-invasive and efficacious method for the induction of both mucosal and systemic immune responses. Recently, a mucosal anthrax vaccine,

Table 1

Summary of biological agents including type, mode/mechanism of action and signs and symptoms of infection

Biological agent	Bacteria, virus or toxin?	Mode/mechanism of infection/intoxication	Signs and symptoms
Anthrax	Rod-shaped, Gram-positive and spore-forming bacterium	*Bacillus anthracis* forms spores under specific environmental conditions. Upon entering the bloodstream, spores are engulfed by macrophages where they germinate, multiply and eventually lead to macrophage lysis (Turnbull, 2002). Bacilli are released into bloodstream and release a toxin comprised of three polypeptides: protective antigen, oedema factor and lethal factor, which cause death through massive oedema and organ failure (Dixon *et al.*, 1999)	Cutaneous: boil-like skin lesion that forms an ulcer with black centre (eschar) (Figure 2); Gastrointestinal: vomiting of blood, severe diarrhoea, acute inflammation of intestinal tract, loss of appetite; Inhalational: cold or flu-like symptoms with (often fatal) respiratory failure. All forms lead to oedema and organ failure resulting in death
Botulism	Neurotoxins of which seven serotypes exist (A, B, C, D, E, F and G). *Clostridium botulinum, Clostridium argentinense, Clostridium baratii* and *Clostridium butyricum* are known to produce botulinum neurotoxins	Botulinum neurotoxin complex contains neurotoxin and other proteins (Inoue *et al.*, 1996; Cai *et al.*, 1999). Activation occurs via a protease that forms a molecule consisting of a heavy chain and a light chain (LC). The LC blocks the release of acetylcholine by inhibiting the fusion of synaptic vesicles with the plasma membrane (Singh, 2000; Simpson, 2004; Montecucco and Molgo, 2005; Singh, 2006)	Food-borne: nausea, vomiting, abdominal cramps, diarrhoea. All forms may include: cranial nerve dysfunction – blurred vision, ptosis (droopy eyelids), diplopia (double-vision), photophobia (sensitivity to light); bulbar nerve dysfunction – dysarthia (difficulty speaking), dysphonia (defective use of voice), dysphagia (difficulty swallowing); descending paralysis: weakness in control of muscles from head to upper and lower limbs; respiratory complications due to upper airway obstruction or paralysis of diaphragm. Symptoms and their severity are dose-dependent
Cholera	Toxin produced by *Vibrio cholerae*	The cholera toxin is an oligomeric complex made up of six protein subunits: a single copy of the A subunit, and five copies of the B subunit (part B) that are connected by a disulfide bond (Zhang *et al.*, 1995). Once part B binds to surface membranes, the toxin is internalized by the cell and part B is released following the reduction of a disulfide bridge. Part A initiates a number of other internal processes that lead to increased adenylate cyclase activity and increased cAMP, resulting in fluid and electrolyte efflux (diarrhoea)	Abdominal pain, diarrhoea, dehydration, kidney failure (in severe cases) (World Health Organization, 2008)
Encephalitis Viruses (Eastern equine encephalitis (EEEV); Western equine encephalitis (WEEV); Venezuelan equine encephalitis (VEEV))	Alphavirus from the *Togaviridae* family. Transmitted by mosquitoes (Figure 3) and uses birds and small mammals as replicating hosts	Single-stranded RNA arbovirus gains access to spinal cord and brain cells via the blood stream or nerves. Entry into brain cells causes damage, ultimately causing cell rupture and death. Immune system response causes cerebral oedema which, in conjunction with the infection itself, causes symptoms of viral encephalitis (Wu *et al.*, 2007b)	General symptoms of encephalitis may include: high temperature, headache, photophobia, general malaise, stiff neck and back, vomiting, changes in personality, confusion, amnesia, seizures, paralysis, coma. EEEV: encephalitis (acute inflammation of the brain) with many survivors developing crippling sequelae: mental retardation, convulsions and paralysis. WEEV: flu-like symptoms, delirium, disorientation and coma. VEEV: fever, malaise, rigors, headache, myalgia
Melioidosis	Gram-negative bacterium *Burkholderia pseudomallei*	Bacteria multiply within the vacuoles of phagocytes following internalization and subsequently induce endosome lysis and cell death (Cheng *et al.*, 2003)	Highly variable, but may include flu-like symptoms such as chills, headache and joint pain. May lead to abscesses in the brain, liver and spleen

Table 1

Continued

Biological agent	Bacteria, virus or toxin?	Mode/mechanism of infection/intoxication	Signs and symptoms
Plague	Gram-negative bacterium *Yersinia pestis*	Following entry to bloodstream via flea bite, *Y. pestis* adhere to and invade epithelial cells (Forman *et al.*, 2008). *Y. pestis* can avoid phagocytosis and proliferates in lymph nodes. Bacterial proteins disrupt host cell protein kinase activity leading to apoptosis of macrophages and invasion of the immune system	Pneumonic: severe bronchopneumonia, dyspnea, cough, chest pain, haemoptysis. Bubonic: prostration, severe malaise, headache, vomiting, chills, cough, abdominal pain, chest pain, development of buboes in groin and/or lymph nodes, tachycardia, hypotension, leukocytosis. Septicaemic: fever, chills, nausea, vomiting, diarrhoea, purpura acryl cyanosis and necrosis (particularly of limbs) (Worsham *et al.*, 2007)
Q fever	Gram-negative bacterium *Coxiella burnetii*	*C. burnetii* is most infectious in immunocompetent animals and humans. The microorganism is engulfed by macrophages and transported systemically, causing histopathological changes in the lungs, liver and spleen (Russell-Lodrigue *et al.*, 2006). After uptake by host cells, proliferation within the phagolysosome leads to rupture of the host cell and infection of a new population of host cells (Hackstadt and Williams, 1981). The bacteria are able to survive inside host cells via their use of a secretion system known as Icm/Dot, whereby they inject effector proteins called Ank proteins into the host (Segal *et al.*, 2005)	Mild to moderate flu-like symptoms including headache, fever, myalgia, arthralgia, anorexia, sweats and acute weight loss. Rash may occur (5–20% of cases), as may varying degrees of pneumonia (presenting with cough and pleural pain) and hepatitis (Parker *et al.*, 2006). Chronic disease (9% of cases) may result in endocarditis, chronic hepatitis, osteomyelitis, septic arthritis, chronic interstitial lung disease or aneurysm (Parola and Raoult, 2007). Factors increasing risk progression from acute illness to chronic disease include: heart valve defects, immunosuppression due to HIV, cancer therapy, transplantation or pregnancy (Fenollar *et al.*, 2001)
Ricin	Toxin – 64 kDa globular glycoprotein lectin	Toxin consists of two chains [ricin toxin A-chain (RTA) and RTB]. RTB binds to galactose-containing glycoproteins on cell surface and triggers endocytic uptake of the toxin (Day *et al.*, 2002). Toxin is relocated to Golgi apparatus via endosomal transport and the two chains are separated. The RTA is moved to the cytosol where it inhibits protein synthesis within the cell (Audi *et al.*, 2005)	Gastrointestinal: nausea, abdominal pain, vomiting, followed by diarrhoea, cramps, dilation of pupils, fever, dehydration, anuria, sore throat, headache, hypotension, heartburn, internal bleeding of stomach and intestines, failure of liver, spleen and kidneys, leading to death within 3 days from circulatory collapse (Furbee and Wermuth, 1997; Reed, 1998)
Smallpox	Variola virus	Upon entry into host lung cells, double-stranded poxviruses replicate in cytoplasm of cells in lymph nodes. Virus then spreads to other lymphoid tissues, spleen, liver, bone marrow and lung. Virus produces pro-inflammatory cytokines, lymphocyte apoptosis and coagulation abnormalities leading to organ dysfunction and multi-system failure (Jahrling *et al.*, 2007)	Fever, tiredness, body aches, headache, backache, prostration, vomiting, maculopapular rash in skin (Figure 4), mouth and throat leading to pustular rash with fluid-filled blisters and scabs. Less commonly, blindness resulting from corneal ulceration and scarring, and limb deformities from arthritis and ostomyelitis may also occur (2–5% of cases) (Behbehani, 1983)

based on a non-toxic mucosal adjuvant (NE) and a recombinant protective antigen (rPA), was developed (Bielinska *et al.*, 2007). Guinea pigs immunized intra-nasally (i.n.) with the vaccine were protected from an intra-dermal (i.d.) challenge ($1000 \times LD_{50}$) of *B. anthracis* Ames spores. Another mucosal anthrax vaccine composed of rPA, MPL (a toll-like 4 receptor agonist) and ChiSys (a chitosan mucoadhesive agent) is available in the form of a dry powder (Klas *et al.*, 2008). The vaccine protects rabbits from lethal aerosol spore challenge up to 9 weeks after a single i.n. immunization.

An anthrax vaccine based on live attenuated *Salmonella* vaccine strain (Ty21a) has also been

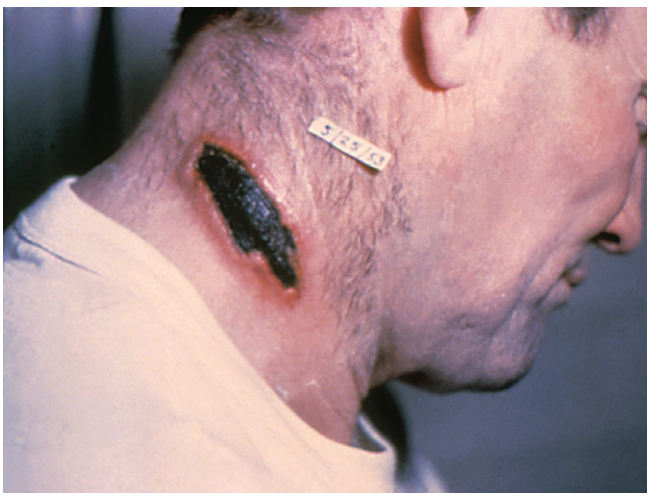

Figure 2
Cutaneous anthrax lesion on the neck (photograph courtesy of the Centre for Disease Control).

Figure 3
Anterior view of the *Culex tarsalis* mosquito, known to spread Western equine encephalitis, in addition to a number of other encephalitis viruses (photograph courtesy of James Gathany, Centre for Disease Control).

reported (Stokes *et al.*, 2007). Administration of Ty21a (p.o.) expressing the full-length rPA conferred significant protection against lethal exposure to aerosolized *B. anthracis* spores in mice. Further modification of rPA by its fusion to two distinct transport proteins (HlyA and ClyA) (Baillie *et al.*, 2008) resulted in significant PA-specific immune responses when mice were immunized with Ty21a expressing the ClyA–PA fusion protein and then boosted with either rPA or AVA. CpG (unmethylated sequences of DNA) oligodeoxynucleotides (ODN) have also been evaluated as an adjuvant for AVA (Klinman *et al.*, 2007). Mice immunized i.p. or i.n.

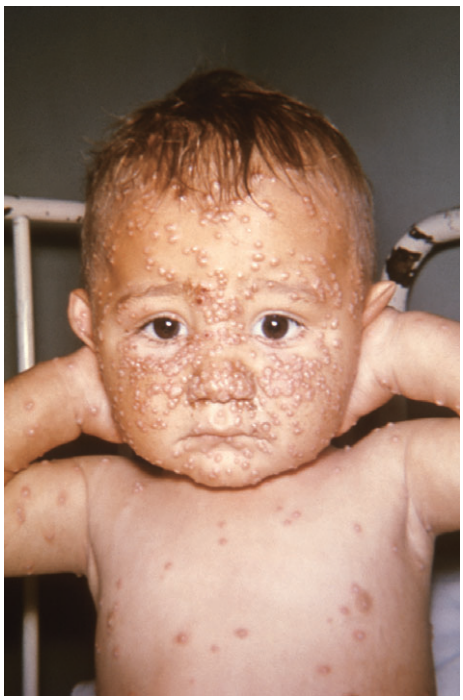

Figure 4
Characteristic maculopapular lesions of the milder form of smallpox (variola minor) on a Brazilian child (photograph courtesy of the Centre for Disease Control).

with AVA + CpG ODN showed significantly increased host immunity to infection via aerosolized anthrax spores, in contrast to animals immunized with AVA alone. Interestingly the enhanced immunity correlates with the induction of strong systemic rather than mucosal immune responses (Klinman *et al.*, 2007).

Protection against anthrax via current anthrax vaccines is mediated largely by antibody (humoral) responses to the protective antigen (PA); however, cellular immunity has been shown to also play an important role (Glomski *et al.*, 2007). Mice immunized with formaldehyde-inactivated spores (FIS) of a non-encapsulated *B. anthracis* strain were then challenged with an encapsulated non-toxinogenic *B. anthracis* strain. Sera, splenocytes and CD4 T lymphocytes were isolated from the FIS-induced mice and administered to naïve mice. The mice were then challenged with the encapsulated non-toxinogenic *B. anthracis* with results indicating that only interferon (IFN)-γ-producing CD4 T lymphocytes provide significant protection against anthrax infection. This study provides the first evidence of protective cellular immunity against encapsulated *B. anthracis*.

A plasmid DNA-based approach has been applied successfully to anthrax vaccine development to boost cellular immunity (Zhang *et al.*, 2008). Vacci-

nation of mice with plasmid constructs expressing either PA or EA1 (an S-layer antigen) produced both Th1 and Th2 cellular responses demonstrating that this approach may be used to generate durable immune responses against anthrax. This method has also been used in conjunction with a replication-defective adenovirus vector in a prime-boost vaccination strategy (McConnell *et al.*, 2007). Mice primed and boosted with plasmid DNA and adenovirus DNA, respectively, were fully protected from anthrax spore challenge. Interestingly the adenovirus-based prime-boost immunization produced 10-fold the anti-PA antibodies than AVA after a single injection.

The toxin components PA and LF are composed of four domains, of which the PA domain 4 interacts with the host cell receptor, while the LF domain 1 binds to PA63 (the active form of PA). Antibodies raised against the PA domain 4 were protective against anthrax infection when tested in mice (Flick-Smith *et al.*, 2002). The PA domain 4 and the LF domain 1 were fused to a thermostable lichenase from the bacterium *Clostridium thermocellum* and then expressed in the plant *Nicotiana benthamiana* (Chichester *et al.*, 2007). Immunization of mice with the fusion protein resulted in high titres of antibodies capable of neutralizing the lethal toxin *in vitro*.

A novel vaccine with the combined function of vaccine and antitoxin has been reported (Manayani *et al.*, 2007). In this vaccine multiple copies of the PA-binding domain VWA of the anthrax toxin receptor ANTXR2 were expressed and displayed on the surface of an insect virus. The resultant chimeric virus particles protected rats from anthrax intoxication, and when loaded with PA, induced a potent immune response against lethal toxin challenge in a single dose without adjuvant.

Previous studies have shown that whole spore-based vaccines are more effective against virulent strains of *B. anthracis* than the current PA-based vaccines (Little and Knudson, 1986; Welkos and Friedlander, 1988; Brossier *et al.*, 2002). However, these vaccines are unlikely to be used in humans because of safety concerns. Mice primed with suboptimal amounts of PA followed by the spore surface antigen BclA were protected from lethal anthrax spore challenge (Brahmbhatt *et al.*, 2007). BclA promotes opsonophagocytosis of spores by macrophages thereby inhibiting intra-macrophage spore germination. More recently, spore surface antigens p5307 and BxpB were identified (Cybulski *et al.*, 2008). Mice immunized with suboptimal amounts of anthrax PA followed by p5307 and BxpB had enhanced protection against lethal anthrax spore challenge compared with animals immunized with PA alone. Although antibodies raised against either antigen reduced the rate of spore germination *in vitro*, both produced enhanced phagocytic uptake and phagocyte-mediated spore destruction in the mice. Holistically, these results demonstrate that spore surface antigens are potential immuno-enhancers to PA-based vaccines.

Catalytic mutants of LF (LF_{E687A}) and EF (EF_{H351A}) have been evaluated in combination with PA for prophylactic use. Studies in mice demonstrated the ability of LF_{E687A} and EF_{H351A}, co-administered with PA, to reduce lethality following lethal anthrax spore challenge (Gupta *et al.*, 2007).

The advances in vaccine development for anthrax over the last 2 years, as outlined above, have been undertaken in preclinical animal studies and more definitive outcomes in human clinical trials are required.

Current treatment for anthrax

The FDA recommends that ciprofloxacin, doxycycline or amoxicillin be used for a period of 60 days post exposure to *B. anthracis* (http://www.fda.gov/). Following the bioterrorism attack of 2001, more detailed guidelines have been produced by the CDC (http://www.cdc.gov/).

The need for novel strategies to treat anthrax infection has been highlighted given that prolonged antibiotic treatment is not suitable for pregnant women and children (Schneemann and Manchester, 2009). Furthermore, antibiotics do not inhibit anthrax intoxication once critical levels of toxin are in the bloodstream. There is also the concern that antibiotic therapy will not be effective against future strains of antibiotic resistant *B. anthracis* (Stepanov *et al.*, 1996; Brook *et al.*, 2001; Schneemann and Manchester, 2009).

The most significant novel therapy has been the development of antibody-based passive immunotherapy against anthrax toxin components, primarily PA and to a lesser extent LF (Bouzianas, 2007). This has been made possible through significant funding from the US government to support the development and commercialization of antibody-based therapy. For example, Human Genome Sciences (HGS) was awarded $165M in June 2006 to develop and deliver 20 000 doses of human monoclonal antibody (mAb) ABthrax, which targets PA, and Elusys Therapeutics Inc. was awarded $12M in September 2007 to produce a high-affinity humanized mAb to PA. Both therapeutics have completed phase I clinical trials.

In addition to the toxin components, the anthrax capsule is also critical for inducing passive immunotherapy because of its role in the early stages of infection. Recently, six mAbs specific to the

capsule have been produced and evaluated for their ability to protect mice against inhalational anthrax (Kozel *et al.*, 2007). Most of the antibodies conferred varying degrees of protection to inhalational anthrax in mice, most likely due to how they interact with the capsule. The most protective antibodies (F26F2 and F26G3) were identified as potential candidates for further development.

A recombinant enzyme called capsule depolymerase (CapD) has been explored as an alternative to antibody-based therapy (Scorpio *et al.*, 2007; Scorpio *et al.*, 2008). CapD functions to degrade the capsule and remove it from the surface of *B. anthracis* cells. Pretreatment of mice with CapD significantly enhances macrophage phagocytosis and neutrophil killing of encapsulated *B. anthracis* cells (Scorpio *et al.*, 2007). CapD confers significant protection against anthrax by promoting *in vivo* phagocytic killing of encapsulated *B. anthracis* cells in mice (Scorpio *et al.*, 2008).

Botulism

For a brief summary of the type, mode/mechanism of action and signs and symptoms of botulism, please refer to Table 1.

Current prophylaxis for botulism

There are no licensed vaccines against botulism currently available to the public. However, the CDC are investigating a pentavalent botulinum toxoid (PBT) vaccine. PBT is derived from formalin-inactivated, partially purified botulinum neurotoxins A–E (BoNT/A–E) (Dembek *et al.*, 2007). The PBT dosing schedule includes a primary series of four injections (0.5 mL at 0, 2, 12 and 24 weeks) followed by annual boosters (Smith and Rusnak, 2007). To date, PBT has been administered to 20 000 at-risk laboratory personnel and 8000 military personnel (Bossi *et al.*, 2004b; Dembek *et al.*, 2007). Studies have indicated PBT to be safe with mild and self-limiting local reactions (Dembek *et al.*, 2007).

The existing PBT (formalin-inactivated toxoid) has several drawbacks that prevent its use in mass vaccination. It is expensive as it requires cultivation of live *Clostridium botulinum* bacteria and there are technical difficulties as formalin inactivation tends to induce structural changes in the toxin itself, thereby implying that immunization may result in low levels of neutralizing antibodies. Jones *et al.* (2008) described for the first time a simple, less-time consuming and novel method for producing an inactivated toxoid that is structurally and antigenically similar to the native toxin. The resulting toxoid exhibits reduced neurotoxic activity by at least seven orders of magnitude, and is capable of raising *in vivo* antibody levels to 600 times those produced by formalin-inactivated toxoids (Jones *et al.*, 2008).

Recombinant protein techniques may be utilized to develop more cost-effective vaccines. Recombinant botulinum neurotoxin heavy chain (HC) has been expressed in *Escherichia coli* and *Pichia pastoris* and assessed for protection against BoNT-induced death. Both studies showed that mice immunized with recombinant HC are protected against challenge with active BoNT. Sera from vaccinated mice also contained high titres of neutralizing antibodies (Webb *et al.*, 2007; Yu *et al.*, 2007a; Yu *et al.*, 2009). Recombinant HC was highly immunogenic and produced high levels of antitoxin antibodies that were effective and may be used as co-treatment for botulism.

The development of a non-catalytic recombinant form of botulinum toxin has also been investigated as a vaccine candidate (Willis *et al.*, 2008). As an example, BoNT/A^{RYM}, a recombinant BoNT/A with two single-point mutations (R365A and Y365F) on the light chain domain renders toxins inactive by cleaving off SNAP-25. BoNT/A^{RYM} has been shown to be highly immunogenic as a vaccine (Pier *et al.*, 2008). Animal studies on mice have shown that when immunized with BoNT/A^{RYM} (1.0 μg), mice survived subsequent challenges of 10 000 times that of the BoNT/A LD_{50} (Pier *et al.*, 2008).

DNA-based vaccines have also been studied as, in contrast to recombinant HC subunit vaccines, they are simple to produce and purify and are easy to store. Plasmid DNA replicon vectors derived from alphaviruses such as Sindbis (SIN) virus and Semliki Forest virus (SFV) are used to boost immune responses (Yu *et al.*, 2007b). The immunogenicity of a plasmid DNA replicon vaccine (pSCARSHc) encoding the HC domain of BoNT/A was compared with a conventional plasmid DNA vaccine (pcDNASHc) encoding the same antigen. pSCARSHc showed greater induction of BoNT/A HC-specific antibodies in mice, resulting in greater protection against BoNT/A than pcDNASHc. The potential use of an adenovirus-vectored recombinant vaccine based on the BoNT/C HC has also been investigated (Zeng *et al.*, 2007). The vaccine elicited a robust immune response against BoNT/C over a prolonged period. This indicated that both types of vaccines can provide protection against BoNTs in mice to varying degrees, and therefore are promising vaccine candidates for botulism.

Current treatment for botulism

Treatment for botulism includes antitoxin therapy and supportive care. Supportive care often includes

mechanical ventilation and feeding by enteral tube. The duration of care varies from a few days to 2–6 weeks (or longer) in severe cases (Dembek *et al.*, 2007). Effective antitoxin therapy can be achieved when it is administered to patients within 24 h of patients exhibiting neurologic signs of botulism (Dembek *et al.*, 2007). The neutralizing antibodies prevent further progression of paralysis by binding to the toxin itself, thereby preventing it from binding to presynaptic membrane receptors. This timely administration of antitoxin therefore minimizes the severity of the disease.

Currently, there are two FDA-approved antitoxin products available: (i) the bivalent botulinum equine antitoxin (BoNT/A and BoNT/B; CDC); and (ii) the human botulism immune globulin [Baby-BIG; California Department of Health Services (CDHS)], for adults and infants, respectively (Dembek *et al.*, 2007). Baby-BIG is the product of pooled plasma from adults immunized with PBT followed by subsequent development for high titres of neutralizing antibodies against BoNT/A and BoNT/B. Therefore, in contrast to adult antitoxin product, Baby-BIG does not pose a risk to anaphylaxis or life long hypersensitivity to equine antigens (Dembek *et al.*, 2007). An investigational human botulinum immune globulin against toxin serotype E is also currently available from CDHS, while CDC has an investigational equine antitoxin product for BoNT/E. With declining titres for BoNT/E, CDC no longer provides the previously available trivalent equine botulinum antitoxin (for BoNT/A, BoNT/B and BoNT/E) (Dembek *et al.*, 2007).

The US Army Medical Research Institute of Infectious Diseases (USAMRIID) currently has two investigational equine antitoxin preparations that target all seven toxin serotypes that produce botulism: (i) equine heptavalent botulinum antitoxin (known as HE-BAT); and (ii) equine heptavalent botulinum antitoxin $F(ab')_2$. To reduce hypersensitivity reactions, the constant domains (Fc fragment) of the equine immunoglobulin molecule are cleaved off. Studies show both heptavalent antitoxin products to be protective *in vivo* (in mice and non-human primates) and *in vitro* with products able to neutralize all seven serotypes (Dembek *et al.*, 2007). Both products would be considered for the treatment of botulism in the case of accidental or intentional incidents.

Side effects of equine antitoxin polyclonal antibodies (pAbs) include serum sickness and hypersensitivity reactions. Other challenges associated with these drugs include limited supply, batch-to-batch variation and importantly, the potential risk of infectious disease transmission, making antitoxins less effective as a prophylaxis treatment for post-exposure populations. Neutralizing monoclonal antibodies (mAbs) have proven to be valuable therapeutic agents when compared with pAbs. mAbs can be produced in unlimited supply, do not require a source of immune donors, are consistent from batch to batch and have no infectious risk.

Cholera

For a brief summary of the type, mode/mechanism of action and signs and symptoms of cholera, please refer to Table 1.

Current prophylaxis for cholera

Prophylactic treatments for cholera have not changed significantly in recent decades. The provision of clean water and proper sanitation are still regarded as the most appropriate measures for cholera control.

There are currently three oral vaccines available for cholera, namely:

1 The WC/rBS vaccine (also known as Dukoral) that is the only internationally available vaccine (licensed in approximately 20 countries) and consists of killed whole-cell *Vibrio cholerae* O1 with purified recombinant B subunit of the cholera toxin (ctx). This vaccine is most commonly administered to travellers; however, not all countries advocate this practice, including the USA. It is administered as two doses given 1–6 weeks apart and provides ~85% efficacy 6 months after immunization. Efficacy and duration of immunity can vary considerably between individuals as it has been known to last up to 3 years in some individuals (World Health Organization, 2008).
2 A variant of the WC/rBS vaccine containing no recombinant B subunit has been licensed in Vietnam and provides similar protection (66%, 10 months). This vaccine is cheaper to produce due to the absence of the recombinant B subunit and an improved formulation is currently undergoing phase III clinical trials in India (Anh *et al.*, 2007).
3 The CVD 103-HgR vaccine (previously and currently known as Orochol and Mutacol, respectively) is a live, attenuated, single-dose vaccine with a protection rate of 95% to *V. cholerae* Classical and 65% to *V. cholerae* El Tor, 3 months after vaccination. Although this vaccine is licensed in some countries, its long-term efficacy remains uncertain (World Health Organization, 2008).

Development of vaccines for cholera is challenging as there are no other animal species who are natural hosts for cholera, nor do they show

significant disease symptoms. Infant mice are susceptible to infection and are used as models for evaluation of potential vaccines, but this only allows for the study of short-term, passive immunity (Nygren *et al.*, 2008). Consequently, vaccine development for cholera has been slow, despite intensive research efforts.

Peru-15, a live, single-dose vaccine manufactured as CholeraGarde by Celldex Therapeutics (previously AVANT Immunotherapeutics), is currently being evaluated in phase II clinical trials (Qadri *et al.*, 2007; Sharma *et al.*, 2008a; http://clinicaltrials.gov/show/NCT00741637). This vaccine is active against the prevalent pandemic strain (biotype El Tor), and studies to date have found it to be safe, immunogenic and protective. The major advantage of Peru-15 is its administration as a single dose, which makes it easier for mass administration.

Intra-nasal administration has been demonstrated to be an effective route of administration for vaccines. Vaccination (i.n.) using live and killed *V. cholerae* without adjuvants has been shown to elicit strong serum and intestinal immune responses in mice (Nygren *et al.*, 2008). Similarly, a single dose (i.n.) of the variant of *V. cholerae* strain, IEM108 (IEM109), confers full protection in rabbits (Yan *et al.*, 2007).

Several *V. cholerae* proteins have immunogenic effects in animals. Mice immunized (i.n., i.g. and i.p.) with the outer membrane vesicles of *V. cholerae* were found to have long-term immunity that was also passed on to offspring (Schild *et al.*, 2008). Hence, these proteins could be potential vaccine candidates for *V. cholerae* and for other bacteria that produce similar proteins. In addition, cellular immune responses elicited by *V. cholerae* flagellin proteins (flaA–flaE) highlight the importance of these proteins in inducing inflammatory responses, thereby informing approaches for the development of future vaccines (Harrison *et al.*, 2008).

The ctx is the main cause of severe disease symptoms and is therefore the target of neutralizing antibodies in immune hosts. Typically, subunit B of this toxin (ctxB) is a useful antigen in vaccines, but it is required in large quantities to elicit a sufficient immune response. The recombinant production of this toxin may be used in vaccines; however, technical difficulties and cost make implementation of the WC/rBS vaccine in poor countries a challenge. Consequently, two genetically engineered strains of *V. cholerae* expressing increased amounts of the ctx were developed (Rhie *et al.*, 2008a,b). A recombinant strain, O395-N1-E1, was found to induce neutralizing antibodies in vaccinated mice and may be an alternate strain for the development of a whole-cell killed vaccine (Rhie *et al.*, 2008a). The protection afforded by these strains is yet to be determined.

Subunit B of the cholera toxin expressed in transgenic rice plants has been shown to be cheap to produce and highly stable at room temperature and is therefore being considered as a potential 'edible vaccine' (Oszvald *et al.*, 2008; Yuki and Kiyono, 2008). MucoRice, the Japanese vaccine candidate, induced systemic and mucosal immunity, remained stable at room temperature for up to 1.5 years and was protected from pepsin digestion *in vitro* (Yuki and Kiyono, 2008). Similarly, transgenic tomatoes expressing the ctx, or a combination of the ctx and the accessory colonization factor subunit A (acfA) or the toxin co-regulated pilus subunit A (tcpA), were produced (Jiang *et al.*, 2007; Sharma *et al.*, 2008a,b). Mice fed transgenic tomatoes expressing the ctx displayed serum and mucosal immunity (Jiang *et al.*, 2007). The protective immunity of these candidates is yet to be determined.

Previous studies have observed a notable level of cross-protective immunity generated by the *E. coli* heat-labile toxin and the ctx. This is due to the homology (~80%) that these proteins share. Transgenic carrots expressing the *E. coli* heat-labile toxin were fed to mice and resulted in systemic and intestinal immunity to the ctx (Rosales-Mendoza *et al.*, 2008). 'Edible vaccines' are still at the infantile stage of development, but demonstrate the potential value of transgenic foods in vaccine development.

Current treatment for cholera

The mainstay treatment of cholera is rehydration therapy (p.o. or i.v.), which often results in full recovery without the need for further treatment. Antibiotics will assist in reducing the duration and severity of illness, but this regime is not implemented in large outbreak scenarios due to cost and the development of antimicrobial resistance. Tetracycline has been the traditional antibiotic of choice for the treatment of cholera; however, widespread resistance has led to more prevalent use of doxycycline, ciprofloxacin, erythromycin, chloramphenicol and azithromycin. Consequently, resistant strains to these antibiotics have also emerged and epidemiological surveillance of circulating strains is of utmost importance in the selection of appropriate antibiotic treatments.

Research on alternative compounds that have antimicrobial activity have been undertaken to combat antibiotic resistance. Peptides derived from granulysin (a protein naturally activated in human killer cells and T lymphocytes in response to infection) have demonstrated anti-*V. cholerae* properties *in vitro* and in suckling mice (da Silva *et al.*, 2008).

Toxicity studies on human peripheral blood mononuclear cells show these peptides to have low *in vitro* toxicity while sialyloligosaccharides, which mimic the cell receptor to which ctx binds, efficiently inhibit ctx (Sinclair *et al.*, 2008). These non-digestible compounds may be useful for cholera management as prophylaxis administered in food and drink. Europium III chloride, a molecule that has low oral toxicity, inhibits the binding of the ctx to receptors (Williams and Jenkins, 2008) while efflux pump inhibitors have been shown to effectively increase *V. cholerae* susceptibility to antibiotics and decrease production of virulence factors (Bina *et al.*, 2009). Thus, these compounds may have future pharmaceutical applications in the treatment of cholera without the use of antibiotics.

Although the benefits of zinc supplementation in patients with acute or persistent diarrhoea have been known since the 1970s, the impact of zinc supplementation on severe diarrhoea, such as that caused by *V. cholerae* had not previously been studied. Trials on zinc supplementation in children with cholera demonstrate that zinc supplementation can significantly reduce the duration of diarrhoea and stool output during cholera (Roy *et al.*, 2008). This suggests that zinc may be administered as a cheap and effective therapeutic for the treatment of cholera and may be particularly useful in situations where rehydration therapy or antibiotics are not immediately available.

The beneficial effect of leaves from the neem plant (*Azadirachta indica*) on gastrointestinal disorders, such as infection with *V. cholerae*, is widely known in indigenous Indian populations. Neem leaf extract was shown to have significant antibacterial, antisecretory and antihaemorrhagic properties in mice infected with *V. cholerae* (Thakurta *et al.*, 2007).

Equine encephalitis viruses

For a brief summary of the type, mode/mechanism of action and signs and symptoms of viral encephalitis infections, please refer to Table 1.

Current prophylaxis for equine encephalitis viruses

There are currently no licensed vaccines available for any of the equine encephalitis viruses (EEV). Vaccines with investigational new drug (IND) status are available for laboratory personnel at risk. They include a live attenuated vaccine for Venezuelan EEV (VEEV) and inactivated vaccines for Eastern EEV (EEEV) and Western EEV (WEEV). However, immunogenicity with these vaccines is often poor and immunity is short-lived.

The role and function of viral structural proteins, particularly the capsid protein, are important in the development of attenuated vaccines. The capsid proteins of EEEV, VEEV and presumably, WEEV, play a part in the inhibition of cellular transcription and the evasion of the host IFN response (Aguilar *et al.*, 2007; 2008b; Garmashova *et al.*, 2007). Attenuation of the TC-83 VEEV vaccine was achieved by replacing the N-terminus of the capsid protein with the SIN virus counterpart (Garmashova *et al.*, 2007). In addition, further elucidation of the complex role that structural and non-structural protein (NSP) genome regions of EEEV play in neurovirulence has provided insight into the future development of live attenuated vaccines and anti-virals (Aguilar *et al.*, 2008a).

Chimeric vaccines utilize a SIN virus backbone expressing either EEEV or VEEV structural proteins. Mice vaccinated with a SIN/EEEV vaccine were protected against live virus challenge and developed high titres of neutralizing antibodies (Wang *et al.*, 2007a). This vaccine has also been shown to have reduced infectivity in mosquitoes, with the impact on dissemination and potential transmission being mosquito species-specific (Arrigo *et al.*, 2008). A SIN/VEEV vaccine has been further evaluated in immunodeficient mice and shown to be highly effective (Paessler *et al.*, 2007). A chimeric VEEV vaccine based on an equine herpesvirus type 1 has been shown to be genetically stable and conferred full protection to mice from lethal challenge with VEEV (Rosas *et al.*, 2008). Further evaluation of the chimeric vaccines in mice may provide the opportunity to identify prognostic markers that correlate with protection against encephalitis.

Adenovirus-based vaccines, such as the human adenovirus serotype 5 (Had5)-vectored WEEV vaccine (Wu *et al.*, 2007a), have been shown to elicit a rapid, long-lasting and robust immune response in mice (Barabe *et al.*, 2007). Mice were protected from challenge with both homologous and heterologous strains of WEEV, including the highly virulent Fleming strain. One of the concerns with adenovirus-vectored vaccines is that existing immunity to the vector can be detrimental for homologous boosting. Therefore, it should be noted that co-administration of adenovirus-vectored VEEV vaccine and CpG can augment antibody responses; however, this is not the case for the transgene product (Perkins *et al.*, 2008).

The development of attenuated VEEV vaccines by mutating the promoter elements in the VEEV genome, without affecting the amino acid sequence of the NSP, has been demonstrated (Michel *et al.*, 2007). V3526 is a live attenuated VEEV vaccine derived by site-directed mutagenesis from a full-

length infectious VEEV isolate. It has been shown to be safe and efficacious in horses (Fine *et al.*, 2007), is not significantly more neurovirulent than TC-83 when tested in non-human primates (Fine *et al.*, 2008) and is able to protect mice from infection of VEEV via mosquito bite as well as i.p. challenge (Charles *et al.*, 1997; Rao *et al.*, 2006). This approach to vaccine development should be conducted with caution as it can produce rapid adoption of mutations in the NSP. Some of the adaptations were observed after only a few rounds of infection, highlighting the plasticity of these viruses and their evolutionary potential. Indeed, this may limit their potential as vaccine candidates against EEV.

A vaccine candidate developed by irradiating 1,5-iodonaphthylazide (INA)-treated VEEV, has been shown to protect mice from lethal challenge. This process inactivates the virus completely and causes the INA to selectively bind to transmembrane proteins in the viral envelope (Sharma *et al.*, 2007). This research may be useful in developing a vaccine that is immunogenic and free from residual virulence.

Interferon is a cytokine involved in the host defence mechanism against viral infection. Strategies to boost the levels of IFN in the host have been investigated to determine if increased IFN levels can be protective against challenge. A hamster model for WEEV infection has been developed to study the effects of exogenous IFN (alfacon-1) and an IFN inducer (Ampligen®) on WEEV disease. The survival rate was 100% when both were administered prophylactically 4 h before viral challenge. Alfacon-1-treated animals had a significantly reduced brain viral titre, with virus loads below the limits of detection. Ampligen® also produced a significant reduction in brain viral titre, despite virus being detected in 60% of animals. In addition, a HAd5 vector expressing murine IFN administered 24 h–1 week prior to WEEV challenge protected mice (Wu *et al.*, 2007b). This vaccine also delayed the progression of WEEV infection and partially protected mice when administered 64 h post lethal challenge.

Despite affording only partial protection to mice administered a lethal challenge of WEEV, a recombinant E2 protein of WEEV has elicited strong humoral and cell-mediated responses when used to immunize mice (Das *et al.*, 2007). Thus, recombinant techniques may also be useful in the development of EEV vaccines.

Current treatment for equine encephalitis viruses

There are no antiviral therapeutics available for the viral encephalitis. Supportive care via i.v. fluids, a respirator, sedatives, analgesics, corticosteroids to reduce brain swelling and anticonvulsants to treat seizures are recommended.

Most antiviral therapy focuses on the inhibition of viral replication, such as RNA interference. This cellular process involves short interfering RNAs (siRNAs) and small double-stranded RNA molecules directing the degradation of complementary messenger RNAs. A combination of four siRNAs targeting conserved sequences of divergent VEEV strains has been demonstrated to inhibit replication of six strains of VEEV *in vitro* (O'Brien, 2007). Interestingly, this work raises the issue of the development of resistance to siRNA as one strain was able to confer resistance to siRNAs.

Peptide-conjugated phosphorodiamidate morpholino oligomers (PPMO) inhibit viral replication through sequence-specific steric blockade of viral RNA. Mice receiving PPMO before and after challenge with VEEV all survived and did not have any detectable virus 2–4 days post challenge. In contrast, mice receiving PPMO only post challenge had partial protection and viral titres in tissue samples were reduced (Paessler *et al.*, 2008).

Antiviral agents that inhibit viral replication were evaluated as treatments for VEEV infection using a lethal mouse model (Julander *et al.*, 2008b). (-)Carbodine was shown to be effective in improving disease parameters even when administered up to 4 days post infection (Julander *et al.*, 2008a). Further, a novel 3-sulphonamido-quinazolin-4(3H)-one derivative, given at a dose below the cytotoxic concentration, was moderately active in inhibiting the replication of VEEV *in vitro* (Selvam *et al.*, 2007).

Identifying agents that interfere with viral assembly is an approach that may provide a new source of antiviral agents. RNA thioaptamers are small RNA sequences that bind to proteins. Several thioaptamers were isolated by *in vitro* combinatorial selection and assessed for their binding affinity to the capsid protein of VEEV. One thioaptamer was shown to have a high binding affinity and specificity for the capsid protein (Kang *et al.*, 2007).

Melatonin has been shown to be effective in treating VEEV infection by decreasing nitrite and lipid peroxidation generated by VEEV infection and increasing IL-1β (a cytokine capable of increasing inducible nitric oxide synthase production) in the brain (Valero *et al.*, 2007). However, the inhibitory effect (if any) of melatonin on VEEV replication is yet to be determined.

A mouse anti-VEEV antibody has been humanized to combat the problem of a human anti-mouse antibody response. The humanized antibody binds to VEEV E2 protein in a dose-dependant manner, and demonstrated strong neutralizing capacity in a standard plaque reduction assay (Hu *et al.*, 2007).

This antibody is likely to be further evaluated as an immunotherapeutic agent against VEEV.

Melioidosis

For a brief summary of the type, mode/mechanism of action and signs and symptoms of melioidosis, please refer to Table 1.

Current prophylaxis for melioidosis

Currently there is no licensed vaccine available for melioidosis. Trimethoprim sulphamethoxazole (TMP-SMX; p.o.) is recommended for post-exposure prophylaxis in the case of a biological attack (Bossi *et al.*, 2004c), and for immunosuppressed patients, as melioidosis is more common and more severe in these individuals (Davis *et al.*, 2003).

Melioidosis prophylaxis focuses on improving our knowledge of the natural history of infection of *Burkholderia pseudomallei* in both animal models and the human host. This includes identification of potential virulence factors using bioinformatics (Haraga *et al.*, 2008) and signature-tagged mutagenesis (Cuccui *et al.*, 2007). It also involves further characterization of protective immune responses, including induction of cell-mediated immunity (Barnes and Ketheesan, 2007), the bactericidal function of macrophages (Barnes *et al.*, 2008), the role of neutrophils in infection (Easton *et al.*, 2007) and the relationship between route of immunization and protective Th1 inflammatory responses (Tan *et al.*, 2008).

A number of antigens have been identified and assessed as potential vaccine candidates. Defence Science and Technology Laboratories (Dstl, UK) have identified 35 *B. pseudomallei* surface proteins using a biotinylation approach and have screened them for immunogenicity using sera from convalescent melioidosis patients (Harding *et al.*, 2007). Nine of these proteins were considered immunoreactive and will be further evaluated as vaccine candidates (Harding *et al.*, 2007). In addition, several proteins of the ATP-binding cassette system of *B. pseudomallei* have been used to immunize Balb/c mice, with significant protection from challenge seen with two of these proteins, LolC and PotF (Harland *et al.*, 2007). LolC also demonstrated enhanced protection when complexed with CpG in adjuvant ISCOM (Harland *et al.*, 2007).

Other vaccine strategies include immunization with attenuated live mutants and low-dose, live immunization. Although immunization with attenuated mutants provided some protection from acute i.p. and i.n. challenge, it did not improve survival against chronic melioidosis nor i.v. infection (Breitbach *et al.*, 2008). Immunization of mice with low concentrations of live *B. pseudomallei* demonstrated enhanced delayed-type hypersensitivity responses when compared with immunization using heat-killed *B. pseudomallei* in complete Freud's adjuvant (Barnes and Ketheesan, 2007).

Murine mAbs specific to *B. pseudomallei* have been used to generate peptide mimotopes. These peptides, conjugated with thyroglobulin and used to immunize Balb/c mice, were shown to delay the time to death in these animals following challenge with live *B. pseudomallei* (Legutki *et al.*, 2007).

Evaluation of amoxicillin/clavulanic acid, doxycycline and co-trimoxazole (p.o.) as both pre- and post-exposure prophylaxis has been performed in Balb/c mice 48 h prior to aerosol challenge, and at 0, 10, 24, 48 h and up to 10 days post challenge (Sivalingam *et al.*, 2008). All animals succumbed to infection if the antimicrobial was administered 48 h or later following exposure. However, when co-trimoxazole was administered within 24 h post infection, all mice survived. As pre-exposure prophylaxis, doxycycline and co-trimoxazole had survival rates of 80% and 100% respectively. All animals receiving amoxicillin/clavulanic acid succumbed to challenge, suggesting this drug is not suitable as a prophylactic against melioidosis (Sivalingam *et al.*, 2008).

Current treatment for melioidosis

The current recommended treatment for severe melioidosis is i.v. administration of ceftazidime or a carbapenem, with or without TMP-SMX, for 10–14 days or longer as clinically indicated (Cheng and Currie, 2005). Meropenem, imipenem and cefoperazone-sulbactuam are also active against *B. pseudomallei* (Simpson *et al.*, 1999; Chetchotisakd *et al.*, 2001; Cheng *et al.*, 2004). TMP-SMX (p.o.) for 12–20 weeks, with or without doxycycline is recommended (Cheng and Currie, 2005). Amoxicillin-clavulanate is an alternative for pregnant women, children and patients with intolerance to the first line therapy but is much less effective (Suputtamongkol *et al.*, 1994). At the Royal Darwin Hospital, the initial phase of treatment for patients with melioidosis-induced septic shock is meropenem plus granulocyte colony-stimulating factor (G-CSF) (Cheng and Currie, 2005).

In vitro antimicrobial susceptibility testing was performed on *B. pseudomallei* isolates collected in Malaysia between 1978 and 2003. The organisms were assessed for susceptibility to imipenem, meropenem, ertapenem, moxifloxacin and azithromycin using the *E*-test minimum inhibitory concentration method. All isolates were found to be sensitive to meropenem and imipenem; however, the majority

of strains were found to be resistant to ertapenem and azithromycin and moxifloxacin, indicating that these drugs should not be used for treatment of melioidosis (Karunakaran and Puthucheary, 2007).

A Taiwanese patient presenting with life-threatening melioidosis, including acute respiratory distress, septic shock and organ dysfunction, was successfully treated with a combination of meropenem and recombinant-activated protein C (rhAPC). Although rhAPC has been reported to reduce septic shock caused by a number of pathogens, this is the first report of rhAPC being used to treat life-threatening melioidosis (Tan *et al.*, 2007).

Novel therapeutics has involved the development of a phage-displayed non-immune human single-chain Fv (scFv) antibody library against whole-killed *B. pseudomallei*, and screening of this library has identified seven different phage scFv antibodies that interact with the organism (Zou *et al.*, 2007). In addition, Defence Science and Technology Organisation (DSTO) has performed panning experiments using phage peptide libraries and shark antibody libraries for potential binders that recognize *B. pseudomallei* OmpA (J. McAllister, pers. comm.) and these are now able to be evaluated as potential therapeutic reagents.

Plague

For a brief summary of the type, mode/mechanism of action and signs and symptoms of plague, please refer to Table 1.

Current prophylaxis for plague

The recommended antibiotic for secondary post-exposure prophylaxis is 100 mg of doxycycline (child >8 years: 2.5 mg·kg^{-1} up to 100 mg) twice a day, or 300 mg ciprofloxacin i.v. twice a day for 9 days, followed by 500 mg (child: 15 mg·kg^{-1} up to 500 mg; p.o.) for an additional 6 days, twice a day. The World Health Organisation (WHO) also recommends 1–2 g·day^{-1} tetracycline (p.o.) at 6 or 12 hourly intervals, or 1.6 g·day^{-1} sulphamethoxazole or trimethoprim (p.o.) at 12 hourly intervals (p.o.) (Poland, 2009).

To improve the efficacy of the current plague subunit vaccine, protective fraction 1 capsular antigen (F1) and the virulence multifunctional LcrV antigen (V) that resides at the tips of type III needle complexes (Baker *et al.*, 1952; Lawton *et al.*, 1963) are currently being engineered as recombinant F1-V proteins (Andrews *et al.*, 1996; Heath *et al.*, 1998; Williamson, 2001; Powell *et al.*, 2005; Goodin *et al.*, 2007). The F1-V fusion proteins together with alum-based delivery have been demonstrated by Dstl and USAMRIID to protect mice against pulmonary *Yersinia pestis* challenge. Currently both vaccines have entered phase I clinical trials and have been reported to be safe, well-tolerated and immunogenic (Heath *et al.*, 1998; Jones *et al.*, 2000; Williamson *et al.*, 2005; Morris, 2007).

Further studies show that formulation of F1-V proteins with adjuvant induces strong humoral and cellular immunity (Glynn *et al.*, 2005; Jones *et al.*, 2006). Four different adjuvants: heat-labile enterotoxin (LT) (R192G), CpG ODN, MPL®TDM and alum, have been administered in combination with recombinant F1-V protein, and have all effectively induced type 1/type 2 antibody responses. The magnitude of antibody response was evaluated in mice immunized via i.n., transcutaneous (t.c.) and s.c. routes. High levels of anti-F1-V $IgG_{1\text{-}2a}$ in both the serum and bronchioalveolar lavage (BAL) were observed with s.c. route producing the greater response (Uddowla *et al.*, 2007). Similarly, a recombinant F1-V protein coupled to a synthetic lipid, a mimetic known as amino-alkyl glucosaminide 4-phosphate (AGP), augmented cell-mediated T_H1 immune responses in rats challenged with lethal *Y. pestis* strain C092. The AGP-based vaccine was administered according to a primary/secondary i.n. prime/boost regime and demonstrated that an initial immunization on day 1 followed by another on day 3, protected 63% of rats by day 7, subsequently achieving 100% protection by 21 days (Airhart *et al.*, 2008).

A bicistronic DNA vaccine (i.n.) co-expressing F1-V fusion protein and a molecular adjuvant, IL-12, protected mice against *Y. pestis* challenge, in contrast to mice immunized with F1-V protein alone. Prime vaccination consisted of a low concentration of the DNA vector coding for IL-12 in conjunction with F1-V. Animals were subsequently boosted with recombinant F1 protein that provided protection from pneumonic plague (Yamanaka *et al.*, 2008). The protective efficacies of flagellin adjuvant fused with F1-V protein and a plant-based oral plague vaccine without adjuvant, against respiratory challenge with *Y. pestis* CO92 have been demonstrated in mice and non-human primates (Mett *et al.*, 2007; Arlen *et al.*, 2008; Mizel *et al.*, 2009).

The enhanced effectiveness of adjuvants in the development of plague vaccines against bubonic and pneumonic plague is evident. However, the major limitation to subunit vaccines is the necessity to administer multiple high dosages to produce robust and prolonged immune protection. With the emergence of *Y. pestis* antibiotic-resistant strains, the development of improved prophylactic approaches are of utmost importance. The

discovery of strains lacking the F1 antigen is an important factor that must be considered in the design of new vaccines.

Yersinia pestis and *Y. pseudotuberculosis* share a close evolutionary relationship. An attenuated *Y. pseudotuberculosis* strain (IP32680; p.o.) was used to immunize mice against bubonic plague and resulted in high antibody responses and protection of 75% (after first dose) and 88% (after second dose) of mice with no obvious adverse effects compared with animals immunized with the live attenuated *Y. pestis* strain EV76 (Blisnick *et al.*, 2008). A mutant live vaccine was constructed by a deletion-insertion in the *lpxM* gene of *Y. pestis* EV NIIEG strain, denoted as *Y. pestis* EV*ΔlpxM*. High protective efficacy of single dosing of *Y. pestis* EV*ΔlpxM* was demonstrated in mice and guinea pigs (Bubeck and Dube, 2007).

An attenuated *Salmonella enterica* strain is frequently employed as a live vaccine vector encoding recombinant F1-V proteins. Vaccination with recombinant F1-V proteins (p.o.) induced specific F1-V specific IgG and IgA antibody titres that protected mice against *Y. pestis* challenge. Oral immunizations provided >80% protection from 1000 × LD_{50} bubonic plague and 100 × LD_{50} of pneumonic plague in mice. Hence, this attenuated *Salmonella*-based vaccine has potential as a plague vaccine (Liu *et al.*, 2007; Yang *et al.*, 2007).

Operons *yadB* and *yadC* operons have also demonstrated novel potential in the plague F1-V vaccine. These virulence factors assist bacterium in the invasion of epithelioid cells. Mice immunized with *yadC* elicited specific IgG_1 antibody responses, and the release of pro-inflammatory cytokine was also observed (Murphy *et al.*, 2007; Forman *et al.*, 2008). Similarly, anti-translocon antibodies YopB, YopD, or a complex of YopBDE, protected mice against lethal challenge with F1-*Y. pestis* indicating that mABs specific for F1, V antigens and the Yop translocon may be useful prophylactic or therapeutic approaches (Eyles *et al.*, 2007; Ivanov *et al.*, 2008).

Vaccines from adenovirus vectors encoding the anti-V antigen produce strong immune responses resulting in 93.3% protection following i.n. *Y. pestis* challenge (Sofer-Podesta *et al.*, 2009). Recombinant V10 protein showed immunogenicity and protected cynomolgus macaques upon challenge with aerosolized pneumonic plague (Cornelius *et al.*, 2008). These studies confirmed that humoral immunity plays an important role in preventing the development of this disease, and recent research has illustrated that cellular immunity also contributes to protection against plague (Philipovskiy and Smiley, 2007; Kummer *et al.*, 2008).

Current treatment for plague

The CDC recommends gentamicin to treat plague patients with 3 to 7.5 $mg \cdot kg^{-1} \cdot day^{-1}$ via i.m. or vascular administration at 8 h intervals. The most effective antibiotic for the treatment of plague, specifically the pneumonic form is streptomycin at 30 $mg \cdot kg^{-1} \cdot day^{-1}$ up to a total of 2 $g \cdot day^{-1}$ every 12 h (i.m.). Alternative antibiotics for specific therapy include: chloramphenicol at 50 $mg \cdot kg^{-1} \cdot day^{-1}$ p.o. or i.v. at 6 h intervals for 10 days (suitable for bubonic and pneumonic plague); tetracyclines 15 $mg \cdot kg^{-1}$ p.o. followed by 25 to 50 $mg \cdot kg^{-1} \cdot day^{-1}$ for 10 days; or ciprofloxacin 400 mg and doxycycline delivered 200 $mg \cdot day^{-1}$ p.o. at 12 or 24 h intervals (Poland, 2009).

Q fever

For a brief summary of the type, mode/mechanism of action and signs and symptoms of Q fever infection, please refer to Table 1.

Current prophylaxis for Q fever

Post-exposure prophylaxis for adults and pregnant or breastfeeding women is a week of either: doxycycline 100 mg p.o. every 12 h, erythromycin 500 mg p.o. every 3 h, clarithromycin 500 mg p.o. every 12 h or roxithromycin 150 mg p.o. every 12 h (Pettineo *et al.*, 2009). Children should receive treatment with the same antibiotic for 1 week at the following doses: doxycycline 100 mg p.o. every 12 h for children up to 8 years of age and weighing >45 kg or 2.2 $mg \cdot kg^{-1}$ p.o. every 12 h if <45 kg; erythromycin 500 mg p.o. every 3 h for children >35 kg or 50 $mg \cdot kg^{-1}$ p.o. every 12 h if <35 kg; clarithromycin 500 mg p.o. every 12 h if >40 kg or 7.5 $mg \cdot kg^{-1}$ p.o. every 12 h if <40 kg; or roxithromycin 8 $mg \cdot kg^{-1}$ p.o. every 12 h (Pettineo *et al.*, 2009).

A whole-cell Q fever vaccine (Q-Vax), consisting of the formalin-inactivated Henzerling strain, is currently licensed for use in Australia, although prescreening for prior immunity is required to prevent adverse reactions due to egg hypersensitivity (Marmion, 2007). An unlicensed, purified Henzerling strain whole-killed vaccine administered via an i.n. device is also available through USAMRIID to immunize occupationally at-risk individuals (Waag, 2007). A chemo-vaccine comprising extracts of *Coxiella burnetii* lipopolysaccharide and protein has also been used to vaccinate laboratory workers and some industrial groups in Eastern Europe (Marmion, 2007).

A phase I chloroform-methanol residue (CMRI) vaccine has been developed as a safer alternative to

the current whole-cell vaccines and is being assessed for safety and immunogenicity in phase I clinical trials (Waag *et al.*, 2008). No antigen-specific antibody could be detected following a single subcutaneous prime; however, after a second booster, significant levels of specific antibody were produced. Peripheral blood cells collected from individuals following the booster also responded to recall antigen *in vitro* in a dose-dependent manner, suggesting that the vaccine is able to prime the immune system to effectively respond to infection (Waag *et al.*, 2008).

A major way of preventing Q fever is through vaccination of animal hosts serving as reservoirs of infection. Immunization of cattle with a monovalent inactivated phase I vaccine has been demonstrated to significantly lessen the probability of susceptible non-pregnant cows becoming shedders of the organism. This highlights the potential of implementing vaccination among non-infected herds to prevent spread of Q fever to humans (Guatteo *et al.*, 2008).

Prophylaxis for Q fever has also focused on better characterization of the mechanisms of vaccine-induced immunity. This includes comparison of formalin-inactivated phase I and phase II vaccines in Balb/c mice. Phase I vaccines conveyed significant protection as well as Th1 dominant immune responses. Although phase II vaccines also induce Th1 immunity, they did not confer measurable protective responses, indicating that phase I lipopolysaccharide is important for host defence against *C. burnetii* (Zhang *et al.*, 2007). The effects of infecting IFN-γ and Toll-like receptor 2 knockout mice with the normally non-pathogenic phase II nine mile strain *C. burnetii* have also been investigated. The febrile response in these immunocompromised animals indicated that both IFN-γ and TLR2 are important in providing protective immunity to *C. burnetii*, and that NMII is capable of causing disease in immunodeficient animals. These results highlight the usefulness of using these animals as models for evaluating vaccine candidates and host–pathogen interactions (Ochoa-Reparaz *et al.*, 2007).

Current treatment for Q fever

Generally acute Q fever is a self-limiting mild or asymptomatic infection that resolves within 2 weeks, therefore treatment is not usually required (Tsironi *et al.*, 2005). However, antibiotic therapy may be warranted to prevent progression to chronic disease. In such cases, the recommended regime is doxycycline 100 mg twice daily for 14 days (Parker *et al.*, 2006).

For chronic Q fever endocarditis, patients should receive a combination of doxycycline (100 mg twice daily) and hydroxychloroquine (200 mg three times daily) for a period of 18 months (Maurin and Raoult, 1999). Quinolones can also be used for patients intolerant to chloroquine (Maurin and Raoult, 1999). Long-term co-trimoxazole therapy (320 mg trimethoprim and 1600 mg sulphamethoxazole for 35 days) is recommended for pregnant women to decrease the risk of placentitis, obstetric complications and maternal chronic Q fever infection (Carcopino *et al.*, 2007).

Treatment of Q fever in pregnant women with doxycycline is contraindicated. Carcopino *et al.* (2007) investigated long-term co-trimoxazole therapy as treatment for pregnant women with Q fever and found that women were protected against chronic Q fever, placental infection and obstetric complications, particularly i.u. fetal death (found to be related to placental infection), when compared with women that had not received the therapy. This finding has led to the recommendation that long-term co-trimoxazole should be used to treat pregnant women with Q fever (Carcopino *et al.*, 2007).

Interestingly, the CDC has assessed the risks and benefits of post-exposure antibiotic treatment following an intentional release of *C. burnetii*. Based on administration of doxycycline to the general population and co-trimoxazole to pregnant women, upper bound probability estimates of adverse effects to prophylaxis indicated that the risk of acquiring Q fever illness outweighed the risk of antimicrobial drug-related adverse effects (Moodie *et al.*, 2008).

Ricin

For a brief summary of the type, mode/mechanism of action and signs and symptoms of ricin intoxication, please refer to Table 1.

Current prophylaxis for ricin

There is currently no licensed ricin vaccine available. Ricin vaccine developments have previously focused on either deglycosylated ricin toxin A-chain (RTA) or formalin-inactivated toxoid (Doan, 2004). Both types of vaccine candidates have been shown to protect against aerosolized ricin poisoning in animals. However, both candidates have shortfalls; for example, formalin inactivation may not completely inactivate the toxin while deglycosylated RTA may lead to local or systemic vascular leak syndrome.

Recently, ricin vaccine development has utilized a recombinant technology approach to produce a non-toxic mutated form of ricin A-chain as a vaccine candidate. The vaccine candidate, RiVax, is a recombinant RTA in which two point mutations, Y80A and V76M, have been incorporated to remove

its toxicity and vascular leak-inducing capacity (Smallshaw *et al.*, 2002; 2005). The vaccine alone has been shown to be non-toxic and immunogenic in mice and rabbits (Smallshaw *et al.*, 2002; 2005) and the first human clinical trial examining three i.m. doses (10, 33 and 100 μg) has been completed (Vitetta *et al.*, 2006). RiVax (i.m.) has been shown to prevent ricin-induced death (up to 10 × LD_{50}) and tissue damage in mice administered ricin by either gavage or via aerosolization (Smallshaw *et al.*, 2007). These findings are relevant to our understanding of treating ricin poisoning, which is most likely to occur following either accidental or intentional contamination of food, water or air.

To improve vaccine efficacy, vaccine formulation, adjuvant use and testing of delivery methods of recombinant ricin vaccines, have been investigated. USAMRIID have developed an optimal formulation for potential vaccine candidate RTA 1-33/44-198, a stable, non-toxic and immunogenic recombinant RTA (Carra *et al.*, 2007). RTA 1-33/44-198 is a RTA derivative containing deletions of the C-terminal sub-domain and an exposed surface loop (McHugh *et al.*, 2004; Olson *et al.*, 2004). Under the new formulation, the vaccine has shown to be highly stable and potent in storage for up to 18 months and has an increased adherence to aluminium hydroxide adjuvant from 15% to 91%. *In vivo* studies in mice also reveal improvement in the vaccine's effective antigenicity by almost threefold (Olson *et al.*, 2004).

Another recombinant RTA vaccine (rRV) prepared by mutating the *E. coli* LT has been assessed in combination with a mucosal adjuvant (Kende *et al.*, 2007). Vaccination of mice (i.n.) with rRV alone offered marginal protection against aerosol-delivered 5–10 × LD_{50} of ricin toxin (Kende *et al.*, 2007). The vaccine alone was only able to stimulate low anti-ricin neutralizing antibody responses. The protection of rRV vaccination was enhanced by co-administration of the mucosal adjuvant LTK63 or LTR72, two mutants of *E. coli* LT. Enterotoxigenic *E. coli*-expressed LT has been shown to act as a potent mucosal adjuvant, thereby inducing a strong immune response against co-administered antigens (p.o., i.n. or i.p.) (Clements *et al.*, 1980; Jackson *et al.*, 1993). However, the toxicity of LT makes it unsuitable for use in human vaccines. However, site-directed mutagenesis has replaced single amino acids within the toxic subunit of LT, thus producing mutants LTK63 and LTR72 with no toxic activity. The study demonstrated that both mutants were effective and safe mucosal adjuvants that can enhance the protection of rRV against aerosol-delivered ricin challenge. However, the study also concluded that rRV with either LTK63 or LTR72 did not protect mice against ricin-related lung injuries (Kende *et al.*, 2007).

Passive prophylaxis, such as antibody therapy, has been shown to be effective in neutralizing ricin intoxication. However, it should be noted that antibody treatment is limited to a few hours following ricin poisoning (Doan, 2004). Although mAbs are effective inhibitors of ricin, with *in vitro* and *in vivo* studies demonstrating the ability of mAbs to neutralize the toxic effects of ricin (Lemley *et al.*, 1994; Furukawa-Stoffer *et al.*, 1999), murine antibodies cannot be used as therapeutics due to side effects (Miller *et al.*, 1983). To address this problem, a chimeric antibody, c4C13, has been constructed by coupling its parent murine antigen-binding variable domains to human constant domains. C4C13 has been demonstrated to neutralize the toxicity of ricin in *in vitro* assays (Wang *et al.*, 2007b), suggesting that this 'humanized' component is a more suitable therapeutic.

To reduce anaphylaxis associated with antibody therapy, despeciated $F(ab')_2$ and Fab' antibody fragments are being developed for therapeutic use. Despeciated antibody fragments are considered to be safe as human products compared with polyclonal antibody (immunoglobulin G, IgG) that contains the species-related constant region, F_c, which is likely to induce the anaphylactic reactions or other side effects. The protective efficacy of anti-ricin sheep IgG and $F(ab')_2$ administered i.v. to mice 2 h following systemic or inhalational challenge with ricin has been demonstrated (Griffiths *et al.*, 2007). The smaller Fab' fragment did not prevent death from ricin intoxication. This study demonstrates the feasibility of effective anti-ricin antibodies and the smaller, less reactogenic fragments $F(ab')_2$ for use following exposure to ricin.

Current treatment for ricin poisoning

Currently, there are no licensed therapeutic antitoxins or antidotes available for ricin intoxication (Challoner and McCarron, 1990). Patient treatment is typically supportive care of symptoms (see Table 1). For oral intoxication, supportive care includes replacement of fluids and electrolytes i.v. to maintain electrolyte balance and prevent dehydration from vomiting and diarrhoea, and monitoring of liver and renal functions. Oral activated charcoal can be considered to reduce gastrointestinal adsorption of the toxin, although adsorption of ricin by charcoal is unknown (Chyka and Seger, 1997). For inhalational exposure, supportive care may include oxygen, bronchodilators, endotracheal intubation and supplemental positive end-expiratory pressure, as required (Audi *et al.*, 2005).

RNA ligands (aptamers) specific to the catalytic RTA have been investigated *in vitro* (Hesselberth *et al.*, 2000). A 31-nucleotide RNA aptamer (31RA) has been demonstrated to bind to RTA, thereby blocking enzymatic activity of RTA (Fan *et al.*, 2008). 31RA also neutralized the effects of ricin on translation inhibition in cell-free and cell-based luciferase assays, and ricin-induced cytotoxicity in *in vitro* assays. Although therapeutic effects of aptamers in *in vivo* models of ricin intoxication are still to be determined, *in vitro* results support the potential use of anti-RTA apatamer as ricin inhibitors.

Smallpox

For a brief summary of the type, mode/mechanism of action and signs and symptoms of smallpox, please refer to Table 1.

Current prophylaxis for smallpox

The global eradication of smallpox as a natural disease poses a major limitation on the ability of scientists to analyse the efficacy and safety of new smallpox vaccines or potential therapeutic agents. Current prophylaxis for smallpox may be divided into four categories: (i) replication-competent; (ii) replication-deficient viruses; (iii) recombinant proteins; and (iv) gene-based vectors. Given that the variola virus is no longer endemic, studies on the humoral and cell-mediated immunity must be presumed from similar orthopoxvirus infections, such as vaccinia, ectromelia, cowpox and monkeypox.

In addition to Dryvax, the first approved vaccine for smallpox since 1931, a second generation live vaccinia cell culture-based vaccine (ACAM2000™) has been developed, registered and licensed in August 2007 (Monath *et al.*, 2004; Greenberg and Kennedy, 2008). ACAM2000™ is only manufactured by the US Government to vaccinate US troops. Phase III clinical trials indicate that neutralizing antibody levels ~1.5-fold lower than for Dryvax were induced in 97% of healthy and vaccinia naïve individuals following vaccination with ACAM2000™. Data also indicated that 1–5 × 10^8 CFU·mL^{-1} of ACAM2000™ was sufficient to elicit cutaneous reactogenicity, immunogenicity and protective activity comparable to Dryvax (Weltzin *et al.*, 2003; Artenstein *et al.*, 2005). Although the homogenicity of ACAM2000™ induces less neurovirulence and cutaneous reaction in animal models, the alarming incidence of post-vaccination myopericarditis is one in 175 for vaccinia-naïve recipients (Weltzin *et al.*, 2003; Frey *et al.*, 2008; Marriott *et al.*, 2008). Further clinical trials are required to better understand the complicated adverse events, such as myopericarditis, in ACAM2000™ recipients.

Modified vaccinia Ankara (MVA) is a replication-deficient and highly attenuated candidate that can be administered as an alternative vaccine against smallpox (Drexler *et al.*, 1998; Slifka, 2005). MVA was registered as a pre-immunization vaccine in combination with Lister vaccine in Germany 1976 and was evaluated in animals and humans during the WHO smallpox eradication campaign (Mayr *et al.*, 1978). The two-step vaccination noticeably reduced the severity of adverse effects, particularly in individuals with immune deficiency and atopic dermatitis, commonly seen with the traditional smallpox vaccine. Importantly, numerous preclinical and clinical studies have demonstrated the safety and high immunogenicity of MVA (Belyakov *et al.*, 2003; Earl *et al.*, 2004; 2008; Meseda *et al.*, 2005; Parrino *et al.*, 2007). However, the long-term protective efficacy of MVA requires further investigation (Ferrier-Rembert *et al.*, 2008).

IMVAMUNE® is an attenuated smallpox vaccine that is genetically stable and does not replicate in human cells. Phase I/II clinical trials in individuals at risk of complication from the first and second generation vaccinia-based vaccines have confirmed a diminishment of serious health risks (Cassimatis *et al.*, 2004; Vollmar *et al.*, 2006). Phase III clinical trials will commence shortly, although preliminary results indicate that IMVAMUNE® is well-tolerated, safe and immunogenic with no myocardial events (Frey *et al.*, 2007; Kennedy and Greenberg, 2009).

Immunogenicity and efficacy by subcutaneous immunization of mice with a combination of MVA and IL-15 (an immune enhancing cytokine) have been demonstrated. This vaccine and cytokine combination induced both humoral and cellular responses, and the antibody responses were fourfold higher than those produced in mice given MVA alone (Perera *et al.*, 2007).

The development of a potential subunit smallpox vaccine consisting of alternative variola protein candidates (H3L, L1R, A27L, A33R and B5R) has recently been investigated. Preliminary data confirms that A27L, B5R and D8L proteins produced in *E. coli* are highly immunogenic and elicit strong protective immunity against viral challenge (He *et al.*, 2007; Berhanu *et al.*, 2008; Golden *et al.*, 2008). A new approach to produce vaccinia virus B5 antigenic domain (pB5) *in planta* afforded mice protection from lethal challenge (Golovkin *et al.*, 2007). Therefore, subunit vaccines may be useful vaccine candidates for the prophylaxis of smallpox.

Current treatment for smallpox

Cidofovir (CDV, VistideTM) is currently the only licensed anti-viral drug approved by the US FDA to treat infections caused by variola and other orthopoxviruses (Jahrling *et al.*, 2005). CDV is regulated under a restricted IND protocol and is often used as the positive control in orthopoxvirus studies in mice (Lalezari *et al.*, 1997). CDV may only be administered when vaccinia immune globulin is not efficacious, although it has been shown to cause nephrotoxicity and is not orally bioavailable. In an effort to address these obstacles, four orally active lipid esters of CDV, HDP-CDV, ODBG-CDV, ODE-CDV and OLE-CDV were synthesized. HDP-CDV (CMX001, Chimerix Inc.) elicited the least nephrotoxicity and is potentially the most effective anti-viral candidate. Hence, it is in a phase I multi-dose clinical trial to evaluate its safety and efficacy (Buller *et al.*, 2004; Parker *et al.*, 2008).

ST-246 (SIGA, Inc.), an orally bioavailable anti-viral agent for orthopoxviruses, targets the F13L gene product that is required for production of extracellular virus. ST-246 is a potent vaccinia virus egress inhibitor that is effective in halting infection by orthopoxviruses. Preclinical studies have revealed the effectiveness of ST-246 (p.o.) following i.n. challenge with orthopoxvirus. In rabbits and squirrels, ST-246 treatment regime was conducted for a period of 10 to 14 days and was found to be safe and efficacious (Sbrana *et al.*, 2007; Jordan *et al.*, 2008; Nalca *et al.*, 2008). Recently, administration of ST-246(R) to non-human primates provided 100% protection with reduced viral load at 3 days post infection following lethal challenge with monkeypox virus. Phase I/II clinical trials have indicated that ST-246 is readily absorbed, well-tolerated and produces no severe adverse effects following a single dose (p.o.) of 2 g in fasting volunteers and 1 g in non-fasting volunteers (Jordan *et al.*, 2008). Hence, ST-246 is a promising candidate as an anti-viral therapeutic in the treatment of smallpox.

Moreover, combinatorial treatment of smallpox with the traditional Dryvax vaccine and ST-246 has demonstrated anti-viral synergy, eliciting protective immunity and diminishing severity of lesions both *in vitro* and *in vivo* (Grosenbach *et al.*, 2008). The combined treatment of ST-246 and CMX001 at doses of 1 and 3 $mg \cdot kg^{-1} \cdot day^{-1}$ has been shown to be synergistically effective in reducing mortality in mice following lethal challenge with vaccinia virus and cowpox virus, when compared with survival rates of animals receiving each drug alone (Quenelle *et al.*, 2007). Further evaluation of these drugs is ongoing in clinical trials, but they are available for emergency purposes (Gupta *et al.*, 2007). A recent case study reports combinatorial treatment involving St-246, CMX001 and the vaccinia immune globulin for a 28-month-old child who developed severe eczema vaccinatum from his father who had been vaccinated with vaccinia virus vaccine (Vora *et al.*, 2008).

Conclusion

Despite decades of research, biological agents continue to be a threat to civilians and military personnel given the lack of prophylaxis and medical countermeasures available for a number of these agents. While there are vaccines commercially available for the prevention of anthrax, cholera, plague, Q fever and smallpox, there are no licensed vaccines available for use in the case of botulinum toxins, viral encephalitis, melioidosis or ricin. Antibiotics are still recommended as the mainstay treatment following exposure (or suspected exposure) to anthrax, plague, Q fever and melioidosis. Anti-toxin therapy and anti-virals may be used in the case of botulinum toxins or smallpox respectively. However, supportive care is the only, or mainstay, post-exposure treatment for cholera, viral encephalitis and ricin – a recommendation that has not changed in decades.

While further development and improvement of current regimes is required, there should be strategic alignment of resources and effort with the risk that biological agents pose (whether that be accidental or deliberate). In addition, the opportunities for international and national government and non-government organizations to collaborate and leverage from each others' work programmes should not be underestimated.

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Conflict of Interest

None of the contributing authors to this review have any conflict of interest to declare.

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