Approaches to Handling High Consequence Pathogens

The thesis is submitted in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy by Publication of the University of Portsmouth.

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Declaration

Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.

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With acknowledgements inevitably I will miss somebody key and that is not good. So, I would like to say a HUGE thank you to anybody that has played any part in my career working with some fantastic microorganisms. Over the past thirty or so years, I have made many friends and worked with the very best people with a passion for science. The portfolio of papers that have contributed to this thesis recognise a whole host of colleagues I have had the pleasure of working with – if you are reading this and think I am talking about you, I most certainly am.

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Abbreviations

ACDP	Advisory Committee on Dangerous Pathogens
AVA	Anthrax Vaccine Adsorbed
AVL	A Virus Lysis (buffer)
AVP	Anthrax vaccine precipitated
BARDA	Biomedical Advanced Research and Development Authority
BEI	Biodefense and Emerging Infections Research Resources Repository
BG	*Bacillus globigii*
CCHF	Crimean-Congo Haemorrhagic Fever
CCTV	Closed-circuit Television
CDC	Centers for Disease Control and Prevention
CL	Containment Level
COSHH	Control of Substances Hazardous to Health
Dstl	Defence Science Technology Laboratory
ECDC	European Centre for Disease Prevention and Control
EF	Oedema Factor
ELISA	Enzyme Linked Immunosorbent Assay
ET	Oedema Toxin
FDA	Federal Drug Administration
GHSAG-LN	Global Health Security Action Group – Laboratory Network
GHSI	Global Health Security Initiative
GLP	Good Laboratory Practice
HCID	High Consequence Infectious Disease
HEPA	High Efficiency Particulate Air filter
HG	Hazard Group
HM	Her Majesty's
HO	Home Office

HSAW	Health and Safety at Work
HSE	Health and Safety Executive
LF	Lethal Factor
LT	Lethal Toxin
MSC	Microbiological Safety Cabinet
NaCTSO	National Counterterrorism Security Office
NHP	Non-human Primate
NHS	National Health Service
NIAID	National Institute of Allergy and Infectious Diseases
Pa	Pascals
PA	Protective Antigen
PCR	Polymerase Chain Reaction
PHE	Public Health England
RA	Risk Assessment
RT-qPCR	Real time quantitative polymerase chain reaction
SAPO	Specified Animal Pathogens Order
UK	United Kingdom
US	United States of America
WGS	Whole Genome Sequencing
WHO	World Health Organisation

Preface

This thesis follows a career that has focussed on working with high consequence pathogens. I started as a research analytical technician working with *Salmonella* bacteriophages, advancing as a research scientist working on *Escherichia coli* serotype O157 (MPhil thesis, 1996), then joined the Special Pathogens Reference Unit as a Senior Project Team Leader taking responsibility for the Anthrax Reference Unit. I subsequently led the response at Porton to the Foot and Mouth Disease Virus crisis in the UK, and then swiftly back to the Anthrax Reference Unit to co-ordinate the response to the Anthrax releases in the United States of America and the subsequent knock-on to the United Kingdom. My successful navigation of these challenges highlighted my capability in this area and resulted in the opportunity to take on the challenge of a previously poorly managed US contracts team of scientists and technicians, that at its peak grew to employing 40+ staff, fourteen projects and funding of over $59M. This wealth of experience has placed me in the unique position of being able to lead the research, clinical and service delivery teams working at the maximum levels of containment for human and animal pathogens.

Abstract

This thesis outlines the classification of biological agents, the regulatory framework for working safely and the security implications of handling high consequence pathogens followed by a detailing of my significant contributions to this field.

My work covers a broad range of pathogens and research questions, yet shares a common theme of developing novel approaches to working in containment: examining the use of bacteriophage as a mechanism for the capture and detection of *Escherichia coli* serotyope O157; characterisation of the anthrax vaccine and its production processes, to inform the understanding and development of current and next generation vaccines; an analysis of environmental anthrax spore levels and their decontamination, contributing to responses in the event of a bioterrorism event; improvements to established but unreliable inactivation methods, thereby allowing viral haemorrhagic fever samples to be taken out of containment whilst maintaining the integrity of molecular material; and innovative approaches to working safely and humanely with a primate model of monkeypox virus has been instrumental in the approval of a new vaccine for smallpox.

This work catalogues my advancement through the containment levels at Public Health England, which has culminated in my current senior management role as the Head of High Containment Microbiology, with responsibility for the ACDP Containment Level 4 *in-vitro* facilities at Porton and Colindale.

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1. Commentary

Approaches to Handling High Consequence Pathogens

1.1. Introduction

1.1.1. ACDP and SAPO Classification of Biological Agents

The UK has two classification schemes for pathogens: the Advisory Committee on Dangerous Pathogens (ACDP) (HSE, 2005) which covers the control and classification of human pathogens, and the Specified Animal Pathogens Order (SAPO) (HSE, 2015) which covers the control and classification of animal pathogens.

The ACDP issues guidelines (HSE, 2001, 2005, 2006) for working with pathogens and it has the status of guidance supporting the Health and Safety at Work Act 1974 and the Control of Substances Hazardous to Health Regulations (COSHH) (HSE, 2013b). Micro-organisms are classified into four hazard groups by the ACDP on the basis of pathogenicity to humans, risk to laboratory workers, transmissibility to the community, and whether effective prophylaxis is available (HSE, 2013a) (Table 1). The hazard group of the pathogen then determines the containment level that has to be used to undertake work with the pathogen. An ACDP hazard group 4 pathogen is the highest categorisation and work with this hazard group must be undertaken at containment level 4. The example pathogens highlighted in bold in Table 1 are pathogens that have been used in work described in this commentary.

Animal pathogens are classified in a similar way to human pathogens with the primary focus, understandably, on the potential harm to susceptible animal species, the potential for the disease to spread from the laboratory and the subsequent economic impact if a release occurred. It is estimated that the 2007 release of Foot and Mouth Disease Virus from facilities in Pirbright, which affected eight farms, cost the government £47 million and industry £100 million (Anderson, 2008). Table 2 describes the criteria for classification of animal pathogens into their respective groups (HSE, 2015).

Table 1: Definition of ACDP Hazard Groups.

ACDP hazard group	Definition	Examples
1	An organism that is most unlikely to cause human disease.	*Staphylococcus epidermis*
2	An organism that may cause human disease and which may be a hazard to laboratory workers but is unlikely to spread to the community. Laboratory exposure rarely produces infection and effective prophylaxis or treatment is usually available	***Legionella pneumophila***
3	An organism that may cause severe human disease and presents a serious hazard to laboratory workers. It may present a risk of spread to the community but there is usually effective prophylaxis or treatment available.	***Bacillus anthracis*** ***Escherichia coli* O157** **Monkeypox virus**
4	An organism that causes severe human disease and is a serious hazard to laboratory workers. It may present a high risk of spread to the community and there is usually no effective prophylaxis or treatment.	**Ebola virus** Crimean-Congo haemorrhagic fever virus

Table 2: Definition of SAPO Hazard Groups

SAPO hazard group	Definition
1	Disease producing organisms which are native to animals in the UK (enzootic) or do not produce notifiable diseases.
2	Disease producing organisms that are either exotic or produce notifiable disease, but have a low risk of spread from the laboratory.
3	Disease-producing organisms which are either exotic or produce notifiable disease, but have a moderate risk of spread from the laboratory.
4	Disease-producing organisms which are either exotic or produce notifiable disease, but have a high risk of spread from the laboratory.

Clearly, some zoonotic pathogens are both human and animal pathogens, and as such have dual classification *e.g. Bacillus anthracis* - ACDP hazard group 3, SAPO hazard group 3; Foot and Mouth Disease Virus – ACDP hazard group 2, SAPO hazard group 4; Nipah virus – ACDP hazard group 4, SAPO hazard group 4. Both

ACDP and SAPO maintain lists of agents and their categorisation which are reviewed and updated on a regular basis.

1.1.2. High Consequence Pathogens

High consequence pathogens are those that are classified in the UK as belonging to ACDP hazard groups 3 or 4 (Table 1); they are also often termed high containment pathogens, and these terms will be used interchangeably throughout this commentary. The National Health Service High Consequence Infectious Disease (NHS HCID) programme defines high consequence pathogens as those that are responsible for infectious diseases that are characterised by: acute infectious illness, the ability to spread in healthcare settings, a high case fatality rate, difficulties in recognition and rapid detection, and often lack of effective treatment. Infections caused by these pathogens frequently require co-ordination at a national level to ensure an effective and consistent response (Pinto-Duschinsky & Jeavons, 2015) and example of this would be the two cases of Monkeypox virus infection in the UK in 2018 (Vaughan et al., 2018) which was managed as an enhanced incident in PHE.

Several high consequence pathogens are included in the Research and Development Blueprint List of Priority Diseases issued by the World Health Organisation (WHO, 2018); this list highlights those pathogens that have the potential to cause a public health emergency and identifies where there is a need for accelerated research and development. All but one of these agents are categorised as ACDP hazard group 3 or 4 and therefore require handling in high containment facilities; some of these are featured in the work described in this commentary.

1.1.3. High Containment Facilities

The requirements for the building, safe operation and maintenance of high containment facilities in the UK are based on the guidance and regulations of COSHH, ADCP and SAPO, and are reviewed in Table 3. Additionally, there are obligations under the Anti-Terrorism, Crime and Security Act 2001 (ACTSA) relating to the access and security arrangements of high containment facilities. The

advanced level of engineering required to meet these containment specifications, and the associated management systems and emergency procedures that establishments housing these laboratories must have in place together result in high building, operation and maintenance costs for these facilities. Consequently, all ACDP containment level 4 facilities in the UK are government owned and funded; UK facilities that can undertake work with ACDP hazard group 4 organisms are the Defence Science and Technology Laboratory (Dstl) at Porton Down (a Ministry of Defence facility), and the two Public Health England laboratories one at Porton Down, Wiltshire and the other at Colindale, North West London.

While there is some overlap between ACDP and SAPO requirements for containment laboratories (Table 3), these differ in their primary aim; ACDP requirements are designed to protect the operator from risk of infection, while SAPO containment is designed to minimise the risk of release to the environment of agents that may have an economic impact. Thus, ACDP4 and SAPO4 containment are not equivalent; SAPO containment level 4 laboratories in animal health laboratories (such as Animal and Plant Health Agency [APHA]) cannot be used for ACDP hazard group 4 pathogens, while ACDP containment level 4 facilities can handle the highest categorisation of both systems if they have the appropriate SAPO licensure.

The ACDP guidelines give very clear guidance on how the laboratory should operate but these only apply to work involving standard microbiology methods. Any work that is non-standard requires further robust risk assessment and may need additional regulatory approval. A view will be taken on new work if it is deemed a significant change from previous work, even if regulatory approval to use such agent has been granted. A recent example of this within my department was a series of experiments involving infection of ticks with Crimean-Congo Haemorrhagic Fever (CCHF) in an ACDP Containment Level 4 *in-vitro* laboratory; this required submission of extensive risk assessments, proof of concept at ACDP Containment Levels 2 and 3 and a walk through of the work with HM Inspector from the HSE.

Table 3: Review of ACDP, COSHH and SAPO containment measures required for containment facilities.

Containment measure	Containment Level		
	2	3	4
Workplace separated from other activities	No	Yes	Yes
Supply and Extract HEPA filtration	No	Single extract only	Single supply, double extract
Restricted access to authorised personnel only	Yes	Yes	Yes
Sealable for disinfection/fumigation	No	Yes	Yes
Negative pressure cascade	No	Yes	Yes, specified for SAPO (-50 to -75 Pa)
Vector control	No, unless animal containment	No, unless animal containment. Yes, if SAPO	Yes
Surfaces impervious to water and easy to clean	Yes, bench	Yes, bench, walls and floor	Yes, bench, walls, floor and ceiling
Safe storage of biological agent	Yes, secured for Schedule 5 pathogens	Yes, secured for Schedule 5 pathogens	Yes, secured.
Observation window or alternative	No	Yes	Yes
Laboratory has own equipment	No	Yes, as far as reasonably practicable	Yes
Infected material, including any animals to be handled in containment	Yes, where aerosol	Yes, where aerosol	Yes
Incinerator	Accessible	Accessible	On-site
Effluent treatment pant	No	Yes, where RA dictates	Yes
Shower on exit	No	No, except where RA dictates	Yes, for SAPO
Autoclave	Yes, in the building	Yes, within suite	Yes, within suite, must be double-ended and interlocked doors
Protective clothing	Yes	Yes	Yes, complete change including footwear
Airlocks	No	No, unless RA dictates	Yes

1.2. Scientific Contribution to the Study of High Consequence Pathogens

A significant portion of my career has involved work with ACDP and SAPO high containment pathogens, both *in vitro* and *in vivo*. This commentary outlines my work on the culture, detection and inactivation of high containment pathogens, followed by a description of my considerable contributions to the study of two particular high containment pathogens, *Bacillus anthracis* and high consequence pox viruses.

1.2.1. Pathogen Culture and Detection in ACDP CL3 and CL4 Facilities

My work on the detection of high containment pathogens began with the development of a rapid detection system for *E. coli* serotype O157 (Roberts, 1996). Briefly, bacteriophages were used to phage type isolates and a cocktail was made of three bacteriophages that between them covered all phage types of *E. coli* serotype O157. These were then grown to high titre and used as an antigen capture for a range of sample types. A biotinylated bacteriophage was then used as a detector for any *E. coli* serotype O157 that was captured on the plate. This could confirm the presence of *E. coli* serotype O157 within several hours as opposed to conventional methods that took several days.

The detection of high containment pathogens historically required containment facilities but with the advancement of molecular techniques, pathogen detection can now be carried out at lower levels of containment. ACDP Containment Level 4 laboratories now are principally used for the culture and propagation of viruses from clinical samples. Inactivation of high-risk clinical samples, isolates and experimental samples containing HG3 and HG4 pathogens is often performed in containment, prior to removal of samples to allow further work to be undertaken at a lower containment level (see section 1.2.2).

The advancements since molecular methods were developed are staggering and have led to an unbelievable amount of data being generated very quickly, particularly in the sequencing field. The 2013–2016 Ebola virus outbreak in West

Africa was the largest and most complex Ebola outbreak since the discovery of the virus in 1976; the number of cases and deaths during this outbreak were considerably higher than all previous known outbreaks combined. A multinational response was mounted to help control the outbreak, and there were several cases of foreign healthcare workers contracting the disease. The UK had three imported cases. My department isolated and cultured the virus from the first of these UK cases; the full virus sequence was rapidly deposited in Genbank for use by the wider research community **(Bell *et al.*, 2014)**.

Culturing pathogens in high containment facilities can be challenging, particularly at ACDP containment level 4. Compliance with the range of regulatory, safety and operational requirements make quite simple tasks difficult: space is limited, especially in cabinet-line-based laboratories; specialised equipment may not be available; sample manipulation using MSCIII gauntlets is difficult. Due to the bespoke nature of each CL4 facility, microbiological methods are hard to standardise between laboratories, and this has led to inconsistencies in data generation between groups. This problem can lead to incorrect conclusions being drawn about pathogen characteristics, the efficacy of therapeutic interventions, or the suitability of animal models being drawn The development of standardised methods of propagation are essential for performing reproducible experiments with high containment pathogens. The US contracts work undertaken under the NIAID program has demonstrated the problems that can be encountered if propagation is not standardised; the program had faced issues with US contractors using *Burkholderia mallei* (personal communication) whereby differing contractors were obtaining different results from performing the same animal studies. Upon investigation, it was found that the challenge material was being grown in different ways, from different sources and different passage numbers were being used at each establishment. As a result, disease presentations, immunological profiles and progression were different at each contractor. As a result of these issues, they funded a repository, the Biodefense and Emerging Infections Research Resources Repository (BEI Resources), that supply all material required to undertake work by their contractors. Where possible, all pathogens are supplied in sufficient quantity for all work to be undertaken, for example the Monkeypox work described in section 1.2.4 (**Tree *et al.,* 2015,** Hatch ***et al.,*** **2013)** and if not possible, stock

vials, reagents and detailed protocols are distributed with clear acceptance criteria to ensure consistency in all facilities.

A considerable amount of recent work by my department has exploited this requirement for consistent pathogen propagation, and we have been successful in winning grants for the production of authenticated Ebola and Marburg virus stocks for the US Government in 2007 (NIAID, 2007), 2013 (NIAID, 2013) and, more recently, in 2017 (BARDA, 2017). These contracts required the production of authenticated master and working virus banks that will be shipped to BEI Resources for dissemination to contractors. This contract is a prime example of where standardisation is key, production of the stocks requires a number of key tests and one of these is the 7U/8U ratio that can change on repeated passage and is thought to affect the pathogenicity of the virus. It has been reported by Trefry *et al.*, 2016 that independent of statistical significance amongst vaccinates, it is the authors' recommendation that future challenges be carried out utilising a high percentage 7U Ebola virus stock in order to mitigate the risk that there is a difference between the two challenge stocks in the context of a vaccine.

1.2.2. Inactivation Studies of High Containment Pathogens

The study of high consequence pathogens, whether for fundamental research, development of new diagnostics, evaluation of therapeutics, or monitoring of clinical specimens from a confirmed patient, requires that infectious material is handled at the appropriate containment level; however, it is highly desirable to be able to work with this material at lower levels of containment for practical and economic reasons. Prior to its removal of material from high containment, infectious material must be inactivated by a method that is both validated for the organism in question and is compatible with procedures that will be carried out downstream. Failure to fully inactivate material before its removal from CL3 or CL4 facilities represents a potentially catastrophic breach of containment, with major implications for the safety of operators and the wider community. Indeed, recent inactivation failures have caused significant concern worldwide, including two incidences of the removal of unsterilised anthrax samples from CL3 (Centers for Disease & Prevention, 2014; Sample, 2014).

Chaotropic salts such as guanidium isothiocyanate or guanidine hydrochloride are a component of the lysis buffers of several commercially available nucleic acid extraction kits, and as such are routinely used for RNA and DNA extraction from clinical samples. Blow and colleagues reported that AVL buffer, a guanidinium isothiocyanate-containing component of the Qiagen QIAamp Viral RNA Mini Kit (Qiagen, 2018), was effective at inactivating a range of different ACDP3 and ACDP4 pathogens, including Ebola and Marburg viruses (Blow *et al.*, 2004). These data have been used as evidence to support the use of AVL buffer for rendering samples safe so that they can be removed to lower containment levels for processing. AVL buffer has since been used extensively for inactivation of high risk samples; AVL buffer alone was relied upon for inactivation of Ebola virus clinical samples before removal from primary containment in a number of diagnostic laboratories during the 2013-2016 West African Ebola virus outbreak (Kerber *et al.*, 2016).

Concerns were raised at a Global Health Security Initiative (GHSI) meeting of the Laboratory Network working group (September, 2013) that nucleic acid extraction buffers may not be completely effective for virus inactivation which would lead to the assumption there were flaws in the Blow study (Blow *et al.*, 2004), for example, inactivated samples were diluted out to overcome the cytotoxic effect of AVL, so experiment would miss low virus titres. Although the GHSI data on which these concerns were based remain unpublished, studies published since have provided evidence of incomplete Ebola virus inactivation by AVL in murine blood, marmoset sera and cell culture media (Haddock, Feldmann, & Feldmann, 2016; Smither *et al.*, 2015). More recently, we have demonstrated incomplete inactivation of Ebola virus in human serum **(Burton *et al.*, 2017)**. Thus, there is now compelling evidence that AVL buffer alone cannot be guaranteed to inactivate Ebola virus. However, it was found that complete inactivation of Ebola virus was achieved following the addition of ethanol to samples in AVL buffer (Haddock *et al.*, 2016; Smither *et al.*, 2015); this is the next stage of the manufacturer's protocol for manual RNA extraction (Qiagen, 2018). It was therefore a recommendation of the GHSI that the ethanol addition step of the nucleic acid extraction procedure should be undertaken prior to removal of Ebola virus samples from containment. As the UK representative on the GHSI working group, I ensured that this recommendation was implemented across the High Containment Microbiology

Department and that local procedures for removal of high hazard material from our CL4 laboratories were updated accordingly.

The addition of ethanol to samples before removing them from primary containment is possible for manual RNA extractions, but this poses problems for automated extraction platforms with which this is incompatible. This has particular implications for diagnostic laboratories, where high-throughput automated systems are preferable. Such automated systems were introduced into the PHE diagnostic laboratories in Sierra Leone during the 2013-2016 Ebola outbreak, when Qiagen EZ1 machines replaced manual extraction procedures. The sample workflow for automated extraction in these laboratories involved the removal of samples from primary containment following incubation with AVL and an additional heat inactivation step (60 °C for 15 minutes) before loading samples onto the automated platforms (Bailey *et al.*, 2016). A potential issue with adopting heat inactivation steps in high-throughput and/or outbreak situations is that continuous temperature monitoring of samples is required to ensure full inactivation. To address this problem, we performed studies to evaluate the suitability of Triton X-100 as a second inactivant **(Burton *et al.*, 2017)**. Triton X-100 is a non-denaturing detergent that solubilises lipid membranes, and has been shown to reduce Ebola virus infectivity without affecting blood chemistry or downstream nucleic acid analysis (Lau,*et al.*, 2015; Lewandowski *et al.*, 2017; Tempestilli *et al.*, 2015). We found that the combination of both AVL buffer and 0.1% Triton X-100 for 10 or 20 minutes completely inactivated Ebola virus in mock clinical serum samples, and that the treatment was compatible with downstream RT-qPCR and next generation sequencing **(Burton *et al.*, 2017)**. This represents a considerable improvement over heat or ethanol treatment as a second inactivation step because it permits consistent treatment of samples and is compatible with the automated extraction platforms widely used in diagnostic laboratories. These findings will be of great benefit to the wider diagnostic community, allowing for the development of standard operating procedures that permit effective downstream sample processing while not compromising operator safety.

1.2.3. Bacillus anthracis

Bacillus anthracis is a gram-positive, spore-forming bacterium that causes anthrax, a severe infectious disease of both animals and humans. As such, it is classified under ACDP and SAPO and must be handled at Containment Level 3 under both sets of regulations. The type of anthrax infection varies according to the entry route of the *B. anthracis* spore: cutaneous anthrax is caused by spore entry through a skin lesion, most commonly following handling of infected animals or animal products; spore inhalation or ingestion (through the consumption of contaminated water or animal products) leads to inhalation anthrax and gastrointestinal anthrax respectively. Cutaneous anthrax is the most common form in humans, and presents as small blisters that develop into ulcers with characteristic black eschars. Cutaneous anthrax causes less severe disease than inhalation or gastrointestinal anthrax, which is associated with more systemic symptoms; however, all types may cause severe disease and death if left untreated. Anthrax cases in the UK are rare. Isolated cases of occupationally-derived anthrax, associated with the handling of contaminated animal products (Anaraki *et al.*, 2008; Pullan *et al.*, 2015; **Sharp & Roberts, 2006**), and sporadic injection anthrax outbreaks have occurred among drug users, associated with contaminated heroin (Grunow *et al.*, 2012; Sykes *et al.*, 2013).

B. anthracis spores are highly resistant to extremes of temperature and humidity, and to treatment with chemical disinfectants; spores can remain viable for long periods in soil, and *B. anthracis* has been recovered from contaminated soil and animal remains after many decades (de Vos, 1990; Wilson & Russell, 1964). These properties have led to anthrax being successfully developed as an effective biological weapon (Jernigan *et al.*, 2002); anthrax weaponisation has been achieved by at least five national bioweapons programs: in the UK, Japan, the US, Russia and Iraq.

The vegetative form of *B. anthracis* produces three primary virulence factors: the bacterial capsule, lethal toxin (LT) and oedema toxin (ET). LT and ET are formed from combinations of the cell receptor component protective antigen (PA) with lethal factor (LF) and oedema factor (EF), respectively. LT and ET are required for the bacteria to evade host immunity and to enable systemic dissemination; to do

this, they must be internalised into the cytoplasm of host cells by endocytosis in a process that is mediated by PA (Friebe, van der Goot, & Burgi, 2016).

An anthrax vaccine is available for humans deemed to be at high-risk of contracting anthrax; cell-free filtrates of the attenuated, non-capsulated Sterne strain of *B. anthracis* (Sterne, 1939) are licensed in the both the UK and US for human use (Turnbull, 1991). The UK vaccine is made by alum-precipitation of antigen from in vitro Sterne cultures and is termed anthrax vaccine precipitated (AVP), to distinguish it from anthrax vaccine adsorbed (AVA) which is licensed for use in the US. AVP has been produced at the Porton site in the UK for over 60 years (historically by PHE and its predecessor organisations, now by Porton Biopharma Limited) and in this time, there has been little change to the manufacturing process. *B. anthracis* Sterne is grown in media supplemented with casmino acids and activated charcoal in order to maximise the yield of PA (Belton & Strange, 1954; Strange & Belton, 1954), which was originally thought to be the principal immunogen of anthrax vaccines (Turnbull, 1991). Five hundred millilitre cultures are grown statically in glass Thompson bottles at 37°C until the culture pH drops below pH 7.6, at which point cultures are harvested and the culture supernatants are pooled and filter-sterilised; this is followed by the addition of alum and pH adjustment to 5.8-6.2. The alum precipitate is allowed to settle under gravity, the supernatant is removed and the precipitate is resuspended in saline.

Despite the demonstrable efficacy of AVP, it was poorly characterised in terms of the components that end up in the final product. A study I authored sought to address this gap in knowledge by elucidating the composition of AVP ***(Hallis et al.*, 2002)**. We used sensitive, specific immunoassays to demonstrate the presence of PA, LF and EF, and surface layer proteins Sap and EA1 in AVP preparations, and used a series of novel *in vitro* functional assays to demonstrate that PA, EF and LF in AVP retained their biological activity. These findings were confirmed a couple of years later in a study that used two-dimensional gel electrophoresis to characterise AVP (Whiting *et al.*, 2004).

The presence of LF and EF differentiates AVP from US-produced AVA, which contains negligible levels of LF and EF (Ivins *et al.*, 1998; Puziss *et al.*, 1963). The differing compositions of AVP and AVA contribute to distinct antibody responses

following vaccination, and there is considerable evidence pointing to a contributory role for antibodies raised against these non-PA components to the protective effect of AVP in animal and human studies (Baillie *et al.*, 2003; Baillie *et al.*, 2010; Dumas *et al.*, 2017; Pezard *et al.*, 1995; Price *et al.*, 2001; Turnbull *et al.,* 1986). It was a concern that the non-PA components of AVP may be immunomodulatory and negatively impact the PA-specific immune response, but the presence of these other vaccine components has since been shown not to adversely impact the PA-induced protective immune response (Baillie *et al.*, 2003). Thus, the inclusion of LF, EF and/or surface layer proteins, rather than solely focusing on PA as a component, in the next generation of anthrax vaccines may improve their immunogenicity.

Beyond a limited study of carbohydrate metabolism and PA production during static culture of *B. anthracis* Sterne (Puziss & Wright, 1959), very little was known about the growth characteristics and physiology of *B. anthracis* Sterne strain under the conditions used for vaccine manufacture, despite decades of successful AVP production. In 2007, we published an extensive study of a range of physiological parameters during AVP production, including growth characteristics, utilisation of substrates and antigen production **(Charlton *et al.*, 2007)** with the aim of providing a set of baseline parameters to inform both current and future vaccine production. Ensuring that our study accurately reflected the conditions during AVP manufacture required a complex study design and the use of the vaccine production facility, which operates at both ACDP and SAPO Containment Level 3. The sampling method for this study involved harvesting whole bottles at desired time-points instead of repeated sampling from the same bottle, since the disturbance of cultures is thought to affect growth and antigen production through disturbance of the pellicle that forms on the surface of the culture supernatant. This study successfully established reproducible growth and metabolism kinetics for *B. anthracis* Sterne under vaccine manufacture conditions.

ELISAs were used to quantify levels of PA and LF across the culture period **(Charlton *et al.*, 2007)**. Furthermore, we demonstrated that levels of PA and LF were near maximal at the time that bacterial culture is harvested during the vaccine manufacture process (providing assurance that current harvest times are

optimal) and showed that these antigens are not degraded during fermentation (as has been reported for other culture methods; (Farchaus *et al.*, 1998)).

The parameters established in the Charlton study (**Charlton *et al.*, 2007)** have been used subsequently in the evaluation of miniature bioreactors for growth of *B. anthracis* (Mukhopadhyay *et al.*, 2010; Mukhopadhyay *et al.*, 2011); these miniature bioreactors have a small footprint and reduce experimental volumes used and are thus particularly suited to the use with ACDP hazard group 3 pathogens in containment, where space is at a premium and small volumes of pathogen are preferable in order to reduce risk to the operator.

Both the Hallis and Charlton studies contribute significantly to the understanding of the AVP production process and the individual components that make up this vaccine, and inform the development of next generation anthrax vaccines. The assays developed in these studies facilitate the use of antigen quantification to provide assurance of AVP batch-to-batch consistency, and could ultimately reduce or replace the *in vivo* tests that are required currently prior to batch release.

The production of authenticated, standardised *B. anthracis* spore batches was required for the delivery of two NIAID task orders that were awarded to test the efficacy of antibiotics in small animals (NIAID, 2003a) and the primate model (NIAID, 2003b). The standard method for the production of *B. anthracis* spores was laborious; it required growing a lawn of *B. anthracis* on sporulation agar using flat bottomed glass medical flats (Turnbull, 1998), which would be left for several weeks before being washed off using diluent and glass beads. The sheer amount of spores required to undertake all the studies required of the two awarded tasks would have taken over twelve months. Early on in my career (in 1988), I was involved in the large scale production of *Bacillus globigii* spores, which were used for bio-tracing and were routinely used to monitor water flows. The growth conditions and sporulation characteristics of *B. globigii* and *B. anthracis* are similar; they are both difficult to spore – sporulation agar is nearly completely dried out before sporulation starts to occurs, in some cases taking up to 6 weeks. The method I was involved in and modified involved the production of 100+ litres of *B. globigii* spores in a large fermenter with multiple feeds at set times, *B. globigii* is an ACDP hazard group 2 pathogen. A similar process was applied to production of

B. anthracis spores. This necessitated scale-down of the procedure so that it could be undertaken in a small fermenter for operation inside a Class III microbiological safety cabinet (MSC), in order to comply with the containment requirements of working with an ACD3 hazard group 3 pathogen and not to contravene the Biological Weapons Convention (BWC, 1972) by producing hundreds of litres of high titre spores. Using this alternative method of propagation, all spore stocks for the task orders were produced within three months and were of high titre and quality for use in subsequent studies. Due to security issues around the nature of this work, this methodology was not published; however, the virulence of the resulting spore stock was presented as a poster at the 7th American Society of Microbiology, Biodefense and Emerging Diseases Research Meeting, Baltimore (Hatch *et al.,* 2009).

The robustness of the *B. anthracis* spore means that the decontamination of contaminated soil represents a particular challenge and we have reviewed the available strategies for environmental sampling and anthrax decontamination **(Sharp & Roberts, 2006)**. This publication included various case studies of the decontamination of specific buildings following incidences of bioterrorism in the US, and the clinical case details of two cases of occupationally derived anthrax in the UK, which I had previously presented at the International Conference on Emerging Infectious Diseases in Atlanta, USA **(Roberts *et al.*, 2002)**. In addition to comprising a comprehensive review of the published literature in this field, this publication included my analysis of the thousands of samples that had been received by the *B. anthracis* reference laboratory, of which I had processed the samples between 1997 and 2006 as part of Environmental and Biosafety Services, and then latterly as the project team leader of the *B. anthracis* reference laboratory, contributing new information to the public domain on the numbers and nature of environmental samples that were being tested in the UK at this time. This publication thus provided a valuable resource for the academic, public health and biodefence communities, and has been widely cited since in *B. anthracis* studies since (see metrics section).

1.2.4. High Consequence Pox Viruses

There are two species of pox virus that must be handled under high containment, monkeypox (ACDP3) and variola virus (ACDP4). This section describes my contribution to the characterisation of monkeypox infection animal models, and evaluation of the toxicity and protective efficacy of a third-generation smallpox vaccine.

The variola virus is the causative agent of smallpox, a devastating disease characterised by distinctive skin lesions that has a fatality rate of up to 30%. Smallpox was declared eradicated by the WHO in 1980 following a global campaign, and following eradication it was recommended that all countries cease smallpox vaccination. Only a fraction of the world's population now retains immunity from previous vaccination, leaving the remainder of the population susceptible to this disease (Henderson *et al.*, 1999). Consequently, the risk of deliberate reintroduction of smallpox in a bioterrorism event, as well as the emergence of monkeypox, would have potentially devastating consequences and there is therefore a need for a safe, effective vaccine to protect against pox infections.

In cases where studies in humans are not possible or are unethical, the US Food and Drug Administration (FDA) permits the approval or licensing of a drug or vaccine on the basis of animal studies, if the animal model is an accurate, well-characterised representation of the disease condition in humans (FDA, 2002): the so-called 'Animal Rule'. This rule has been applied to studies testing the efficacy of smallpox vaccine, since working with variola virus is both unethical and impossible given the obvious risks of reintroducing an eradicated disease. There is consequently great demand for surrogate models of smallpox infection in humans. In addition to being a clinically relevant human virus in its own right, monkeypox virus presents with similar clinical symptoms to smallpox in humans and results in lethal systemic infection in primates. **Tree *et al.*, 2015** has contributed significantly to the characterisation of monkeypox virus infection of cynomolgus macaques as a smallpox model, building on previous evidence indicating its suitability for simulation of smallpox infections (Cann *et al.*, 2013; Chapman *et al.,* 2010; Zaucha *et al.*, 2001). A range of challenge routes have been studied in

cynomolgus macaques, including subcutaneous (Nagata *et al.*, 2014; Saijo *et al.*, 2009), intravenous (Buchman *et al.*, 2010; Earl *et al.*, 2004; Earl *et al.*, 2008; Hirao *et al.*, 2011; Huggins *et al.*, 2009; Johnson *et al.*, 2011; Jordan *et al.*, 2009), intrabronchial (Johnson *et al.*, 2011) and intratracheal (Goff *et al.*, 2011; Stittelaar *et al.*, 2006; Stittelaar *et al.*, 2005). The natural route of infection for smallpox in man is through close contact with an infected person via the oropharynx or nasopharynx (Fenner *et al.*, 1998), and furthermore, deliberate release of either smallpox or monkeypox is considered likely to be by aerosol to permit rapid dispersion across a large area. Despite this, there was comparably limited data obtained using this infection route (Barnewall *et al.*, 2012; Nalca *et al.*, 2010; Zaucha *et al.*, 2001) and the initial stages of monkeypox infection had not been previously investigated in detail. We therefore sought to characterise the early pathogenic events that occur during aerosolised infection of cynomolgus macaques (**Tree *et al.*, 2015**), through clinical observations and the determination of associated viral loads, immune responses and pathological changes.

The establishment of a reliable, reproducible macaque model of aerosolised monkeypox virus infection required a considerable investment in containment facilities at PHE Porton, and involved both the improvement of existing methods and the development of novel approaches for carrying out animal studies in high containment. Under my direction an entirely new building was designed solely for these studies, and included a new laminar flow system to permit handling of infected animals, specialist aerobiology equipment including plethysmography, and telemetric capability and advanced CCTV to enhance subject monitoring. Planning permission for this was granted by Wiltshire Council in 2006 (Application: S/2006/1259). A major limitation of animal studies using aerosolised routes of infection is that they often struggle to meet the accuracy and reproducibility obtained using other challenge routes. The new aerobiology equipment in this facility enabled us to deliver accurate doses for virus challenge and we were able to match the levels of accuracy seen with other routes of infection.

The data we generated during this study supported and expanded on previous work using this infection model (Zaucha *et al.*, 2001) and demonstrated similarities to the clinical presentation of smallpox in humans (Fenner et al., 1998). This work thus contributed to both our understanding of the progress of monkeypox infection

and to providing further characterisation of the smallpox animal model, thereby supporting its application in future smallpox intervention studies. Our study was reviewed by the US government and we were granted approval to use this animal model for studying human pox infections on the basis of the FDA Animal Rule.

Some countries have stockpiled smallpox vaccine for use in event of a bioterrorist attack; in the UK, the stockpiled vaccine is the second-generation Lister vaccine, which is composed of live vaccinia virus. In certain individuals, this vaccine has serious or even life-threatening side-effects, and it has been estimated that in a public health emergency approximately a quarter of the population would be at risk of developing complications (Kemper, Davis, & Freed, 2002). Modified vaccinia Ankara (MVA) has been attenuated by hundreds of passages in cell culture and has subsequently lost the ability to replicate effectively in humans (Earl *et al.,* 2004). Third-generation MVA vaccines have been shown to be comparatively safe with none of the complications of first- and second-generation smallpox vaccines, especially for patients with HIV or atopic dermatitis, which are contraindicated for the first and second-generation vaccines (Earl *et al.*, 2004; Kennedy & Greenberg, 2009; Mayr *et al.*, 1978; Stickl *et al.*, 1974).

IMVAMUNE is a third-generation vaccine manufactured by Bavarian-Nordic (Martinsried, Germany) that is derived from a strain of MVA. It has been supported through to licensure by the US Government, the National Institute of Allergy and Infectious Diseases (NIAID) for animal studies and subsequently the Biomedical Advanced Research and Development Authority (BARDA) clinical trials. My group has played an essential part in providing evidence supporting the licensure of IMVAMUNE **(Hatch *et al.,* 2013; Tree *et al.*, 2016)**, using the aerosolised macaque monkeypox virus model described above **(Tree *et al.*, 2015)**. **Hatch *et al.*, 2013** describes a pivotal study assessing the protective efficacy of either one or two (prime-boost; administered 28 days apart) doses of IMVAMUNE against a subsequent aerosol monkeypox virus challenge, and evaluated its performance against the second-generation vaccine ACAM2000. We showed that the use of a prime-boost regime (but not a single dose) of IMVAMUNE provided complete protection from subsequent challenge with monkeypox, demonstrating stimulation of both neutralising antibody and cell-mediated immune responses. This IMVAMUNE prime-boost regimen is well-tolerated in human subjects, with the

second dose significantly boosting antibody responses (Frey *et al.*, 2013), supporting our observation that two doses of vaccine were required for maximal efficacy.

The administration of two doses of IMVAMUNE 28 days apart is the optimal dosing schedule for this vaccine; however, in event of a deliberate smallpox release, this dosing regimen would be not be effective at providing rapid protection of the population. To give maximum protection in an emergency situation, an accelerated vaccination schedule or, ideally, a single dose of vaccine would be desirable. Frey and colleagues evaluated the use of a compressed schedule of vaccination (two doses given, 7 days apart) and found this was not as effective at stimulating antibody responses in humans as when given 28 days apart (Frey *et al.*, 2013). The same group evaluated the effects of administration of a single, high-dose (5 x 10^8 $TCID_{50}$, compared to the standard 1 x 10^8 dose) in a phase II clinical trial (Frey *et al.*, 2014). Prior to this study being carried out, we conducted a good laboratory practice (GLP) toxicity study testing the effects of this high dose in New Zealand white rabbits to contribute to the safety assessment for the use of this vaccine regimen in humans **(Tree *et al.*, 2016)**. We established that a repeated high dose of vaccine was safe in this rabbit model and that this dose elicited no adverse events, supporting its use if required in an emergency situation. The high dose was subsequently shown to be well-tolerated in human subjects although the high dose gave inferior antibody responses relative to the standard dosing regimen *(Frey et al.*, 2014).

Our monkeypox and IMVAMUNE studies **(Hatch *et al.*, 2013; Tree *et al.*, 2015; Tree *et al.*, 2016)** have provided crucial evidence supporting the IMVAMUNE licensure package submitted to the US FDA. Bavarian-Nordic is currently seeking approval for use of IMVAMUNE in the US and our studies are key to their FDA submission (personal communication, BARDA). These studies also have implications for protection of the human population against monkeypox, which has emerged in West and Central Africa since the cessation of mass smallpox vaccination (Durski *et al.,* 2018; Hutin *et al.*, 2001; Meyer *et al.*, 2002; Rimoin *et al.*, 2010). The first cases of monkeypox virus infection in the UK were very recently diagnosed, following two unrelated imported monkeypox cases from Nigeria (Vaughan *et al.*, 2018). IMVAMUNE is available in the EU under the

tradename IMVANEX and has been used as part of the response in the UK and is indicated in the rapid risk assessment issued by the European Centre for Disease Prevention and Control on the 21st September 2018 (ECDC, 2018).

The principles of the '3Rs' (Replacement, Reduction and Refinement) were developed over 50 years ago and provide a framework for performing humane animal research: Replacement refers to methods which avoid or replace the use of animals in an area where animals would otherwise have been used, Reduction refers to any strategy that will result in fewer animals being used and Refinement refers to the modification of husbandry or experimental procedures to minimize pain and distress. Since then the 3Rs have become embedded in national and international legislation and regulations on the use of animals in scientific procedures. The application for a project licence to undertake the monkeypox studies **(Hatch *et al.*, 2013; Tree *et al.*, 2015)**, in accordance with the Home Office Animals (Scientific Procedures) Act 1986, undertook to refine and reduce the number of animals used. We achieved refinement by way of using of implanted telemetric devices that could allow for the remote monitoring of the animals, more robust and accurate data with no manipulation of the animals and the provision of additional measurements that could be used as euthanasia criteria. We were able to reduce the number of animal subjects in our studies by using the latest aerobiology equipment linked to real time plethysmography, to reproduce aerosol dosing with accuracy not previously possible in these sorts of experiments; fewer animals were therefore needed to ensure the studies were robust. The licence had an animal allocation of that allowed for a number of repeat experiments but due to the robust aerobiology data and information obtained, no repeats were needed and the programme of work was completed with a significant reduction in subject numbers. We refined our procedures to employ the most up-to-date CCTV technology to permit monitoring of the animals without having to enter the room, thereby minimising animal disturbance and distress. The building, containment system, telemetry and all necessary equipment was funded by NIAID (NIAID, 2003c). The technological and procedural advances introduced during the course of the IMVAMUNE and monkeypox studies have demonstrably and positively impacted the way animal containment studies are performed at PHE Porton in many ways: reproducibility of delivered aerosol doses has reduced the number of test animals required; telemetry and advanced CCTV have improved the quality of life of study

animals; and the use of veterinary blood analysers, a first for PHE, gave a greater wealth of data that was not previously possible. Together, these all fulfilled an obligation to reduce animal numbers and refine the studies, whilst capturing the maximum possible amount of data on the efficacy of interventions. Leading the team responsible for so many advancements in the way animal studies are carried out in containment and the consequent improvement in the welfare of animal subjects has been a particularly rewarding part of my career.

1.3. Future Work

PHE is committed to building a suited ACDP containment level 4 facility when it relocates its two scientific centres, Porton and Colindale, and its headquarters function to a new campus at Harlow on the former GlaxoSmithKline (GSK) New Frontiers Science Park (https://www.gov.uk/government/news/government-invests-350-million-to-create-world-class-public-health-labs-in-harlow). Currently, the UK utilises cabinet lines for *in-vitro* work due to regulatory constraints but a tri-partite group was set up with a view to amending the UK guidelines to allow for suited working systems at ACDP containment Level 4. The new build facility at PHE Harlow will contain a new animal facility capable of handling animals up to ACDP and SAPO Containment Levels 4, along with a suited and cabinet line ACDP and SAPO Containment Level 4 *in-vitro* laboratories. I was part of the tri-partite group that consisted of dstl, HSE and PHE which has rewritten the ACDP Containment Level 4 guidelines to include suited systems of work which are due to be issued in late 2018, early 2019.

Additionally, I am the PHE Senior User on the Science Hub programme for the new high containment facility to be built in Harlow. Transition to PHE Harlow is key for my department, I need to be able to maintain business as usual for a critical capability, ensure competent cohort of staff in the new facility and have available a cadre of cabinet line and suit trained individuals. The training for suited systems takes many years and I have instigated a programme of training with the Public Health Agency of Canada to have staff trained at their National Microbiology Laboratory in Winnipeg.

Our vision for the new facility was presented at the WHO Consultative Meeting on High/Maximum Containment (Biosafety Level 4) Laboratories Networking at the International Agency for Research on Cancer, Lyon, France 13-15 December 2017 were I was invited to present for the UK on the proposed new facility at Harlow and separately on engineering challenges faced when running high containment facilities (Proceedings of meeting to be published on the WHO website in due course. Draft issued). None of this would have been possible without the expertise and knowledge I have gained working with the challenges around handling high consequence pathogens.

1.4. Concluding Comments

This thesis has brought together my published papers, which involve working with a range of pathogens from ACDP hazard group 3 and 4. These are the culmination of differing strategies to allow the handling of high consequence pathogens for culture, detection, testing of interventions, and inactivation to release them from the restrictions of containment work. This has allowed significant advances in being able to work, understand, detect and treat infections with these pathogens.

The papers follow a career that has grown into the leadership of research, clinical and service delivery working with the most dangerous pathogens known to man, at both the national and international level. The strategies employed have been instrumental in the approval of a new vaccine for smallpox, an understanding of anthrax background levels and decontamination, looking at bacteriophage as a detection strategy and potential therapeutic, understanding of vaccine processes and helping the advancement of modern molecular techniques by allowing samples to be removed from containment while maintaining the integrity of molecular material.

2. References to the Commentary

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3. Academic contributions by Allen Douglas Glen ROBERTS

Articles are listed in chronological order. Contributions that are part of this submission are listed in **bold**.

1. Walker, J. T., Roberts, A. D., Lucas, V. J., Roper, M. A., and Brown, R. G. (1999). Quantitative assessment of biocide control of biofilms including Legionella pneumophila using total viable counts, fluorescence microscopy, and image analysis. *Methods Enzymol, 310*, 629-637.

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4. Metrics, contributions and original versions of the presented body of work.

Metrics, contributions and original versions of the publications submitted in the support of this PhD by publication are listed in chronological order.

Original article

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Contributions by ROBERTS, A.D.G.

Study design

Microbiological experimental work

Data analysis

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Dev Biol. Basel, Karger, 2002, vol 111, pp 321-326.

Novel In vitro Functional Assays for the Determination of Anthrax Toxin Components

B. Hallis, N.J. Silman, S. Noonan, R. Baker, A.D.G. Roberts, C.P. Quinn, T. Marks, C. Wiblin, G. Lloyd, A. Robinson, M.J. Hudson

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Key words: Anthrax toxin, vaccine, protective antigen, lethal factor, oedema factor.

Abstract: The characterisation and evaluation of the UK licensed human anthrax vaccine depends on several in vivo tests that determine its safety and potency. Assays for the determination of functionally active and/or immunoreactive toxin components and S-layer proteins have been developed and applied to the characterisation of anthrax vaccine. These technologies may support production of consistent and effective vaccines, and may ultimately reduce the requirements for in vivo testing.

INTRODUCTION

Anthrax is a disease caused by the gram-positive spore-forming bacterium, *Bacillus anthracis*, an organism capable of causing infection in a range of mammals including humans. The disease results from entry of spores via a skin lesion, inhalation or ingestion; the pneumonic and gastro-intestinal forms of the disease are more severe than the cutaneous form [1].

Acellular anthrax vaccines comprising sterile culture filtrates of the attenuated Sterne strain have been licensed for use in both the U.K. and U.S.A. [2]. The U.K. licensed anthrax vaccine is an undefined mixture of cellular components from in vitro cultures of the toxigenic, non-capsulated *B. anthracis* Sterne $34F_2$ strain, precipitated in the presence of potassium aluminium sulphate (alum). However, the exact composition of the product is not known, as only limited characterisation studies have been performed.

The main virulence components of *B. anthracis* are a poly-glutamic acid capsule and two binary anthrax toxins, lethal toxin and oedema toxin formed from combinations of lethal factor (LF) and oedema factor (EF) with the cell receptor component protective antigen (PA). LF is a Zn-dependent endopeptidase active on

MAP kinase kinases (e.g. MEK-1) and EF is a potent calmodulin and Ca-dependent adenylate cyclase.

Cell-free cultures of *B. anthracis* contain PA and, because expression of the three toxin component genes is co-ordinately regulated, LF and EF are also likely to be present. The other major protein components of a cell-free culture filtrate include one or both of the S-layer proteins. The vaccine may, of course, contain many other *B.anthracis* proteins, including secreted and lysis products, peptidoglycan, nucleic acid and carbohydrate.

The aims of this study were to characterise the U.K. licensed vaccine and to measure the presence and/or functionality of the three anthrax toxin components and two S-layer proteins.

Materials and Methods

Extraction of vaccine

Proteins were released from the aluminium salts precipitate of batches of the vaccine using sodium citrate (0.1M, pH 7.5 at 4°C overnight) followed by dialysis.

ELISAs for the detection of antigen (capture ELISAs)

Antigen capture ELISAs were performed using conventional methodology. Briefly, appropriate purified antibodies raised in rabbits and specific for PA, LF, EF, EA1 or Sap were coated on a microtitration plate at 10µg/ml at 4°C overnight. Plates were blocked, washed and then incubated with vaccine or reference samples. Antigen was detected using antibody-enzyme conjugate, developed, stopped with 2M sulphuric acid and read at 450nm.

cAMP assays for EF adenylate cyclase

Adenylate cyclase activity was determined as described by Leppla (3) except that cAMP was measured using a BIOTRAK enzyme immunoassay (EIA) kit (Amersham-Pharmacia Biotech). EF, with Ca and calmodulin, was incubated for 60min at 30°C in the EIA and cAMP measured according to the kit instructions.

Macrophage lysis assays for LF and PA

The macrophage lysis assay was performed essentially as described by Quinn et al [4] using RAW 264.7 monocyte/macrophage cells. For assay of LF, PA was added at 1µg/ml in pre-warmed growth medium. LF or vaccine antigen was then added at varying concentrations. The resulting LT was added to the cells and incubated for 3h. Cell viability was determined after incubation using the MTT tetrazolium dye assay [5]. For the detection of PA, the assay was performed as described but using a fixed concentration (0.1 µg/ml) of LF.

LF functional assay using synthetic peptide substrate

A synthetic peptide representing the N-terminal 60 residues of human MEK-1 was synthesised (Sigma-Genosys); this peptide includes the LF cleavage site. The peptide (10 µg/ml) was bound by drying overnight at 37°C on to a microtitre plate. Coated plates were fixed with methanol, washed and blocked. Vaccine samples containing LF were diluted in assay buffer and incubated. Cleavage products were detected using antisera produced against cleaved peptide, and visualised using a second antibody-enzyme conjugate. The assay was developed and read at 450nm.

Results

Antibody capture ELISAs for anthrax vaccine components

ELISAs for the detection of PA, LF, EF, EA1 and Sap were developed. Each uses a purified immunoglobulin G (IgG) as the capture antibody and the same IgG

labelled with horseradish peroxidase as the second antibody. The ELISAs were antigen-specific, with no cross-reactivity, and sensitive (typical detection limits 1-2ng/ml; data not shown). The ELISAs were used to quantify immunoreactive toxin components and S-layer proteins in vaccine extracts. Figure 1 shows a typical ELISA titration for LF in the vaccine; other components give similar results.

Determination of adenylate cyclase activity of EF

The adenylate cyclase activity of EF was determined using a commercial EIA kit and had a detection limit of 60 pM. The activity of purified recombinant EF was determined (Fig. 2). The data indicate that recombinant EF was calcium and calmodulin-dependent, and functionally active. Moreover, recombinant EF exhibited similar specific activity to native material (data not shown). This assay has been applied to extracted vaccine batches and functional adenylate cyclase activity demonstrated (data not shown).

Macrophage lysis assay for LF and PA

The macrophage cell lysis assay [4] was used to compare the activity of recombinant PA and LF with their native counterparts (Fig. 3). The optimum time

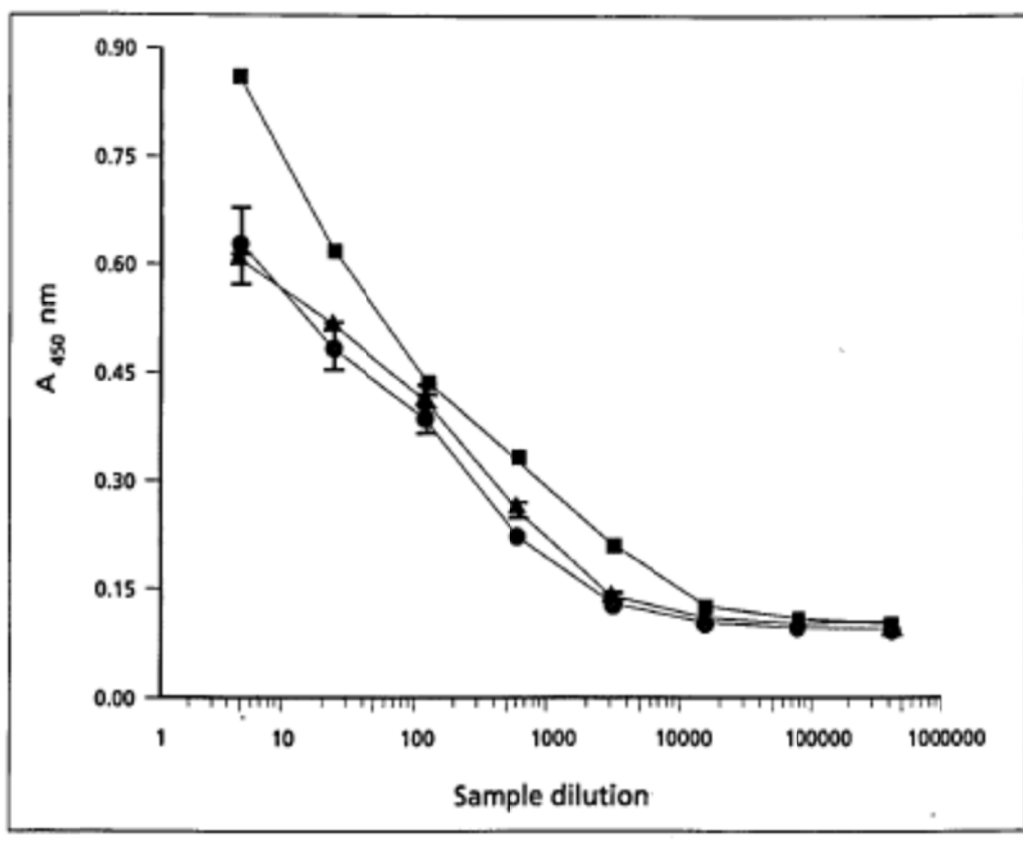

Fig. 1: Detection of LF in an extracted anthrax vaccine using antigen capture ELISA.
Assays were performed on two extracted vaccine batches with recombinant LF (rLF) as the reference standard. Data are from one single experiment, performed in triplicate. Data points are shown as the mean ± SEM values. rLF (■), extracted vaccine batch number 1 (▲), extracted vaccine batch number 2 (●).

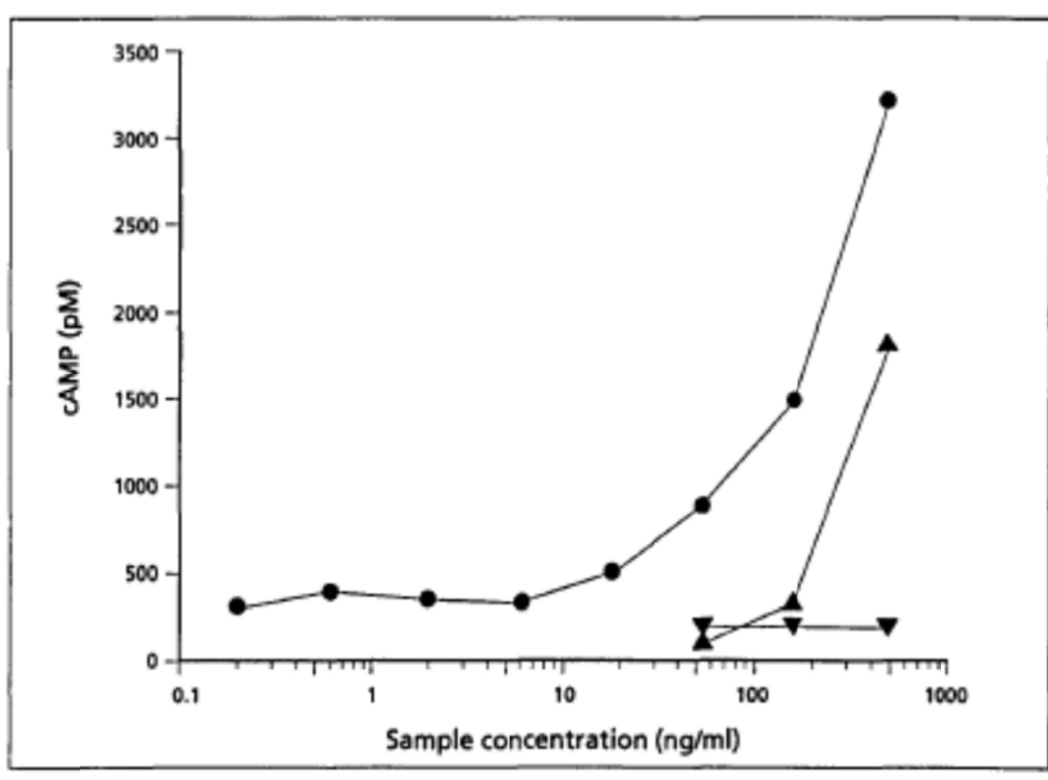

Fig. 2: Assay of adenylate cyclase activity of recombinant EF.
Adenylate cyclase activity of rEF was determined as described in the Materials & Methods. Controls without calmodulin or calcium (presence of EGTA) were performed to confirm calmodulin dependence. Recombinant EF (●), Recombinant EF minus calmodulin (▼), Recombinant EF plus EGTA (▲).

for incubation of PA and LF with the macrophage cells was determined and found to be 2.5-3.0 hours, thereby providing a satisfactory balance between assay duration and sensitivity. The assay may be used with a fixed concentration of PA to determine functional LF and vice versa. The optimised assay was used to determine the functionality of both PA and LF extracted from the vaccine. In the presence of a constant PA (1µg/ml), LF activity was demonstrable after extraction from the vaccine. In contrast, when the assay was performed with a fixed concentration of LF (0.1µg/ml), PA extracted from a limited number of vaccine batches was found to be inactive in this assay (data not shown).

In vitro cleavage assay for LF endopeptidase activity

LF endopeptidase activity may be determined in vitro by the measurement of cleavage of one of its intra-cellular substrate MEK-1 using a synthetic polypeptide representing the N-terminal 60 amino acids of MEK-1. Polyclonal antisera raised against both cleaved ends were screened for cross-reactivity against uncleaved MEK-1. This assay has been used to determine the functionality of LF present in the vaccine (Fig. 4). These data clearly demonstrate that LF activity can be determined in the two vaccine batches. This assay may be used in combination with the antigen ELISA to determine the percentage functionality, based on the expected cleavage activity of a known concentration of LF.

Discussion

Specific ELISAs for key vaccine components (PA, LF, EF, Sap & EA1) have been developed and used to quantify these proteins in extracted vaccine samples. The assays are sensitive, robust and reproducible with typical detection limits of 1-2ng/ml. Currently, licensing regulations do not require biochemical quantification of vaccine components; however application of the assays described here could facilitate the use of antigen quantification to determine vaccine batch-to-batch consistency, etc. Assays to measure functional activity of the anthrax toxin components have also been successfully developed and applied to vaccine extracts. The macrophage cell lysis & MEK-1 cleavage assays confirmed the presence of active LF in extracted vaccine batches. Interestingly, LF retains its activity despite having been immobilised on an aluminium salts precipitate, several years storage at 4°C, followed by citrate extraction for assay. The activity of EF was also demonstrated in vaccine extracts. In contrast, PA functionality was not demonstrable in extracts, although these are very preliminary studies. The macrophage cell lysis assay has been applied to measurement of PA activity using several different preparations of rPA, all of which exhibit stoichiometric activity in the assay.

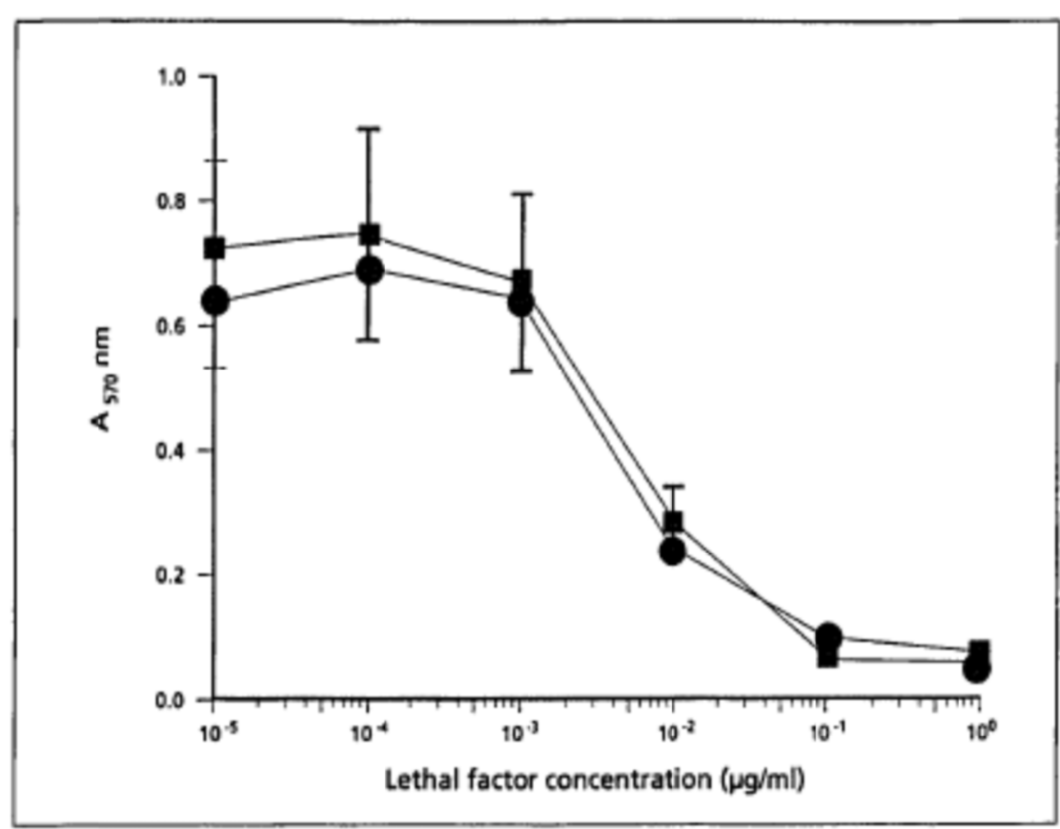

Fig. 3: Assay of rLF activity by macrophage cell lysis.
The activity of two preparations of rLF was determined using a fixed concentration of rPA at 1μg/ml. Dilutions of two rLF preparations were made and applied to the cells. The assay was performed as described by Quinn et al. (1991). Data are the mean of independent determinations and are shown ± SEM.

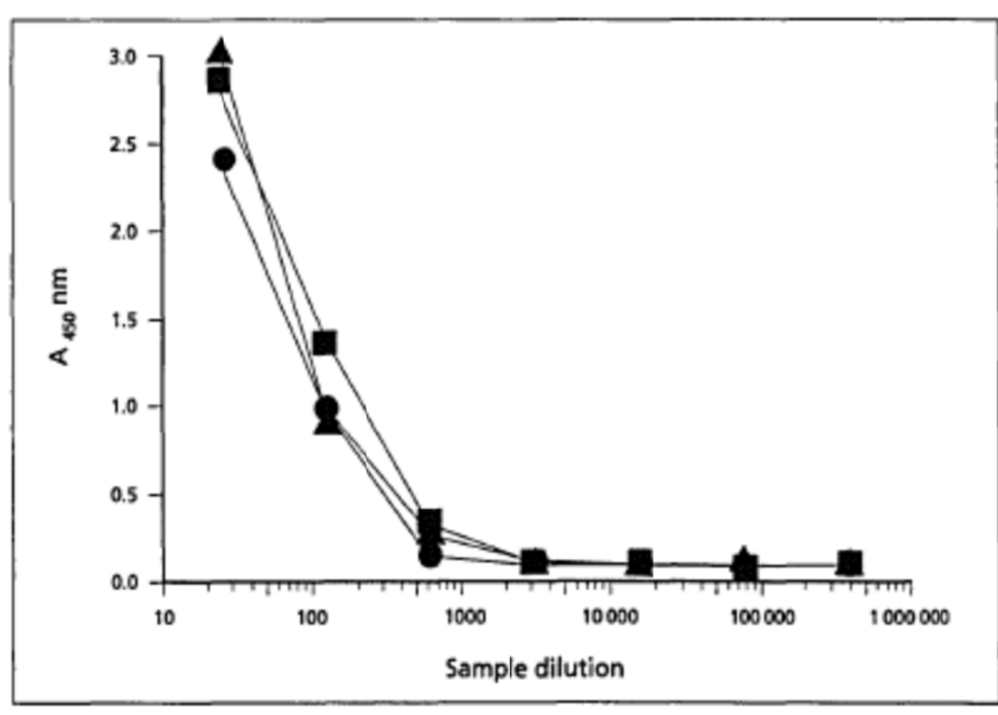

Fig. 4: Assay of LF functionality using the MEK-1 cleavage assay.
Two vaccine batches were extracted and the LF activity determined using rLF as a reference standard. Data are from one experiment, performed in triplicate. Data are shown as the mean ± SEM. rLF reference standard (■), extracted vaccine batch 1 (▲), extracted vaccine batch 2 (●).

Conclusions

A range of in vitro assays has been established for the characterisation of the UK licensed Anthrax Vaccine. These immunological and functional assays, which do not depend on the use of animals, are being evaluated for the support and characterisation of anthrax vaccine manufacture. Such assays could ultimately replace or reduce the in vivo tests currently required for the batch release and quality control of anthrax vaccines.

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Environmental anthrax testing
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Review
Anthrax: the challenges for decontamination

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Abstract: Anthrax remains endemic in many parts of the world with regular infections of livestock presenting a consequent risk to public health. In the United Kingdom anthrax has diminished as a significant threat to human health with only sporadic outbreaks in farm animals derived from ingestion of spores from soil at sites associated with previous outbreaks and the burial of carcasses. Occupationally-derived anthrax, associated with industries involved in the processing of animal products, has historically had an impact on the occurrence of outbreaks of infection. The introduction, in 1965, of vaccination for workers in high-risk occupations contributed significantly to the eradication of the disease from the UK. During 2001 the deliberate release of anthrax spores in the USA, disseminated through the postal system, resulted in the infection of 22 people, five of which resulted in death through inhalational anthrax. At that time anthrax was unheard of in many clinical practices and there was a lack of training and preparedness to handle such incidents; the emergency resulted in medical and public health personnel across the world having a significantly raised awareness of both the organism and the clinical symptoms of infection, and the new threat posed by bioterrorism. In the USA, the immediate public health emergency was followed by the legacy of contaminated buildings and facilities. There had been little previous systematic study of the issues surrounding sampling and decontamination of areas contaminated with *Bacillus anthracis*. The decontamination of large complex buildings and the equipment they contained required the urgent development and validation of new procedures for both sampling and decontamination.
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Keywords: anthrax; bioterrorism; public health; *Bacillus anthracis*

INTRODUCTION

While recent interest in *Bacillus anthracis* has concentrated on its exploitation as a weapon of bioterrorism, anthrax remains a significantly important, naturally occurring disease, world-wide.[1,2] Data from the Office for International Epizootic Control (OIEC) indicate that anthrax is endemic in Asia, Africa, Europe, America and Australia.[3]

During the past year, many outbreaks involving both livestock and humans have been reported to the Programme for Monitoring Emerging Diseases (ProMED).[4] Outbreaks were reported in north-eastern Zimbabwe in November 2004 and in January 2005 in Masvingo, resulting in the death of 60 cattle and infection of some 200 people, with three fatalities. In 2004 an outbreak in the Gonarezhou National Park killed over 1500 animals comprising kudus, buffaloes and antelopes. In Botswana an outbreak of anthrax occurred in the Chobe National Park, resulting in the deaths of 120 animals including elephants, buffalo and hippo.

In 2004 the deaths of cattle and sheep were reported from Victoria and New South Wales in Australia, cattle in Saskatchewan in Canada and pigs, sheep, red deer and cattle in Basilicata, Italy.[4] In Kursk, Russia, meat from an infected bull calf was presented for sale at market but was intercepted and confirmed as infected with anthrax by the local veterinary laboratory.[4] The slaughtering and eating of infected beef resulted in seven people in Tajikistan becoming infected, 20 being hospitalised in Krygyzstan with cutaneous anthrax and eight admitted to hospital in Tbilisi, Georgia. Other outbreaks infecting either livestock or humans or both were reported in Turkey, South Africa, United States, Indonesia, Peru, India and Finland.[4]

ENVIRONMENTAL FACTORS INFLUENCING SURVIVAL AND OUTBREAKS OF INFECTION

Transmission

Anthrax is primarily a disease of herbivores, although other mammals and some avian species can become infected. The organism can be isolated in large numbers from the blood or tissues of recently-dead animals. As the carcass decomposes (particularly in warmer climates) putrefactive bacteria out-compete *B. anthracis* and may eventually eliminate the infective organism from the carcass. Confirmation of anthrax may then depend on the isolation of spores which have contaminated the soil through terminal discharges

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from the carcass.[5–7] Vegetative cells within the carcass die rapidly, killed by putrefaction, when released in body fluids, sporulation occurs following exposure to air. The latter process is delayed if the temperature is below 20 °C.[8]

Animals are usually infected by ingesting soil-borne spores in contaminated food or water. Spores can be picked up directly from the soil through grazing and particularly during periods of drought when animals are forced to forage much closer to the ground.[8] Other routes of infection include biting insects, which transmit the organism between animals,[9–11] or from ingestion of vegetation contaminated by deposition from infected carcasses.[12] De Vos reported that anthrax epidemics in 1960 and 1970 in the Kruger National Park coincided with an explosive population of blow-flies.[12] Carnivores and scavengers appear relatively resistant to anthrax[13] with ingested spores passing through the gastrointestinal tract and contaminating the soil through the deposition of faeces.[9,14,15]

Survival

The spores survive for long periods in the soil and are highly resistant to heat, cold, humidity, chemical disinfectants, and long dry periods. The island of Gruinard off the north-west coast of Scotland was deliberately contaminated by the exploding of small devices containing anthrax spores during the Second World War; soil remained contaminated for over 40 years prior to its successful decontamination.[16–18] Viable spores have been recovered from soil samples stored in the laboratory at room temperature (10–38 °C) for over 60 years[19] and in the Kruger National Park, *B. anthracis* has been recovered from bones estimated to be 200 years old.[12]

Studies of the distribution of spores on Gruinard Island, 40 years after their initial deposition to the surface, indicated that most remained in the top 4 cm of topsoil.[16] There appeared little evidence of natural redistribution of spores from the point of deposition on the surface and movement was considered to be due to rainfall and surface build-up of soil humus. Data from the Kruger National Park also indicate that redistribution was mainly due to rainfall with a decrease in the number of cases of anthrax occurring during the rainy season.[12]

There are however conflicting opinions regarding the ability of spores to germinate and multiply in soil as vegetative cells. Van Ness[20] considered that spores in heavily contaminated areas could be eliminated through biological competition or soil acidity. From studies of epidemiology of anthrax in the US he observed that anthrax outbreaks were more likely where the soil pH was greater than 6.0 and the ambient temperature was above 15.5 °C. Factors such as an appropriate temperature and soil moisture content were considered prerequisites for outbreaks to occur with the involvement of the vegetative state. He concluded that epidemiological evidence indicated the occurrence of anthrax in areas where the organism survived ecological competition with other microorganisms; animals became infected following a period when biological conditions favoured the multiplication and sporulation of the organism in soil.

Van Ness proposed that micro-environments such as depressions in which water had stood long enough to kill the grass, or dried-up water courses and hillside run-offs where organic matter had accumulated, provided suitable 'incubator environments' for germination and multiplication of *B. anthracis* spores. Infection of animals was observed to occur later through ingestion of spores when the earth became dry and dusty.

Alkaline or calcareous environments also appeared to be associated with areas of high infectivity. During 1957, outbreaks in Oklahoma were only associated with limestone areas, and absent from areas of shale and sandstone hills. Other geological studies of epidemics in the Mississippi valley and coastal regions of Louisiana and Texas also indicated that outbreaks were frequently associated with calcareous soils, rich in nutrient. Areas of high anthrax infectivity were also shown to closely parallel the cattle drive trails of the 1800s.[20]

In laboratory culture, *B. anthracis* is more fragile and less able to survive in simple environments than most other *Bacillus* spp. In water, survival of vegetative cells was negligible after 24 h, indicating that sporulation is an essential survival mechanism.[21]

The factors that favour survival in soil have been evaluated by a number of groups and the conclusions are not always consistent. Minett and Dhanda seeded both sterilised and natural soil with a known inocula of spores and incubated samples in flasks at 25 °C.[22] An increase in the number of spores was recorded in sterilised soil where the water content was in the region of 10–20%. No increase was observed in the natural soil.

The addition of blood to soil samples from the island of Gruinard resulted in a significant increase in spore numbers when incubated for 7 days at 22 °C and 37 °C, the pH of the acidic soil was raised from 4.5 to 7.2. Soil cores supplemented with faecal pellets from rabbits also showed a small increase in numbers when incubated at 37 °C. It was considered that the death of an animal in a contaminated area during a period of warm weather could provide a favourable environment for the germination of spores, followed by growth and development of increased concentrations of spores at the soil surface; on Gruinard with its acidic soils such occurrences were not observed.[16]

While the incubator theory of Van Ness remains controversial, outbreaks of anthrax do appear to be seasonal and the factors favouring outbreaks appear to have some regional variation. Areas of high contamination appear to be associated with soils above pH 6.0, soils with a high nitrogen content, rich in organic matter, high calcium levels, ambient temperatures higher than 15.5 °C and dramatic

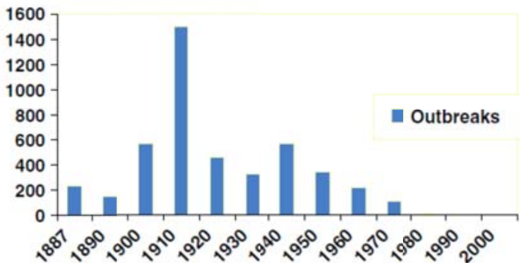

Figure 1. Anthrax outbreaks in livestock in Great Britain 1887–2000.

changes in climate, such as abundant rainfall following a prolonged drought.[20,23]

A contrasting view suggests that the low level of survival of anthrax spores in New Zealand is due to the high competitive microbial activity.[24]

OUTBREAKS IN THE UK

In the UK outbreaks of anthrax are now relatively rare and, when they do occur, are often the result of imported bone meal or animal hides. The Anthrax Order of 1886 made anthrax a notifiable disease in the UK and in 1887, 236 outbreaks were recorded across 51 counties involving some 640 cattle, sheep and pigs. Major outbreaks occurred across 80 counties during 1909 and 1910 involving 3500 cattle, sheep, pigs and horses. Since those peak years there has been a steady decline (Fig. 1), but as recently as 1977, 139 outbreaks were reported across 29 counties, involving 153 cattle, 13 pigs and one dog.[25] Since 1987 outbreaks have been in single figures.

Patterns of farming and those industries associated with animal products such as tanning, and bone meal manufacture and the use of horse-hair plaster in building construction have all had an impact on the occurrence of outbreaks of infection.

In the UK anthrax has diminished as a significant threat to human health. The occurrence of spores in the soil is associated with a diminishing number of historical hot-spots of activity. Sporadic outbreaks in farm animals are derived from ingestion of spores from soil at sites associated with their presence from previous outbreaks and the burial of carcasses on the site, or associated with the processing of animal products such as tanneries.

In 1994, three cows on a farm in Gloucestershire, UK, were infected by anthrax spores thought to have been left in the soil after another animal with the disease had been buried with quicklime 52 years earlier.[26] In 1996 three cattle died in an outbreak in Wiltshire, presumed to have been the result of ditch-dredging work which disturbed spores which had lain dormant for decades. The spores were considered to have been carried along a stream from an abandoned leather works on the outskirts of Westbury, where infected hides were reported to have been buried in the past. The farm was about a mile-and-a-half downstream from the works.[27]

The last confirmed case of anthrax in the UK was in 2002 on a farm in the Wrexham area and was the first outbreak in the UK since an outbreak in Lanarkshire in 1997. The case was confirmed following the sudden death of a dairy cow on a farm which had suffered three previous anthrax cases in the last two decades.[28]

The Anthrax Order of 1991 replaced the revised Anthrax Order of 1938.[29] It included anthrax as a notifiable disease under the Animal Health Act with any occurrence or suspicions of disease to be notified to the DEFRA (previously MAFF - the Ministry of Agriculture, Fisheries and Food) Divisional Veterinary manager. The order provides for a veterinary inquiry to determine the existence of disease and the action to be undertaken at infected sites. Movement of animals is controlled and local authorities have the power to dispose of carcasses by incineration at the site of infection or by means approved by the Divisional Veterinary Manager.[30]

Anthrax became a notifiable industrial disease under the Factories Act in 1895, and in 1960 became a notifiable disease under the Public Health Act. Data relating to outbreaks at certain factories since 1895 are available but information relating to morbidity in the general population is only available from 1961. Between 1961 and 1980, 122 cases of occupationally-associated anthrax were reported, resulting in six fatalities. During the same period, 26 cases not associated with occupational exposure were reported and resulted in six fatalities.[31]

Following the introduction of vaccination in 1965, for workers considered to be at risk, there was a significant reduction in cases associated with occupations involving the handling of animal wool, hair and bristle fibres. Other outbreaks reported were associated with the meat trade, the handling of bone-meal, farming and horticulture, dock workers and sailors, and the handling of leather goods. The majority of the non-occupationally-derived infections were due to the handling of bone-meal as a garden fertiliser.[31]

The last two deaths in the UK both occurred in 1974 with one patient suffering haemorrhagic septicaemia and generalised infection with *B. anthracis* and the other with gastrointestinal and pulmonary anthrax. Both were considered to be associated with the use of bone-meal fertiliser. Since 1981, 16 possible cases of anthrax have been notified under the Public Health (Control of Disease Act, 1984).[32] One case was considered to be misdiagnosed and one was de-notified, leaving 14 cases on the register. All of these were cases of cutaneous anthrax, five were associated with the meat trade and others with imported wool and leather, bone-meal fertiliser, or handling animal products abroad. The last two cases reported to the HSE under RIDDOR (Reporting of Injuries, Diseases and Dangerous Occurrences Regulations) were in the financial years 1991/92 and 2000/01.[33,34]

B. anthracis still presents a challenge to public health in many parts of the world. In the UK however the threat of a bioterrorism incident appears to present more risk to public health than the occasional and diminishing threat from contaminated soil and animal products.

Recent cases in the UK

The United Kingdom Anthrax Reference Unit at the Health Protection Agency Laboratory, Centre for Emergency Preparedness and Response, Porton Down, provides for the diagnosis of anthrax or detection of *B. anthracis*. Over the past seven years a wide range of samples have been received for analysis from environmental health officers, consultants, clinicians and developers. Samples received include soil, horse-hair plaster, horse-hair, bristle from shaving brushes, bone, wool, sacking, flakes of paint, dust and debris, filters, sewage and sludge, fatty deposits, flesh deposits, meat, culture isolates, serum, blood, heroin, swabs and culture media. Since 1995 there has been a steady increase in the number of environmental samples received for analysis (Fig. 2). During the last quarter of 2001, enhanced surveillance for *B. anthracis* in the UK, due to the bioterrorist-related incidents in the US, resulted in a significant increase in the number of samples received for analysis (Fig. 3). During this period two cases of occupationally-derived anthrax were identified.

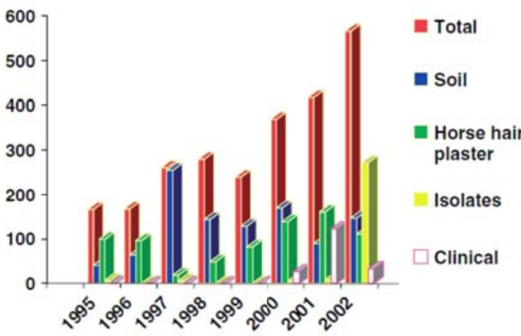

Figure 2. Samples received by the UK Health Protection Agency *B. anthracis* reference laboratory for analysis for the presence of *B. anthracis* spores from 1995 to 2002.

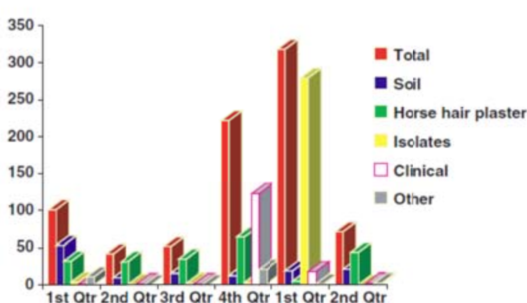

Figure 3. Samples received by the UK Health Protection Agency *B. anthracis* reference laboratory (2001 and the first half of 2002) for analysis for the presence of *B. anthracis* spores before and during the period of heightened alert following the deliberate release of anthrax spores in the USA.

A 55-year-old man who handled animal skins and hides was notified to the Communicable Disease Surveillance Centre (CSDC) with a diagnosis of cutaneous anthrax which was occupationally acquired. He presented to his general practitioner (GP) with swelling around a painless scab on his right forearm. He was treated with antibiotics for suspected impetigo but returned to his GP after a further four days when the scab became necrotic. At this stage the case was notified to Nottingham Health Authority as suspected anthrax and swabs were taken from the healed area by the consultant in communicable disease control. This was after a further three days of antibiotics. The swabs revealed no pathogens but a serum sample taken a week later showed antibodies to protective antigen (PA) and lethal factor (LF) specific for *B. anthracis*.[35]

A second case involved a 50-year-old man who returned from Africa with malaria and a skin lesion, he reported flu-like symptoms to his GP. His travel history indicated Rickettsial infection and he was treated with antibiotics. The GP requested serological analysis for Rickettsia and three samples were supplied over 25 days. After 36 days a request for serological analysis for Rickettsia and anthrax was requested. A positive serological response to both PA and LF, which are specific for anthrax, was confirmed. Before, and on his return from his fishing holiday in the Gambia, he was employed in building renovation work on an old water-mill. He had removed large amounts of horse-hair mixed with old plaster and wore no protective clothing. Environmental sampling indicated positive identification for anthrax by the polymerase chain reaction (PCR).[36]

During the period from 1981 to October 2001 only 14 cases of anthrax infection were registered, in the 3-month period October 2001–December 2001, two cases were recorded. This was a period of heightened surveillance in the UK and raises the question that cutaneous anthrax is under-diagnosed in the UK, with anthrax skin lesions being treated as unidentified bacterial infections which are generally successfully treated by administration of antibiotics.

SAMPLING AND DETECTION STRATEGIES

Where contamination of a site is suspected, environmental sampling is required to determine the degree and extent of contamination. This enables decisions to be made on the likely degree of human exposure, the on-going risk to human health and provides information to support the development of decontamination strategies. Environmental sampling generally results in a heterogeneous range of samples containing a range of micro-organisms depending on the sample's source. In soil, depth, vegetation, the rhizosphere and water content all influence microbial distribution. Where large areas such as fields or former industrial sites are

involved sampling may be carried out using a statistically random manner using a grid system.[37] Turnbull has discussed problems and strategies for sampling.[38]

Personal protective equipment (PPE)

First responders and teams carrying out environmental sampling or decontamination are at risk from exposure to *B. anthracis*; a risk assessment of the site and the degree of operator protection must be made prior to entry. Emergency responders generally need greater levels of protection since they may be responding to incidents involving unknown agents and be exposed to agents which have been aerosolised; PPE will include respiratory devices, protective clothing, gloves and overshoes.[39,40]

Face and respiratory protection should be provided using a powered air-purifying respirator with full face-piece and high-efficiency particulate air (HEPA) filters. The constant flow of clean air into the face-pieces ensures contaminated air cannot enter gaps in the face-to-face-piece seal. These respirators also give operators greater mobility and field of vision. Disposable protective clothing with integral hood and boots not only protects the skin but can eliminate the likelihood of transferring contaminated dust to places away from the work site. Disposable rubber shoe coverings with ridged soles made of slip-resistant material over the booties of the disposable suit will reduce the likelihood of slipping on wet or dusty surfaces. All PPE should be decontaminated immediately after leaving a potentially contaminated area. Protective clothing should be removed and discarded before removing the respirator.

Disposable lightweight nitrile or vinyl gloves protect hands from contact with potentially contaminated dusts without compromising dexterity. A thin cotton glove can be worn inside a disposable glove to protect against dermatitis, which can occur from prolonged exposure of the skin to moisture in gloves caused by perspiration.[39,40]

Sampling on Gruinard

Gruinard Island off the west coast of Scotland was heavily contaminated with spores of *B. anthracis* during biological weapons trials during the Second World War. Small bombs containing a slurry of spores were detonated, mostly suspended six feet above ground but one was dropped by an aircraft. The trials resulted in extensive contamination and routine sampling and analysis from 1948 to 1972 confirmed the persistence of viable spores.

In 1979 extensive sampling was carried out to establish the distribution of the spores prior to the development of a decontamination strategy.[16,17] The northern end of the 211 ha island was marked out in a 200 m grid system with marker posts and the more heavily contaminated southern half in a 100 m grid. The latter was further subdivided by the positioning of marker posts in the centre of each square. Soil core samples were taken at each marker by pushing a hollow tube (350 mm long and 25 mm internal diameter) into the ground to a depth of 300 mm. In the laboratory the cores were transferred to glass jars and shaken with twice their weight of 0.1 M phosphate buffer (pH 7.0) for 3 min on an orbital shaker. After heating to 60 °C for 1 h to destroy vegetative cells, the large particles were allowed to sediment before spreading 0.2 cm^{-3} of the supernatant fluid to nutrient agar plates. The large numbers of aerobic spore-formers recovered required dilution of the soil extract to enable colonies *of B. anthracis* to be distinguished. These were sub-cultured and purified before carrying out confirmatory tests such as phage sensitivity and penicillin sensitivity. The mouse LD_{50} test was used to confirm identity and virulence of isolates.

Dilution of the extract before plating reduced the chance of recovering spores at low concentrations and subsequent testing used the selective medium (polymixin–lysozyme–EDTA–thallous acetate, [PLET]) agar developed by Knisley[41] which incorporates polymixin to inhibit growth of saprophytic spore-formers. Recoveries were also improved by mixing the soil with twice its weight of distilled water, agitating for 5 min in a domestic blender, and straining the soil slurry through a double layer of cotton gauze. The spreading of 0.2 cm^3 undiluted samples to PLET agar plates enabled detection levels of three spores per gram of soil.

The study indicated that nearly all the viable spores recovered were found in the top 6 cm of soil, considered in part due to the low accumulation of humus on the surface delaying the rate at which they were buried.[16]

Sampling of buildings

Where the contamination of buildings has occurred it is essential to consult with building engineers to consider airflow patterns and the design and location of heating, ventilation and air-conditioning systems in the building. Since ventilation systems often recirculate air to other parts of the building these should be closed down immediately to prevent any further air-borne spread of spores. Access control to the contaminated area is essential and the affected area can be sealed using a dust barrier such as impervious lightweight plastic sheeting sealed into place using a suitable adhesive tape. Air vents and ducts should be similarly sealed to prevent further dispersal. Where particle size is less than 10 μm, spores will remain suspended in the air for some time, enabling spread to adjacent areas.[42,43] Personnel entering the area must follow a safety and infection control regime which may include vaccination and prophylactic antibiotic therapy.[44]

A sufficient number of samples must be taken to ensure the sampling is representative of the situation. Bulk sampling of materials such as areas of carpet and furniture can be made, however these will require double-bagging and do present an increased hazard to laboratory staff when being handled. Collection

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of samples by vacuuming can be carried out using suitably-designed HEPA vacuum cleaners. Home or industrial cleaners will result in further dispersal of spores. The HEPA filter exhaust can be preferably vented outside of the contaminated area via a long hose to avoid re-aerosolisation of spores within the contaminated area. Dust collected should be transferred to suitable containers for transfer to the laboratory. Alternatively, samples can be collected on a suitable cellulose ester filter contained in a suitable cassette unit. Filters can then be returned to the laboratory within the housing for subsequent transfer to selective media.

All material removed from the infected area must be double-bagged and transported in appropriate safety containers. The surface of the first bag must be cleaned with an appropriate and approved disinfectant prior to transfer to a second clean bag for transport. All equipment used will need to be appropriately decontaminated after use.[42,43]

Surface sampling

Surface sampling methods for the recovery of spores of *B. anthracis* had not been developed and validated prior to the contamination of buildings in the US during October 2001. Initial sampling protocols used in postal facilities at The Southern Connecticut Processing and Distribution Center indicated no evidence of *B. anthracis*. Dry synthetic swab samples were initially taken at random sites in the facility. During a second sampling ten days later, surfaces where letters, flats (large envelopes and flat packages) and parcels were processed were swabbed, along with three of the 13 digital bar-code sorting machines and five air circulating units. Four days later wet synthetic swabs were taken from letter-cancelling and sorting machines, flat- and parcel-sorting machines and five vacuum filter units. All of these samples showed no evidence of *B. anthracis*.[45]

A fourth sampling strategy in the facility was based on epidemiological data which was derived following the death of a 94-year-old woman in Connecticut whose mail had been processed by the facility. The sampling strategy was focused on the machines likely to have processed mail delivered to the patient's address and wet synthetic wipes were used together with HEPA vacuum collection as an alternative to wet and dry swabs. Six samples of the 212 collected yielded positive *B. anthracis,* two from the vacuum-collected samples and four from wet wipe samples. Further sampling using these methods indicated the presence of spores in collecting bins for the carrier route to the patient's home.

This sampling regime required large numbers of samples to determine the presence of spores and its effectiveness was enhanced by the provision of epidemiological data (the potential route and sorting machinery associated with the contaminated mail).[45] The use of wet and dry swabs had provided no evidence for the presence of *B. anthracis* although comparative data at the same positions were not available.

An investigation of the Brentwood Mail Processing facility in Washington was carried out in December 2001, shortly after the Connecticut study.[47] Comparative studies were made using dry and wet swabs, wipes and HEPA vacuum sock sampling. Sample areas at each site were divided into three and sampled with swabs, wipes and HEPA vacuum sock sampling. Good agreement was observed between results obtained with HEPA vacuum and wipe samples but there was poor correlation with swab samples. Wet and dry swabs failed to detect spores in greater than 33% and 66% of sites respectively, where spores were detected by HEPA vacuum or wipes.[46]

Wipe samples collected following HEPA vacuum samples and HEPA vacuum-collected after wipe samples indicated that neither method completely removed all spores from the surface sampled.[46] Swab samples were collected using sterile rayon (non-cotton) swabs. Wet swabs were moistened by dipping into phosphate buffered saline (PBS) at pH 7.2 and swabbing the surface by moving the swab back and forth with several horizontal then several vertical strokes. The swab was rotated during sampling to ensure the whole surface was used. Wipe samples were collected on 7.6 cm × 7.6 cm rayon gauze pads pre-moistened with 5 cm^{-3} of sterile distilled water. The surface was thoroughly wiped back and forth using several vertical strokes and then after folding the exposed pad, making several horizontal strokes over the same area with the other side of the pad.

B. anthracis was extracted from swabs and wipes by adding 20–30 cm^{-3} of 0.3% (w/v) Tween 20 in PBS in a tube and vortexing for 3 min. After allowing 3 min to settle, the swab or wipe was removed and the tube centrifuged to sediment the contents which were then re-suspended in 2 cm^{-3} of 0.3% (w/v) Tween 20 in PBS prior to microbiological analysis. The HEPA vacuum socks were added to a vessel containing 20–30 cm^{-3} of 0.3% (w/v) Tween 20 in PBS and placed on a shaker for 30 min. After allowing the contents to settle the extract was treated similarly to the extract from swabs and wipes.[46]

The use of rapid detection strategies

Alternative methods for rapid detection and identification include both antisera- or PCR-based strategies which can provide results within minutes and are used for on-site detection of environmental contamination.

Samples collected from the Brentwood sorting office were analysed using both on-site PCR and the culture technique.[47] Of 107 samples analysed, 95 (89%) were negative by both methods. Of six identified as positive by the culture method, two were positive by PCR. Of eight identified as positive by PCR, two were positive by culture. These were not part of a formally validated sampling strategy, however there was poor correlation between the two methods. Poor agreement of the on-site PCR method may have been

due to concentration of spores on the surfaces, or sample collection and preparation procedures, sample splitting and methods for removing the sample from collection material. PCR- and immune-based methods have the disadvantage of not distinguishing between viable and non-viable spores. They can produce positive results for samples which culture methods define as negative and are therefore not appropriate in evaluating the success of disinfection techniques that do not remove non-viable spores. The Centers for Disease Control and Prevention (CDC) consider that PCR- or antisera-based results themselves should be confirmed using standard culture methods before making public health decisions.[47]

Validation of sampling strategies

Concern over the lack of validation of many of the procedures used was expressed by the US Government Accountability Office (GAO).[48] The GAO reviewed the sampling activities undertaken by the US Postal Service (USPS), the Environmental Protection Agency (EPA), and the Centers for Disease Control and Prevention to detect anthrax in 286 postal facilities. They expressed concern that the strategies employed could not reliably exclude the presence of anthrax contamination in the facilities sampled. Of the 286 postal facilities tested, 23 tested positive, in two (West Palm Beach, and Wallingford, Connecticut) the first tests were initially negative but follow-up tests were positive; the Wallingford facility did not indicate the presence of anthrax until the fourth round of testing. In a building in West Trenton no anthrax was found in three rounds of tests, even though a postal worker had contracted cutaneous anthrax. The main points arising from the report were:

- A targeted sampling strategy was mainly used, collecting samples predominantly from areas considered the most likely to be contaminated. The GAO considered that probability sampling to achieve 'wide-area coverage' would have provided greater statistical confidence in the negative results.
- The report was critical of the methods used to collect samples, indicating that none were tested in advance and the agencies 'had no information available for reliably choosing one method over another and no information on the limits of detection to use when evaluating negative results'.
- Transportation of samples to the laboratory followed the appropriate regulations designed to protect workers and the public, but did not take account of factors which could compromise their biological integrity and lead to false-negative test results.
- There was a lack of definitive scientific information regarding the extraction efficiency of spores from swabs, casting additional doubt on the reliability of negative results.
- The agencies used any of four different preliminary tests and three confirmatory tests to identify anthrax in extracted samples. The GAO considered that the number of different tests used, combined with differences at other stages of the sampling process, increased the level of uncertainty about the results.
- The report said, 'The sampling strategy used by the agencies could not provide any statistical confidence with regard to the basic question: Is this building contaminated?'

The GAO report concluded that 'because the agencies did not use an empirical process to validate their testing methods, the agencies had limited information available for reliably choosing one method over another and no information on the detection limit to use when evaluating negative results'. 'The lack of validation of agencies' activities, coupled with limitations associated with their targeted sampling strategy, means that negative results may not be reliable'; they recommended that the secretary of homeland security ensure that pathogen detection methods are validated and environmental testing for pathogens by different agencies is coordinated.[48]

Aerosols

As a bioterrorist weapon inhalational anthrax is of greatest concern due to the relative ease with which spores can be dispersed in an aerosol. Spores dispersed in the air, either as tiny droplets of liquid or as dry particles, with a steady wind blowing, have the potential to cause a large number of casualties.[49,50] The air-borne spread of anthrax is very much determined by the particle size; spores will settle on various surfaces and on the ground and may be re-aerosolised by activity in the area, causing secondary dangers of infection.[50] Particles 1–2 µm in diameter penetrate into the lungs and are difficult for the lungs to eject.[51–54]

There is significant uncertainty relating to the number of spores required to constitute an infectious dose. Studies of the levels to which workers in goat-hair processing mills in the US were exposed indicated that approximately 1300 *B. anthracis* spores in particles 5 µm in diameter or less may be inhaled by workers during an 8-h shift without inducing infection in non-immunised workers.[55] Carr and Ray[56] examined the carriage of spores of *B. anthracis* in the nose and throat of 100 mill workers involved in the processing of goat hair.[57] No spores were recovered from throat swabs, however 7% of nose swabs and 7% of pharyngeal washes showed evidence of spores. No individual yielded spores from both the nose and pharyngeal washings.[56]

Inhalation studies using cynomolgus monkeys indicated an LD_{50} for aerosolised anthrax spores of around 5000–8000 colony forming units. Fatality in such experiments was typically 20–80%. The data derived from earlier studies of occupational exposure in processing mills and from studies with monkeys indicate the risk to humans of a low exposure to spores would not appear to be significantly dangerous.[51,55] During the recent incidents in the US however the

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source of anthrax spores resulting in two of the fatalities from inhalational anthrax remained unidentified after epidemiological investigation and environmental sampling.[58,59] Fennelly *et al*.[59] modelled the exposure of postal workers to spores and concluded that a number of factors had an impact on the risk of air-borne infection. These included the virulence of the organism, the production and removal of the infectious aerosol, pulmonary ventilation rate, duration of exposure and host susceptibility factors. Ventilation of the indoor environment to dilute the concentration of air-borne spores was considered an important determinant of the risk of infection.[59] Peters and Hartley[55] reviewed the data carried out on cynomolgus monkeys and considered that while an LD_{50} was related to 4000–8000 spores, an LD_{10} could be related to 50–98 spores and an LD_1 to 1–3 spores. While the extrapolation used may not be valid it did indicate that where a large population is given a low exposure to *B. anthracis* spores a small percentage may be susceptible through increased susceptibility to infection.

DECONTAMINATION STRATEGIES

Decontamination of Gruinard Island

Prior to the emergency in the US in October 2001 the island of Gruinard was the main example of successful decontamination of an area contaminated with anthrax spores.[18] As part of the development of a strategy for the decontamination of the island a number of different sporicidal disinfectants were evaluated in the laboratory and formaldehyde, glutaraldehyde, peracetic acid and dodecylamine used in trials on the island for the treatment of contaminated soil. In both closed and open plots dodecylamine had little effect in reducing the number of spores, however formaldehyde and gluteraldehyde and peracetic acid were successful in eliminating or significantly reducing spore numbers.[18]

Full-scale decontamination was initiated by spraying the vegetation with herbicide and then burning off after seven days. The site was then covered with 50 parallel runs of lay-flat horticultural irrigation tubing which delivered the biocide through pores on the upper surface. The lines of tubing were pegged at 900 mm apart. Seawater was pumped from the sea and mixed with formaldehyde to give a 5% (w/v) concentration. The area was divided into 40 treatment plots of about 1000 m^2 and each was irrigated for 10 min at a time until all plots had received 50 dm^3 of solution per m^2. At sites of higher contamination commercial grade formalin (38% formaldehyde) was injected to the depth of the bedrock through perforated brass tubes 700 mm apart; 1.75 dm^3 was inoculated per 50 mm of soil depth. Two months after treatment, soil cores were taken from 78 points, 58 of which had previously indicated the highest levels before decontamination and 20 selected at random. Only three of the 58 points of high contamination and three of the sites randomly sampled showed evidence of contamination. Positions that remained contaminated in the vicinity of the detonation point were found to contain 10^6 viable spores per gram at a depth of 50 cm. Further treatment involved spiking with stainless steel rods to the bedrock and then irrigation with 10 dm^3 of formalin. Further sampling six months after the end of the decontamination indicated the absence of spores even from areas which were originally heavily contaminated. Two months after decontamination the area was fertilised and re-seeded with grass. Forty sheep were allowed to graze on the island for five months and none showed any evidence of infection.

Decontamination at Sverdlovsk

The particle size is a key factor in determining the air-borne spread of anthrax. Spores will settle on various surfaces and on the ground and may be re-aerosolised by activity in the area, causing secondary dangers of infection. A major incident, resulting in the air-borne release of *B. anthracis* spores, occurred in 1979 in the Soviet Union.[60] In April and May of 1979, an anthrax epidemic broke out among residents in the city of Sverdlovsk (now Ekaterinberg) in the former Soviet Union. When news of the epidemic reached the west, Soviet officials claimed that the outbreak stemmed from supplies of contaminated meat. It was 13 years later that the Russians confirmed the epidemic had been the result of an accident at a bio-weapons manufacturing plant. The release was due to a breakdown in maintenance schedules when a new replacement exhaust filter was not fitted to drying and milling equipment. When the equipment was brought back into use after a weekend shut-down spores were released into the atmosphere.[61,62]

The wind-borne spread of anthrax caused a 6-week epidemic that claimed approximately 66 lives. Outbreaks in farm animals also occurred in villages down wind of the plume[62] and 47 000 of the 59 000 residents considered to be eligible (out of a potentially exposed population of 1.2 million), were given prophylactic immunisation.[49] Initially only minor efforts were made to clean the area and it was surprising that such a small number fell ill. The dead were placed in chloramine before burial. Eighteen of the victims were employed at a ceramics factory down-wind of the plume, this was decontaminated by spraying inside and out with chloramine.[63] The decontamination strategy involved spraying streets, pavements and trees with disinfectant. Firemen scrubbed roofs and walls of buildings with caustic solutions. Topsoil in contaminated areas was removed and buried, contaminated dirt roads were resurfaced with asphalt and the police shot stray dogs.[63]

Decontamination of buildings and facilities associated with the handling of animal products

Whilst workers in occupations associated with the risk of anthrax infection are vaccinated against possible infection, the buildings associated with these occupations may present potential risks. Demolition of

buildings or those undergoing change of use, which have previously been involved in the processing of animal products, may present particular hazards. The conversion or renovation of old buildings where horse-hair has been previously used to bind plaster have been associated with the presence of spores of *B. anthracis*. Any renovation or demolition work associated with older buildings where its use is suspected should undergo a risk assessment and environmental sampling. The use of appropriate personal protective equipment is essential and consideration should be given for the need to vaccinate workers.

Many of the early cases of occupationally-derived anthrax in the UK were derived from imported wool and animal hides. The work of Dr William Frederick Eurich, Professor of Forensic Medicine at the University of Leeds, during the early part of the 20th century concentrated on reducing the level of occupationally-derived anthrax. His research led to the discovery of a method to sterilise wool with formaldehyde with no detriment to the wool or workers' health. This led to the opening of a Government disinfection station at the port in Liverpool in 1921, where much of the contaminated material was brought into the country.[64]

In the late 1970s the redundant government wool-disinfecting station in Liverpool was due for demolition and presented particular problems of safety to workers and the general public. Although empty for some time, during the 1950s workers had contracted anthrax through the handling of contaminated, imported animal hides. After securing the site, 1.5% formaldehyde solution was sprayed onto the bricks and fittings of the part of the building that was to be demolished. The material was then crushed in a jaw-crusher before spraying again and loading via a conveyer belt into skips. Once loaded onto vehicles the skips were sheeted down and again sprayed with formaldehyde before burial in landfill. Vehicles were sprayed after unloading, before returning to the site. All timber was removed from the building and burned on site. All operatives were inoculated against anthrax and wore protective overalls and facemasks. All protective clothing was sterilised daily after use then washed before burning.[65]

Decontamination of a textile mill in the USA used vaporised formaldehyde (37% formaldehyde by weight) delivered through some of the windows of the building. The volume delivered was based on the size of the room and aimed to give a level greater than $0.5-1\ \text{cm}^3\ 0.028\ \text{m}^{-3}$. An approximately equal volume of water was vaporised simultaneously to maintain a relative humidity close to saturation. The building was closed and all the heating systems and fans turned on to maintain a temperature of above 26.6 °C. Biological indicators using an avirulent anthrax strain were used to determine the efficiency of the process and sampling using swabs was carried out prior to and after decontamination.[66]

Laboratories

Fumigation with formaldehyde is a generally accepted procedure for reducing microbial contamination in laboratories where the handling of dangerous micro-organisms has been carried out. Its effectiveness however is highly dependent on the physical conditions prevailing at the time; the area should not be regarded as decontaminated until its effectiveness has been confirmed through the use of appropriate biological indicators (spore strips). Fumigation should only be carried out by staff that are wearing approved protective equipment and have undergone an approved and appropriate training course. The ventilation system should be turned off and the room isolated from the ventilation system. The room should be left to fumigate for 6 h followed by a further 6 h for vaporisation of the fumigant to be completed. The levels of formaldehyde vapour in surrounding areas should be monitored and if greater than 1 ppm these should be evacuated. After removal of the biological indicator strips the room should be allowed to ventilate using the normal room ventilation system (Health Protection Agency standard operating procedures).

Decontamination of buildings in the USA post-October 2001

During September and October 2001, envelopes containing anthrax spores were mailed to news media companies and government officials in the USA, leading to 22 cases of anthrax infection (11 inhalational and 11 cutaneous) and resulting in five deaths.[67] Two letters posted in or around Trenton, New Jersey and post-marked September 18th were addressed to Tom Brokaw at NBC news and the editor of the *New York Post*. Two further letters addressed to Senator Tom Daschle and Senator Patrick Leahy were posted in or around Trenton and postmarked October 9th.

The letters posted on September 18th were initially processed at the Trenton Mail Processing and distribution centre in Hamilton, New Jersey and sent to the Morgan Central Postal Facility in New York City for sorting and delivery. Both of these facilities and five others associated with the Hamilton facility were later demonstrated to have environmental samples positive for anthrax. Evidence of spores was also found at two other media facilities, the American Broadcasting Company and the Columbia Broadcasting Company, although no evidence of other *B. anthracis*-positive letters was recovered: neither were any contaminated envelopes recovered from other sites in New York.

The letters mailed on October 9th were again processed at the Trenton facility in Hamilton before transfer to the US Postal Service Brentwood Mail Processing and Distribution Center in Washington, DC. The envelopes were processed through high-speed sorters at both facilities, probably resulting in aerosolisation of the spores. These and other cross-contaminated letters were then transported to various government mail facilities including the Sterling Mail

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facility in Loudoun County, Virginia which is the central mail receiving and distribution facility for the Department of State and distribution centre for all mail to US embassies throughout the world.[67,68]

The first cluster of nine cases of infection began about four days after the posting of the first two letters and were in five media company employees in New York, two postal workers from New Jersey and two media company employees from Florida.[69]

The second cluster began approximately five days after the posting of second two letters and included five postal workers from DC. Two cases from New York City in the second cluster were found to have handled the letter of September 18th posted to the New York Post when it was moved in mid-October prior to its identification. Three cases from New Jersey were postal workers and one was a book-keeper in a nearby commercial office building, environmental samples positive for anthrax were recovered from the work-sites of all four. The source of one case of inhalational anthrax in a woman working in a hospital stock room was not clearly identified and considered due to cross-infected mail. A 94-year-old woman in Connecticut who was not identified until November 14th also appeared to have been exposed to cross-infected mail. Environmental samples from the Wallingford sorting office in Connecticut were positive for *B. anthracis*.[67]

In addition to the immediate public health emergency during 2001 there was a legacy of contaminated buildings and facilities needing decontamination. The US EPA, working with a number of private contractors, the US Postal Service and the CDC, provided technical support for the monitoring and development of on-site clean-up strategies and clean-up validation. The costs for the environmental decontamination programme have continued to escalate and are now estimated to run into hundreds of millions of dollars. The Hart Senate Office Building on Capitol Hill, where the letter addressed to Senator Tom Daschel was opened,[70] was decontaminated in December 2001.[71] Initial costs for the clean-up of Capitol Hill were estimated at $5 million but the EPA eventually spent over $27 million from its Superfund project.[71]

Chemical disinfection procedures

The decontamination of buildings of significant complexity with regard to design and the equipment they contained required the development and validation of new procedures. Clean-up procedures required rigorous validation to gain the confidence of staff that were due to return to work in previously heavily contaminated buildings.

The EPA granted crisis exemptions for the use of a number of unregistered disinfectants for emergency use against anthrax in different decontamination strategies and at different locations. State or federal agencies were required to submit a written request describing the antimicrobial product(s) to be used; how, when and where they would be used; data demonstrating efficacy of the product for the intended purpose; and how human health and safety would be protected.[72] All decontamination strategies required extensive post-treatment environmental sampling to confirm that the treated areas were free from anthrax spores. The chemicals granted exemption certificates by EPA are discussed in the following paragraphs.

Paraformaldehyde

Paraformaldehyde was identified as being potentially effective for use on the surfaces and crevices of electronic and mechanical equipment. When heated, paraformaldehyde releases formaldehyde gas, which may be used as a decontaminant. Paraformaldehyde was authorised for decontamination of spores from a mail sorting and stamping device located at the Department of Justice mailroom in Landover.

Ethylene oxide

Ethylene oxide was authorised for the fumigation of items retrieved from congressional offices that were potentially contaminated with anthrax. It was also authorised for use by the US Department of Justice (DOJ) in order to test the fumigation process for mail received by the DOJ that was potentially contaminated with anthrax.

Registered liquid bleach products

Registered liquid bleach products contain sodium hypochlorite, used as a cleaner and to kill bacteria, fungi, and viruses. The EPA confirmed the sporicidal activity of bleach using the AOAC Sporicidal Activity Test (modified)[73] and issued a crisis exemption for the limited sale, distribution, and use of EPA registered bleach products for use against anthrax. Under this crisis exemption, only registered bleach products could be sold or distributed to employees of EPA, other federal, state, or local government agencies, and the US Postal Service for use in anthrax decontamination. Conditions of use were limited to application to hard surfaces only, with the bleach solution close to, but not above, pH 7 and 5000–6000 ppm with treated surfaces remaining in contact with the bleach solution for 60 min.

Methyl bromide

Methyl bromide, a broad spectrum pesticide used to control insects, weeds, rodents, and pathogens, is currently being phased out due to its contribution to the destruction of the ozone layer. EPA issued a crisis exemption to conduct limited testing to determine whether methyl bromide could be used for anthrax decontamination. Following review of the laboratory efficacy data to support the crisis exemption request, EPA determined that methyl bromide had the potential to be effective for use in a decontamination programme but an evaluation of the efficacy and feasibility of applying methyl bromide in buildings was required. A crisis exemption was issued for the limited sale, distribution, and use of methyl bromide

for use in a vacant mobile home by the University of Florida for carrying out an evaluation study.

Vaporised hydrogen peroxide

Vaporised hydrogen peroxide (VHP) is an antimicrobial pesticide registered by EPA as a low-temperature sterilant to kill bacterial spores on environmental surfaces in enclosed areas such as scientific workstations, isolators, pass-through rooms, medical and diagnostic devices, and for other biological safety applications. The EPA indicated that available data using the AOAC Sporicidal Activity Test[73] suggested that vaporised hydrogen peroxide would significantly reduce bacterial spore populations under specific conditions that include pre-cleaning, concentration, and contact time. A crisis exemption for the limited sale, distribution, and use of registered VHP for use against anthrax spores was issued for Vaprox™ Hydrogen Peroxide Sterilant for use by the General Services Administration and its contractors for anthrax decontamination at Building 410 in the Anacostia Naval Yard, Washington, DC.

Peroxyacetic acid

Peroxyacetic acid, like hydrogen peroxide, is an oxidising agent and when formulated as a liquid is used as a disinfectant and sanitiser, and is usually applied as a spray, or as a mop-on solution for eradication of bacterial spores, fungi, fungal spores, and viruses.

Available data using the AOAC Sporicidal Activity Test[73] suggested that hydrogen peroxide and peroxyacetic acid would reduce bacterial spore populations under specific conditions including surface type, concentration and contact time. EPA issued crisis exemptions for the limited sale, distribution, and use of four registered products containing both hydrogen peroxide and peroxyacetic acid and also one for a product containing only hydrogen peroxide. Applications of the five pesticide products was limited to specific buildings or treatment sites identified by EPA or other federal, state, or local governmental authorities, or the United States Postal Service.

Chlorine dioxide gas

Chlorine dioxide gas was first registered by the EPA in the 1980s as an antimicrobial pesticide. It was registered for the decontamination of manufacturing and laboratory equipment environmental surfaces and clean rooms. It is also registered for the washing of fruit and vegetables, disinfecting meat and poultry and decontamination of drinking water systems. It was used by the EPA for the decontamination of the Hart Building during November and December 2001. In March 2002 the crisis exemption for liquid chlorine dioxide was amended to specify its use to decontaminate hard surfaces only with a contact time of at least 30 min at room temperature.

Decontamination of the Hart Senate building

In the first major decontamination project, following the releases of anthrax in the US, the EPA used chlorine dioxide for the fumigation and decontamination of the Hart Building. After extensive environmental monitoring the building was prepared for fumigation by constructing isolation barriers in the 3000 sq. ft office suite to contain the gas. The EPA used mobile monitoring equipment to assess the concentration of any escaping gas which was found to be well below the 25 parts per billion exposure level considered acceptable. Prior to fumigation the humidity level was raised and then fumigation with chlorine dioxide carried out for a period of eight hours. Chlorine dioxide gas was then removed using sodium bisulfite. Three thousand test strips of surrogate spores considered to have a higher tolerance to chlorine dioxide than *B. anthracis* spores were exposed during the fumigation process. Initial problems involved maintaining the humidity at the required level and the concentration of gas across the building to ensure saturation point was reached to ensure killing of the spores. Fumigation was repeated to remove viable spores still detected in the heating and ventilation systems. The EPA also cleaned other offices and areas in the Hart building using different strategies employing chlorine dioxide liquid, foams and HEPA vacuum.[74–76] The Hart building was opened again in January 2002.[75]

Decontamination of Brentwood Sorting Office

The Brentwood sorting Office was the largest of the US facilities to be contaminated, extending to some 700 000 sq. ft. Decontamination of the site involved sealing all doors and windows, including more than 100 dock-doors and 235 skylights. All movable items including tubs, trays, and rolling equipment in the building were removed and cleaned using a bleach solution and all surfaces were decontaminated with bleach. After many trials and building on the experience from fumigation of the Hart building, chlorine dioxide gas was pumped into the building through the heating and air-conditioning system to kill the remaining spores. Extra gas emitters were installed in the building to increase the amount of gas in the building and mechanical equipment was run to assist in air flow and expose all parts to the gas.[77]

The gas was made on-site and maintained in the building from 12 to 48 hours. The temperature was controlled at 24 °C with 75% relative humidity. Monitors were placed throughout the building to ensure gas levels reached 750 parts per million and over 8000 spore test strips were used to monitor the effectiveness of the process. After fumigation the gas was pumped out of the building and shipped to a disposal company and the building aired. Secondary decontamination was carried out in areas suspected to be heavily contaminated by vacuuming with high efficiency particulate air vacuums to remove any residual dust or residue left from fumigation and followed by wiping surfaces with a

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bleach solution. The building was then re-sampled to confirm decontamination before re-occupation.[78–80] All personnel involved in the clean-up wore chemical protective overalls, gloves and boots to protect the skin and respirators to protect against inhalation of spores. As a precaution all workers involved in clean-up operations were given antibiotics and maintained on them for 60 days following their last day on the site.[81] The FDA commissioned a health-survey of all 1200 workers employed on the Brentwood site during the decontamination process.[82]

The Trenton processing and distribution centre was decontaminated during the summer of 2003 using the techniques initiated at the Hart building and extended at Brentwood.[83] The Sterling Mail Facility which handles all mail for the State Department was also due to be decontaminated during 2003 by the USA Army Corps of Engineers rapid response programme. The decontamination strategy involved initially disposing of as much of the contents of the building as possible to reduce the exposed surface for fumigation, cleaning selected hot spots with a chlorine solution and fumigating the whole building with vaporised hydrogen peroxide.[84,85]

LESSONS LEARNED

Anthrax spores provided a significant challenge for the development of reliable sampling and decontamination strategies; the experience gained however provides a strong evidence base from which to handle any future bio-threats. While different bio-threat agents present their own range of specific issues and problems, preparedness to deal with these types of emergency depends upon good planning, regular exercises and training.

Following the events of 2001 the US Government Accountability Office (GAO) reviewed the public health response to the anthrax incidents and the lessons learned to improve public health preparedness at the local, state and federal level.[86]

The Centers for Disease Control and Prevention (CDC) was placed under major pressure in supporting the demands from local and state officials and in coordinating the federal public health response in the face of the rapidly unfolding incidents. Dealing with the large amounts of information coming into the centre and communicating with public health officials, the media, and the public placed a significant strain on resources.[86]

Local and state public health officials indicated that pre-existing planning, exercises, and previous experience had helped promote a rapid and coordinated response, problems arose through not fully anticipating the extent of coordination needed among responders and in not having all the necessary agreements in place to put the plans into operation rapidly. Public health officials had difficulty reaching clinicians to provide them with guidance and sustaining the capacity of the public health workforce and clinical laboratories over a longer period would have been difficult to maintain. Three general lessons for public health preparedness were:

- The benefits of planning and experience.
- The importance of effective communication, both among responders and with the general public.
- The importance of a strong public health infrastructure to serve as the foundation for responses to bioterrorism or other public health emergencies.

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ORIGINAL ARTICLE

A study of the physiology of *Bacillus anthracis* Sterne during manufacture of the UK acellular anthrax vaccine

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Abstract

Aim: To analyse the growth of *Bacillus anthracis* during simulations of the UK anthrax vaccine manufacturing process.

Methods and Results: Simulated vaccine production runs were performed using the toxigenic, acapsulate Sterne $34F_2$ strain of *B. anthracis* in semi-defined medium. After rising during the logarithmic growth phase, the pH of the culture starts to fall at about 18 h from pH 8·7 to reach <7·6 at 26 h, coincident with consumption of glucose and optimal production of protective antigen (PA; 7·89 g ml^{-1}, SD 1·0) and lethal factor (LF; 1·85 g ml^{-1}, SD 0·29). No increased breakdown of toxin antigens was seen over the 26–32 h period. When glucose was exhausted, amino acids (principally serine) were utilized as an alternative carbon source. Sporulation was not observed during the 32 h.

Conclusions: PA and LF, the principal constituents in the UK anthrax vaccine, undergo little degradation during vaccine fermentation. The vaccine manufacturing process is robust and reproducible.

Significance and Impact of the Study: This is the first detailed analysis of the manufacturing process used for the UK acellular anthrax vaccine; insight gained into the process will support continued and safe vaccine manufacture.

Introduction

In the early 1950s, Wright and others succeeded in producing an anthrax vaccine in a protein-free, chemically defined medium which protected rabbits and guinea pigs against virulent spore challenge. When precipitated with potassium aluminium sulphate (alum), this antigen could also induce a degree of protective immunity in mice (Wright *et al.* 1954). Further work by Belton and Strange (1954) improved yields of antigen using a chemically defined medium supplemented with casamino acids, yeast extract and charcoal, which was thought to increase the potency of the final product by adsorbing or removing undefined toxin inhibitors. Concentrated antigen protected rabbits and monkeys when challenged with *Bacillus anthracis* Vollum M.36 spores via an intramuscular or aerosol route.

Anthrax vaccine has been manufactured at the Health Protection Agency's Centre for Emergency Preparedness and Response (previously Microbiological Research Establishment, later Centre for Applied Microbiology and Research) for nearly 50 years, and since 1979 under a Product Licence held by the UK Secretary of State for Health. During that time, there has been little product development or changes to the manufacturing process. In brief, production of the UK-licensed anthrax vaccine precipitated (AVP) involves growth of cultures of the toxigenic, noncapsulated *B. anthracis* $34F_2$ Sterne strain (Sterne, 1939) in a chemically defined medium supplemented with casamino acids and activated charcoal. Cultures are grown statically in 500 ml volumes in Thompson bottles at 37°C until the pH of selected culture bottles falls below pH 7·6. At the end of the growth period (approx. 24 h), the cultures are harvested and the pooled supernatants filter-sterilized. Alum solution is added and pH adjusted to 5·8–6·2. The precipitate is allowed to settle under gravity at 5°C. A 15-fold concentration (by volume) of the alum precipitate is effected by aspiration and this precipitate is diluted 1 : 3 with saline to provide a 'fivefold' concentrate of AVP i.e. the antigen

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used for vaccine formulation. Although AVP is superficially similar to the US acellular vaccine (BioThrax, previously Anthrax Vaccine Absorbed, Emergent Biosolutions, Lansing, MI, USA), the manufacturing process is very different, being a stirred-vessel anaerobic fermentation followed by absorption of the culture supernatant proteins onto Alhydrogel adjuvant; the Sterne-like seed stock strain used (V770-NP1-R) is reported to produce negligible amounts of lethal factor (LF) and oedema factor (EF) (Winberry *et al.* 2001).

Only limited studies have been performed on the growth of *B. anthracis* Sterne under conditions similar to those used in vaccine manufacture. Puziss and Wright (1959) performed an early study on carbohydrate metabolism and protective antigen (PA) production in cultures of *B. anthracis* grown statically. Culture growth was estimated visually and PA in culture filtrates was determined by immunization of guinea pigs and subsequent challenge with spores of *B. anthracis* Vollum. Individual antigens were not quantified and the relative contribution of these to protective efficacy could not be determined from the data collected. Puziss and Wright claimed that levels of 'PA' peaked at around 38 h, a time corresponding to glucose limitation. After this time levels of 'PA' declined.

There have been no reports detailing the physiology of *B. anthracis* Sterne during the UK vaccine production process, in particular on the kinetics of growth, substrate utilization, production of antigens, the possible contribution of sporulation to toxin production, and the effect of proteolytic enzymes on vaccine antigens. We have undertaken an extensive study of the physiology of *B. anthracis* Sterne under the conditions used during vaccine manufacture and are using this information to support continued production of the licensed UK acellular anthrax vaccine. In addition, these data will inform the debate on the relative immunoprotection afforded by the proposed second-generation anthrax vaccines currently in human clinical trials, all of which are based solely on adjuvanted recombinant PA.

Materials and methods

Cultivation and sampling of *Bacillus anthracis* 34F$_2$ 'Sterne'

Bacillus anthracis 34F$_2$ 'Sterne strain' was cultivated as described in the current AVP Product Licence Application (PL 1511/0058). Briefly, a spore suspension seed stock was diluted in sterile water (1 : 10) and heated at 60°C for 60 min. The spore suspension was held at 4°C overnight before being diluted to a concentration of 2×10^4 spores per millilitre with 'addition medium' (60 g l^{-1} $NaHCO_3$; 20 g l^{-1} glucose; 10 mg l^{-1} $MnSO_4 \cdot 4H_2O$). Thompson bottles containing 450 ml of basal medium (5·956 g l^{-1} casamino acids; 0·518 g l^{-1} KOH; 69·5 mg l^{-1} activated charcoal; 52 mg l^{-1} DL-serine; 25 mg l^{-1} $MgSO_4 \cdot 7H_2O$; 24·5 mg l^{-1} $CaCl_2 \cdot 6H_2O$; 20 mg l^{-1} L-cystine; 0·167 mg l^{-1} thiamine hydrochloride) were sterilized by autoclaving at 121°C for 15 min and then warmed to 37°C prior to inoculation with 50 ml of diluted spore suspension. Thompson bottles were incubated statically at 37°C for up to 32 h; a culture method which has historically been used to produce anthrax vaccine in the United Kingdom. Samples were taken throughout the time course. At each time point, three bottles were killed for sampling and analysis and designated A, B or C to aid analysis of data, effectively performing three runs in parallel. Culture pH was measured immediately after sampling. From each bottle a number of replicate samples was taken and assayed as described below. Viable counts were performed immediately; culture supernatant (0·2 μm filtrate) was stored frozen (−20°C) in aliquots until analysed.

Viable cell and spore counts

Total viable counts were determined by plating serial decimal dilutions (100 μl aliquots) onto tryptone soya agar (TSA, Oxoid, Basingstoke, UK) plates in duplicate and incubating at 37°C for 48 h. Spore counts were determined by heating the dilutions from the total viable counts at 60°C for 30 min to kill vegetative cells. The dilutions (100 μl aliquots) were plated onto TSA plates in duplicate and incubated at 37°C for 48 h. Plates with 30–200 colonies were used to calculate the viable counts and spore counts of the sample.

Glucose determination

The Amplex Red Glucose/Glucose oxidase kit (Molecular Probes, Eugene, OR, USA) was used to determine glucose levels in filter-sterilized culture supernatant.

SDS–PAGE gels and Western blot analysis for PA, LF, EF, Sap and EA-1

Culture supernatants were analysed by SDS–PAGE using 4–12% Bis-Tris precast gels (Invitrogen, Paisley, UK). Staining was performed using SimplyBlue Safestain (Invitrogen) according to the manufacturer's instructions. Gels were scanned using a CCD image analyser (Image Master VDS-CL, Amersham Pharmacia Biotech, now GE Healthcare, Little Chalfont, UK). Western blotting of gels was performed by electro transfer of the proteins to nitrocellulose membrane. The immunoblotting conditions used were as described by the manufacturer (Tobin transfer reagent, Invitrogen). Blots were blocked using PBST

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[phosphate buffered saline (PBS) 0·1% (v/v) Tween 20] with 2% (w/v) BSA (Sigma, Poole, UK) and probed with rabbit polyclonal hyperimmune antisera (anti-LF and anti-PA produced in-house by repeat immunization of rabbits with recombinant PA or LF and Freund's incomplete adjuvant; anti-Sap (surface array protein) and anti-EA-1 (extractable antigen-1) were kindly provided by DSTL, Porton Down, Salisbury) or goat polyclonal antisera (anti-EF, List Biological Laboratories Inc., Campbell, CA, USA), and detected using goat anti-rabbit horseradish peroxidase-conjugate (Sigma) or rabbit anti-goat horseradish peroxidase-conjugate (Sigma).

Quantification of PA and LF

Quantification of immunoreactive PA and LF in samples of filtered culture supernatants was undertaken using specific capture enzyme-linked immunosorbent assays (ELISAs). These assays have been extensively characterized to determine the optimum concentrations of antibodies and conjugates, and incubation times (King *et al.* 2006). Polyclonal antibodies [immunoglobulin G (IgG)] specific for PA or LF were purified from hyperimmune rabbit sera (generated in house by the immunization of rabbits with highly purified recombinant PA or LF), diluted in PBS to a final concentration of 2 μg ml^{-1} and coated onto microtitre plates (Immulon II, Thermo Fisher Scientific, Basingstoke, UK) at 100 μl per well. The plates were incubated for 18 ± 2 h at 4°C and washed three times using PBST before they were incubated with blanking buffer (PBST and 5% foetal calf serum) for 60 ± 10 min at 37°C with continuous shaking.

Samples containing PA and/or LF were serially diluted in blanking buffer and 100 μl per well added to the plates. After 60 ± 10 min at 37°C with continuous shaking, the plates were washed as above. Antibody-enzyme conjugate [anti-PA or anti-LF conjugated to horseradish peroxidase (HRP)] was prepared by first activating the HRP with sodium periodate and then using reductive amination to conjugate the activated enzyme to IgG [essentially as described by Hermanson (1996)]. Conjugate was diluted in blanking buffer and added (100 μl per well) to the plates. The anti-PA-HRP and anti-LF-HRP conjugates were used at a final dilutions of 1 : 5000 and 1 : 30 000, respectively. The plates were incubated for 1 h at 37°C with continuous shaking and then washed three times with PBST. The substrate solution (TMB Liquid Substrate System for ELISA, Sigma) added and incubated at 37°C with continuous shaking (25–30 min for the PA ELISA and 20 ± 2 min for the LF ELISA). The reaction was then stopped with 2 mol l^{-1} sulphuric acid (50 μl per well) and the absorbance measured at 450 nm using a Multiskan MS plate reader (Thermo Labsystems, Basingstoke, UK).

Macrophage cell lysis assay

The macrophage lysis assay was established essentially as described by Quinn *et al.* (1991). Optimal assay conditions were determined experimentally. J774A.1 monocyte/macrophage cells were obtained from ECACC (HPA, CEPR) and maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) with 3% (v/v) L-glutamine, 10% (v/v) foetal calf serum and penicillin/streptomycin solution (Sigma) at 0·5 IU ml^{-1} and 0·5 μg ml^{-1}, respectively. The cells were routinely grown in 75 cm^2 flasks at 37°C in a humidified 5% (v/v) carbon dioxide (CO_2) atmosphere. Cells were harvested by scraping growing cultures into pre-warmed (37°C) DMEM buffered with 10 mmol l^{-1} HEPES, pH 7·4 (DMEM/HEPES) and adjusting the cell density to 4×10^5 cells per millilitre. The cell suspension was plated at 200 μl per well in 96-well culture plates and cultured at 37°C in 5% CO_2; cells were allowed to settle and attach for 18 ± 1 h. Before use in the assay, plates were checked to ensure cells were greater then 80% confluent.

Samples and standards were prepared in dilution plates, diluted in 5% foetal calf serum in DMEM/HEPES. Medium and detached cells were removed from the assay plates by gentle aspiration and replaced (100 μl per well) with samples/standards. After a 3 h ± 10 min incubation period with toxin, cell viability was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) tetrazolium dye assay (Mosmann, 1983). 4·5 mg ml^{-1} MTT (Sigma) in PBS was diluted in 1 : 5 in DMEM/HEPES and 50 μl per well added to assay plates to effect a final concentration of 0·3 mg ml^{-1}. Incubation was continued at 37°C, 5% CO_2 for 60 ± 10 min to allow uptake and oxidation of the dye by viable cells. The medium was aspirated and replaced by 100 μl per well of 0·5% sodium dodecyl sulphate (w/v), 25 mmol l^{-1} HCl in 90% isopropyl alcohol and the plates shaken to dissolve the MTT (30 min). After visual inspection to ensure dissolution of MTT crystals, MTT absorption at 570 nm was determined using a Multiskan MS plate reader (Labsystems).

Amino acid analysis

Amino acid composition analysis was performed using high-pressure liquid chromatography (HPLC) analysis (Alta Bioscience, University of Birmingham, Birmingham, UK).

Results

Viable counts, spore counts and pH

Figure 1 shows the changes in culture pH, viable cell and spore counts throughout the bacterial growth phase of

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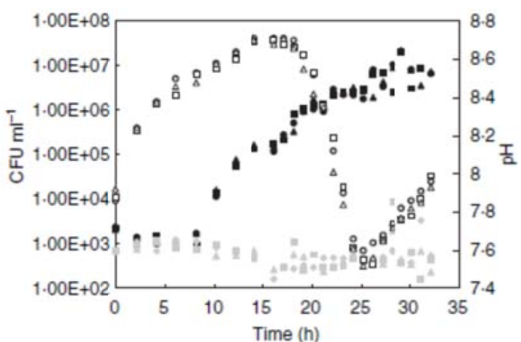

Figure 1 Viable counts, spore counts and pH determined for each sample throughout the time course. Viable counts are represented by solid black shapes; spore counts are represented by solid grey shapes; pH values are represented by open shapes. Samples from bottles A, B and C are represented by circles, squares and triangles, respectively.

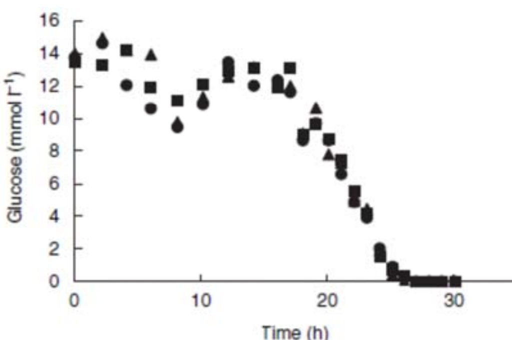

Figure 2 Glucose concentration of filter-sterilized culture supernatant from samples taken throughout the time course. Samples from bottles A, B and C are represented by circles, squares and triangles, respectively.

the vaccine manufacturing process. Replicate Thompson bottles harvested at the same time point gave comparable results, indicating a greater degree of consistency than might be expected given the sampling method used (single bottle sacrifice). Following a gradual rise in pH over the first 18 h, the culture pH shows a dramatic fall, concomitant with late exponential phase of vegetative cell growth. After 25 h the pH again begins to rise, and continues to rise until the end of the experiment. The spore counts remained constant throughout the 32 h studied here and indicate that approx. 30% of the spores used to inoculate each bottle do not go on to germinate. This general pattern of growth was observed in two additional studies of simulated manufacturing fermentations performed over a 2-year period (data not shown) and growth kinetics, pH and glucose data were essentially super imposable.

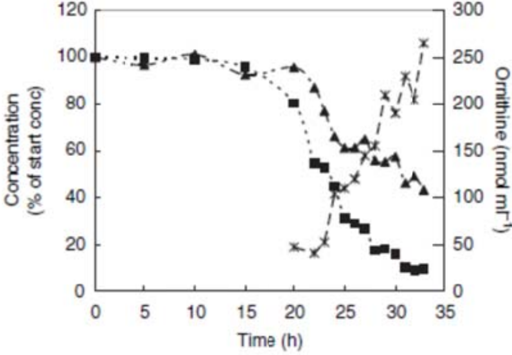

Figure 3 Serine (■) and arginine (▲) utilization and ornithine (✱) production by *B. anthracis* Sterne 34 F_2. Levels of amino acids are represented graphically as a percentage of their start concentrations (serine 2560 nmoles ml^{-1} and arginine 832 nmoles ml^{-1}).

Glucose utilization

Glucose in the culture was exhausted after approx. 26 h as determined using the Amplex Red glucose assay, concomitant with the observed fall in pH. Data are shown in Fig. 2.

Amino acid utilization

As glucose levels in the culture medium start to decrease at around 18–20 h, amino acids began to be utilized as additional carbon sources, but not to the extent that they are likely to become growth limiting. The principal amino acid utilized was serine, the decline of which paralleled that of glucose; arginine, glycine and tyrosine were utilized to a lesser extent. Figure 3 shows serine and arginine utilization, together with the increase in ornithine after 20 h growth. Ornithine production is consistent with the metabolism of amino acids, especially arginine. The fact that *B. anthracis* produces a range of extracellular proteases would seem to indicate that it will have the opportunity to utilize amino acids as alternative carbon sources. Other bacilli possess amino acid degradative enzymes which are subject to glucose repression; the activity of these enzymes is detected at the onset of stationary phase (Deutscher and Kornberg, 1968), and it would seem reasonable to assume that the same is true for *B. anthracis* and that amino acids are utilized by the bacterium as it adapts to nutrient-limited growth conditions.

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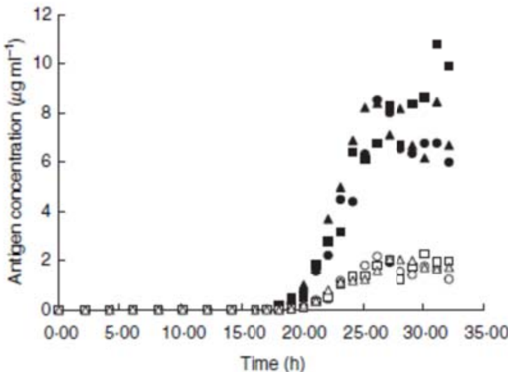

Figure 4 LF and PA levels in samples of filter-sterilized culture supernatants from cultures of *B. anthracis* Sterne $34F_2$. LF data are represented by open shapes, PA data by filled shapes. Samples from bottles A, B and C are represented by circles, squares and triangles, respectively.

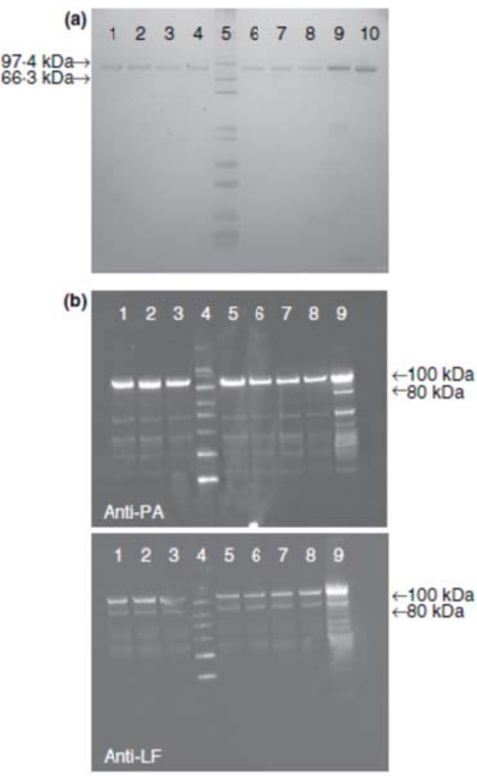

Figure 5 Antigen profiles of culture supernatants on a Coomassie-stained protein gel (a) and Western blots (b) probed with anti-PA and anti-LF antisera. Standards of PA and LF were loaded at a concentration of 1 μg per well. Only the relevant PA or LF standard was included on the blots. Markers on the gel were Mark 12 protein standards (Invitrogen) and on the blots were Magic Mark XP (Invitrogen). 20 μl of culture supernatant was loaded for each sample (approx. 10 μg protein). (a) Lanes 1: 26 h, 2: 27 h, 3: 28 h, 4: 29 h, 5: marker, 6: 30 h, 7: 31 h, 8: 32 h, 9: LF standard, 10: PA standard. (b) Lanes 1: 26 h, 2: 27 h, 3: 28 h, 4: marker, 5: 29 h, 6: 30 h, 7: 31 h, 8: 32 h, 9: standard.

Quantification of PA and LF

PA and LF present in the culture supernatants were determined using antigen-capture ELISA; results are shown in Fig. 4. Mean PA and LF levels in the culture supernatant were approx. 7·89 (SD 1·00) and 1·85 (SD 0·29) g ml^{-1}, respectively, at 26 h. This corresponds to the approximate point at which the cultures in the multiple Thompson bottles of a manufacturing run would have been harvested, and is marked by a fall in the pH below 7·6.

SDS–PAGE and Western blot analysis

Samples of filtered supernatant were analysed by SDS–PAGE and the proteins visualized and recorded using image analysis. Replicate gels were Western blotted and probed with IgG purified from hyperimmune antisera raised against PA, LF, EF, Sap and EA-1. HRP-conjugated second antibodies were used to detect immunoreactive proteins. As ELISA data were comparable for each of the triplicate bottle sets, only the 'Series A' samples were analysed by SDS–PAGE and Western blotting. PA was first detected in Western blots in the sample at 17 h, 1 h prior to its detection by ELISA. The other antigens were detected slightly later: LF was first detected at 19 h; EF Sap and EA-1 were all detected at 21 h. Close examination of Coomassie stained SDS–PAGE gels of 26 to 32 h samples shows that no substantial degradation is evident. Western blotting shows no increased breakdown of any of the antigens examined over this time period (data for PA, LF and EF are shown in Fig. 5). While a number of smaller bands are present, the intensity of these relative to the major, full-length antigen band remains constant. This indicates that proteolysis of these principal vaccine antigens is not a significant problem under the growth conditions used. Earlier samples in the time course show predominantly full-length antigen on Western blotting, but it is thought that this is more likely to reflect the lower protein loadings at the earlier time points, rather than increased degradation at later points. A commercially available protease substrate was used and demonstrated that proteases were present at extremely low levels – activity was detected only after prolonged (overnight) incubation at 37°C (data not shown). The detection limit of this assay calculated using trypsin was 50 ng ml^{-1}.

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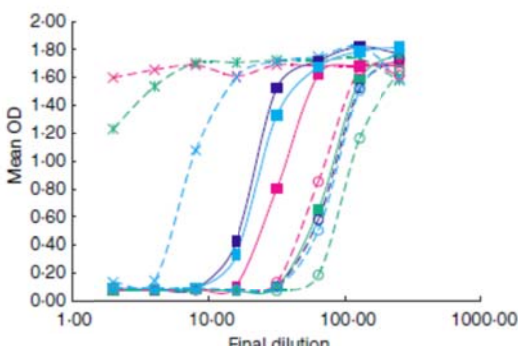

Figure 6 Analysis of Series A time course samples in macrophage cell lysis assay. 18 h; 19 h; 20 h; 21 h; 22 h; 23 h; 24 h; 25 h; 26 h; 27 h; 28 h.

Macrophage cell lysis assay of lethal toxin activity

The lethal toxin activity in the culture supernatants from each of the 'Series A' samples from throughout the time course was determined in a macrophage cell lysis assay. LT activity was first detected at 19 h and showed a trend towards increasing activity over the next few hours, consistent with the increased PA and LF levels in the culture supernatants detected by antigen ELISAs. No significant decrease in activity was observed, even towards the end of the time course (Fig. 6). Although the 24 h sample appeared to have a lower ED_{50} than expected, this reflects the values obtained in the PA ELISA, which indicates that the Series A sample at 24 h contained much less PA than the Series B or C sample, indicative of the potential variation introduced by the sampling method used.

Predilution of each sample to the same concentration of PA (as determined from ELISA data) allowed direct comparison of the specific activity of the samples which remained essentially constant from 24 to 32 h (Fig. 7). The ratio of PA : LF in the samples was approx. 4 : 1 throughout. Taken together with the Western blot data, these observations confirm that the LF and PA in the culture supernatants remain intact, even at later stages in the fermentation, and retain functionality in being able to combine on the macrophage cell surface to form lethal toxin which can and then be internalized and kill the macrophages.

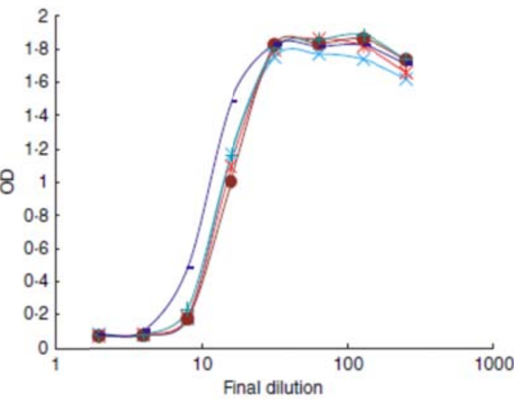

Figure 7 Macrophage cell lysis assay on time course Series A samples prediluted to equivalent starting concentrations of PA (2 μg ml^{-1}). Starting LF levels in the samples (calculated from the ELISA data) were 0·38, 0·56, 0·51, 0·45, 0·41 μg ml^{-1} for the 22, 24, 26, 29 and 32 h.

Discussion

The main rationale for this study was to generate baseline data profiling the kinetics of growth, substrate utilization, production of PA and LF, and relationship between pH and antigen production in the UK acellular anthrax vaccine production process. To this end, three dedicated simulation manufacture runs were carried out in the manufacturing facility. These simulation runs were treated in exactly the same manner as normal vaccine production runs with the exception of the periodic sacrificing of bottles for sampling. Individual bottle sacrificing was considered to be the best available method for sampling, as repeated sampling from the same bottle was not possible without disturbing the growing cultures, a phenomenon believed to affect growth and antigen production; pellicle/surface growth in the Thompson bottles is a notable feature of the process, and disturbance of this pellicle during the incubation period results in culture supernatant of reduced vaccine potential. This indicates a potential link between growth and the liquid/gas interface and head-space gas composition. Importantly, the critical requirement for pellicle growth may indicate the occurrence of density dependent signals linked to quorum sensing, and we are currently investigating this possibility; experiments have been performed where spent culture supernatants added to growing cultures of *B. anthracis* Sterne are shown to stimulate growth and antigen production (T. Mukhopadhyay, unpublished data). Additionally, *B. anthracis* Sterne is known to possess a functional *luxS* system and toxin synthesis is diminished after treatment with furanone inhibitors of quorum sensing (Jones and Blaser, 2003; Jones *et al.* 2005).

Analysis of the data obtained for culture pH, viable cell and spore counts, glucose utilization, PA and LF levels showed good conformity across all three simulation runs

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despite the potential for variability inherent to the practice of single bottle killing. Indeed, these profiles are consistent with those obtained in similar experiments performed in the same manufacturing facility over several years, demonstrating the robust nature of the vaccine production process despite its rather low-tech nature.

Growth kinetics showed a sigmoidal profile reaching late exponential phase after about 18 h with a viable cell count peaking with the onset of stationary phase at about 23 h growth, reaching approx. 10^7 viable cells per millilitre (Fig. 1). Interestingly, viable spore counts remained relatively constant between 10^2 and 10^3 counts per millilitre throughout the whole period of investigation, indicating that a fixed proportion of the spores (around 30%) present in the inoculum failed to germinate during the production process, although they are clearly viable and germinate on TSA; it seems likely that specific germination triggers are absent from the chemically defined production medium. In addition, this observation suggests that sporulation does not occur to any appreciable level during the fermentation process. This may simply be because of the fact that the necessary sporulation triggers, such as severe medium nutrient depletion, have not occurred at the 32 h time point at which sampling stopped. We know that sporulation is not initiated immediately on the onset of nutrient limitation and indeed is suppressed until alternative responses to starvation have been exhausted. Additionally, readily utilizable nitrogen sources strongly inhibit sporulation (Stephens, 1998) and although in the vaccine fermentation *B. anthracis* Sterne has switched to amino acid metabolism, these nitrogen sources have not been used up. Furthermore, the relatively low cell densities achieved at the end of the vaccine fermentation period will not favour sporulation (Grossman and Losick, 1988).

The pH of the medium shows an initial rise to approx. 8·7 coincident with the logarithmic phase of culture growth; however, the pH then shows a dramatic fall to below 7·6 as the culture reaches the end of logarithmic growth (Fig. 1), coincident with depletion of glucose (Fig. 2). The pH drop is believed to be caused by acid produced as a result of glucose metabolism. After approx. 25 h, the culture switches to extensive amino acid utilization (Fig. 3) and the resulting pH rise is consistent with the release of ammonium ions resulting from the metabolism of amino acids. Historically, the fall in pH to a value below 7·6 has been used as the end-point indicator for harvesting the production batch, when production of vaccine antigens in the process is presumed maximal. Glucose depletion is used as the harvest indicator in the US fermentor-based anthrax vaccine manufacture process (Puziss *et al.* 1963); nonetheless, both the parameters are coincident, reflecting their value as harvest indicators.

Little or no degradation of antigens in the samples was observed, even over the 26- to 32-h time period. This observation is noteworthy as it has been reported that high levels of protease activity are associated with an asporogenic derivative of *B. anthracis* Sterne (ΔSterne-1) if grown in a high yeast extract medium in the absence of higher levels of protecting tryptone (Frachaus *et al.* 1998). This adds credence to the use of a more defined type of medium in the UK-licensed vaccine manufacture process and the exact point of harvest.

Antigen levels during the manufacturing fermentation process were determined for PA and LF using sensitive and specific antigen-capture ELISA methods. These assays showed that PA and LF levels were close to maximum at the theoretical time of harvest. This study clearly demonstrates the presence of significant amounts of LF in culture supernatants used in the manufacture of the UK acellular anthrax vaccine, in contrast with the US vaccine strain, *B. anthracis* V770-NP1-R, which is thought to produce only minimal levels of LF and EF (Puziss *et al.*, 1963; Ivins *et al.* 1998). This is of particular importance to vaccine efficacy as several recent studies have implicated roles for LF (and also EF) in protective immunity studies using mice, in addition to the dominant role that PA plays in humoral immunity (Pezard *et al.* 1995; Price *et al.* 2001; Singh *et al.* 2002). As it is now becoming clear that components other than PA in the UK anthrax vaccine may well be contributing to the overall immunoprotection afforded, it will be of interest to discover further the identity and significance of components other than PA and LF in the vaccine. It is intended to perform further analysis on these samples to quantify the EF, oedema toxin (ET; PA EF) and S-layer proteins which were detected in these samples using Western blotting. The functional and immunoassays required to perform this analysis are currently being qualified in our laboratory. This information could also be used in the rational design of new recombinant anthrax vaccines.

In conclusion, this study has defined a baseline set of key physical, biochemical and microbiological parameters during growth of *B. anthracis* $34F_2$ Sterne strain in the UK-licensed anthrax vaccine manufacture process. Furthermore, it has been established that these parameters show good conformity within batches over a significant time period. This work has generated a basic understanding of the production kinetics and biophysical parameters within the culture system used for the production of anthrax vaccine.

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Original article

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Assessment of the Protective Effect of Imvamune and Acam2000 Vaccines against Aerosolized Monkeypox Virus in Cynomolgus Macaques

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To support the licensure of a new and safer vaccine to protect people against smallpox, a monkeypox model of infection in cynomolgus macaques, which simulates smallpox in humans, was used to evaluate two vaccines, Acam2000 and Imvamune, for protection against disease. Animals vaccinated with a single immunization of Imvamune were not protected completely from severe and/or lethal infection, whereas those receiving either a prime and boost of Imvamune or a single immunization with Acam2000 were protected completely. Additional parameters, including clinical observations, radiographs, viral load in blood, throat swabs, and selected tissues, vaccinia virus-specific antibody responses, immunophenotyping, extracellular cytokine levels, and histopathology were assessed. There was no significant difference ($P > 0.05$) between the levels of neutralizing antibody in animals vaccinated with a single immunization of Acam2000 (132 U/ml) and the prime-boost Imvamune regime (69 U/ml) prior to challenge with monkeypox virus. After challenge, there was evidence of viral excretion from the throats of 2 of 6 animals in the prime-boost Imvamune group, whereas there was no confirmation of excreted live virus in the Acam2000 group. This evaluation of different human smallpox vaccines in cynomolgus macaques helps to provide information about optimal vaccine strategies in the absence of human challenge studies.

Variola virus, the etiological agent of smallpox, is highly contagious and causes disease with a high mortality rate (1). Endemic smallpox was eradicated through a successful global immunization campaign by the World Health Organization more than 30 years ago (2), with the final natural case of smallpox recorded in Somalia in 1977 (3). Since the eradication, widespread vaccination against this pathogen has been discontinued, and so the majority of the world's population currently lacks protective immunity (4). As a consequence, the use of variola virus as a biological weapon poses a current major public health threat. Other orthopoxviruses, for example, human monkeypox, cowpox virus, and a variety of vaccinia virus-like viruses (5–8), also threaten public well-being. These orthopoxviruses are naturally occurring and usually spread to human beings by zoonotic infection. Since all of these orthopoxviruses pose a risk to public health, there is a renewed effort to develop and stockpile medical countermeasures such as safe, effective orthopoxvirus vaccines and therapeutic agents.

The traditional calf-lymph derived, smallpox vaccines (e.g., Dryvax) used in the eradication of smallpox are based on replicating vaccinia virus. They are highly efficacious; however, their use is associated with rare but severe side effects, particularly in immunocompromised individuals (9, 10). Adverse events include progressive vaccinia, eczema vaccinatum, myo/pericarditis, Stevens-Johnson syndrome, fetal vaccinia, encephalitis, and occasionally death (11). Second-generation smallpox vaccines, for example, Acam2000, have subsequently been developed and licensed. These vaccines are produced using the Lister-Elstree or New York City Board of Health vaccinia virus strains in qualified cell cultures according to Good Manufacturing Practice standards (12, 13). Although these qualified vaccine preparations are cleaner and appear to be as effective as earlier vaccines, there are still adverse events following vaccination (11). Thus, if these vaccines were used today, in a public health emergency, it is estimated that 25% of the general population would be at risk of developing complications (14).

Third-generation smallpox vaccines, such as Imvamune, manufactured by Bavarian Nordic (Martinsried, Germany), are currently being developed as safe and effective vaccines without the complications associated with traditional smallpox vaccines (15). Imvamune is based on a strain of the modified vaccinia Ankara (MVA) virus, which is a highly attenuated, replication-deficient strain of vaccinia virus. It was generated by more than 500 passages of vaccinia virus in chicken embryo fibroblasts, during which time it acquired multiple deletions and mutations and lost the capacity to replicate efficiently in people and most mammalian cells (16). In Germany, in the 1970s, MVA was tested in ~120,000 people. It was given as a preimmunization vaccine in combination with the Lister vaccine (a second-generation vaccine). Several high-risk groups were vaccinated, including young children with skin conditions (15–18), and there were no reports of serious adverse events using this two-step inoculation process (15).

It is not feasible to assess the protective efficacy of single or multiple doses of Imvamune vaccine in phase III human clinical trials because smallpox is no longer endemic in any part of the

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world. In order to progress licensing of effective medical countermeasures for biodefense, such as Imvamune, the U.S. Food and Drug Administration (FDA) has published the "Animal Rule" (19). This rule permits the approval or licensing of drugs and biological compounds based upon results obtained from an animal model that appropriately replicates the human condition. In the past, macaques have been used in studies employing both variola virus and monkeypox virus in order to model the ordinary disease presentation of smallpox infection in people (1). Since there are difficulties with working with variola virus, monkeypox virus infection in macaques has now become an acceptable surrogate model for human smallpox disease (20, 21), provided an appropriate dose and route of challenge such as aerosolization is used (1). Thus, this animal model is supported by the FDA and may provide valuable information on vaccine efficacy that could be used to aid licensing.

The purpose of the present study was to evaluate the protective effect of either a single dose of Imvamune, a prime and a boost of Imvamune, or a single dose of the licensed vaccine Acam2000 against disease following an aerosolized severe or lethal dose of the central African strain (Zaire 79) of monkeypox virus in cynomolgus macaques. Humoral and cell-mediated responses to vaccination were also examined.

MATERIALS AND METHODS

Experimental animals. Twenty-four captive bred, healthy, cynomolgus macaques (*Macaca fascicularis*) of Mauritian origin (12 male and 12 female) were obtained from a United Kingdom breeding colony for use in the present study. All of the animals weighed between 2.5 and 4.5 kg and were between 2 and 4 years of age at challenge. The monkeys were negative for neutralizing antibodies to orthopoxvirus prior to the start of the study. Animals were housed according to the United Kingdom Home Office *Code of Practice for the Housing and Care of Animals Used in Scientific Procedures* (1989) and the National Committee for Refinement, Reduction, and Replacement (NC3Rs) *Guidelines on Primate Accommodation, Care and Use, August 2006.* If a procedure required the removal of a primate from a cage it was sedated by intramuscular (i.m.) injection with ketamine hydrochloride (10 mg/kg; Ketaset, Fort Dodge Animal Health, Ltd., Southampton, United Kingdom). All procedures were conducted under a Project License approved by the Ethical Review Process of the Health Protection Agency, Salisbury, United Kingdom, and the United Kingdom Home Office. None of the animals had been used previously for experimental procedures.

Vaccines. Acam2000 Smallpox (vaccinia) vaccine was obtained from Acambis, Inc., Cambridge, MA. The freeze-dried vaccine was reconstituted in 0.3 ml of diluent, according to the manufacturer's instructions. Imvamune, modified vaccine virus Ankara-BN (MVA-BN), was manufactured by IDT Biologika GmbH (Germany) and was supplied by Bavarian Nordic A/S, Denmark, as a homogenous suspension. It was diluted in the diluent provided (Tris-buffered saline [TBS]) to give a final concentration of 2×10^8 50% tissue culture infective doses ($TCID_{50}$)/ml. The negative control for the experiment was TBS, the diluent used for the Imvamune vaccine.

Four treatment groups of six cynomolgus macaques were established. The first group of animals (TBS negative control) were inoculated with 0.5 ml of TBS 28 days prior to challenge. The second group of animals (Acam2000 ×1) were vaccinated with one dose of Acam2000 vaccine (2.5×10^5 to 12.5×10^5 PFU) at the same time. Both the TBS and the Acam2000 vaccines were delivered by scarification to the midscapular area with the use of a bifurcated-end needle. In the third group (Imvamune ×1), animals were vaccinated once with Imvamune (10^8 $TCID_{50}$ in a total volume of 0.5 ml) 28 days prior to challenge via the subcutaneous route. In the fourth group (Imvamune ×2), animals were vaccinated via the subcutaneous route with an Imvamune primer dose of 10^8 $TCID_{50}$ in a 0.5-ml total volume, 56 days prior to challenge, and an Imvamune booster dose (10^8 $TCID_{50}$ in a 0.5-ml total volume) 28 days prior to challenge. The distribution of male and female macaques in the study was as follows: the TBS negative control animals were male ($n = 6$), the Acam2000-vaccinated animals were female ($n = 6$), the Imvamune ×1-vaccinated animals were male ($n = 6$), and the Imvamune ×2-treated animals were female ($n = 6$). Each group of animals was kept separate to avoid cross contamination and/or spreading of the vaccine.

Monkeypox virus challenge strain. Monkeypox virus strain Zaire 79, NR-2324, was obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources, Manassas, VA). On the day of challenge, stocks of virus were thawed and diluted appropriately in minimum essential medium containing Earl's salts (Sigma, Poole, United Kingdom), 2 mM L-glutamine (Sigma), and 2% (vol/vol) fetal calf serum (Sigma).

Aerosol exposure and sampling. Monkeys were challenged with a target dose of 10^5 PFU of monkeypox virus using the AeroMP-Henderson apparatus; a flexible, highly configurable system in which the challenge aerosol was generated using a six-jet Collison nebulizer (BGI, Waltham, MA). The aerosol was mixed with conditioned air in the spray tube (22) and delivered to the nose of each animal via a modified veterinary anesthesia mask. Samples of the aerosol were taken using an SKC BioSampler (SKC, Ltd., Dorset, United Kingdom) and an aerodynamic particle sizer (TSI Instruments, Ltd., Bucks, United Kingdom); these processes were controlled and monitored using the AeroMP management platform (Biaera Technologies, LLC, Frederick, MD). To enable delivery of consistent doses to individuals each animal was sedated and placed within a "head-out" plethysmograph (Buxco, Wilmington, NC). The aerosol was delivered simultaneously with a measurement of the respiration rate. A back titration of the aerosol samples taken at the time of challenge was performed to calculate the presented/inhaled dose. The challenge was performed on 2 days and the mean presented dose on each day was 2.1×10^5 and 3.1×10^5 PFU/animal (the overall mean presented dose was 2.6×10^5 PFU).

Antibody concentrations, cellular-immune populations, and cytokines were monitored in the blood pre- and postchallenge. After challenge, the viral loads were monitored in the blood and throat. For the latter, a flocked swab (Copan Diagnostics, Murrieta, CA) was gently stroked six times across the back of the throat in the tonsillar area.

Lung imaging. Thoracic, dorsoventral, and ventrodorsal radiographs (SP VET 3.2; Xograph Imaging Systems, Ltd., Tetbury, United Kingdom) were acquired at day 9 postchallenge using Xograph mammography film. Lung pathology was evaluated by two consultant thoracic radiologists blinded to the animals' vaccination and clinical status, using a predetermined scoring system.

ELISA. Samples of blood were taken at various time points throughout the study. Serum was isolated and assayed for immunoglobulin G (IgG) serum antibodies to vaccinia virus using an enzyme-linked immunosorbent assay (ELISA). Maxisorp 96-well plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with a preparation of commercially prepared psoralen/UV-inactivated, sucrose density gradient-purified vaccinia virus (Lister strain; Autogen Bioclear UK, Ltd., Wiltshire, United Kingdom) in calcium carbonate buffer at 2.5 μg/ml. Unbound antigen was removed by washing the plates three times. The plates were blocked with blocking buffer (phosphate-buffered saline [PBS], 5% milk powder [Sigma], 0.1% Tween 20 [Sigma]) for 1 h at room temperature with shaking. Unbound blocking solution was removed by washing three times. Fourfold serially diluted serum samples (starting at 1:50) were added to the plate for 2 h at room temperature with shaking. Unbound antibodies were removed from the plate by three washes. The plates were then incubated for 2 h with shaking with horseradish peroxidase-labeled anti-monkey-IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Unbound detection antibody was removed by five washes and then developed using an ABTS [2,2′azinobis(3-ethylbenzthiazolinesulfonic acid)] peroxidase

substrate system (Kirkegaard & Perry). The development of the ELISA was stopped using the ABTS stop solution (Kirkegaard & Perry). ELISA titers were calculated and compared to a vaccinia virus immune globulin standard (BEI Research Repository Resource, Manassas, VA), which was used to convert the titer into arbitrary international units (AIU)/ml.

Flow cytometry. Whole blood was collected at time points throughout the study by using heparin as the anticoagulant. Antibodies to CD3e, CD4, CD20, and CD16 (BD Biosciences, Oxford, United Kingdom) and to CD8a (Invitrogen, United Kingdom) conjugated to R-phycoerythrin (PE)-cyanine dye (Cy7), allophycocyanin, PE, fluorescein isothiocyanate, and PE-Texas Red, respectively, were incubated with the blood for 30 min at room temperature. The red blood cells were removed from the whole blood by lysing them with Uti-Lyse reagent (Dako, Cambridgeshire, United Kingdom). Flow count beads (Beckman Coulter, High Wycombe, United Kingdom) were added to provide a standard to enable cell counts per μl of blood, before being acquired on the flow cytometer. The data were collected on an FC500 flow cytometer (Beckman Coulter) and analyzed with CXP analysis version 2.1 software (Applied Cytometry Systems).

Luminex analysis of cytokines. The concentrations of interleukin-6 (IL-6) and gamma interferon (IFN-γ) were determined in serum samples using a NHP 23 Plex kit (Merck Millipore, Billerica, MA) according to the manufacturer's instructions. Samples were acquired using a Luminex 200 system (Luminex, Austin, TX), and the data were analyzed using the Xponent software (version 3.0). The concentration of each cytokine in the serum was calculated based on a comparison with the corresponding standard curve generated using purified cytokines from the kit.

Monkeypox virus plaque assay. During the course of the study, EDTA-treated blood and throat swabs were collected and frozen at −80°C and, at necropsy, tissues were collected and snap-frozen in liquid nitrogen. Prior to testing, the tissue was thawed and homogenized in PBS by using a Precellys24 tissue homogenizer (Bertin Technologies, Villeurbanne, France). The titers of live infectious virus in the tissues, blood, and throat swabs were determined by plaque assay. Samples were incubated in 24-well plates (Nunc/Thermo Fisher Scientific, Loughborough, United Kingdom) with Vero E6 (ATCC CRL-1586; American Type Culture Collection, Manassas, VA) cell monolayers under MEM (Life Technologies, Foster City, CA) containing 1.5% carboxymethyl cellulose (Sigma), 5% (vol/vol) fetal calf serum (Life Technologies), and 25 mM HEPES buffer (Sigma). After incubation at 37°C for 72 h, the samples were fixed overnight with 20% (wt/vol) formalin-PBS, washed with tap water, and stained with methyl crystal violet solution (0.2% [vol/vol]; Sigma).

PRNT assay. Samples of blood were collected at designated time points prior to challenge and neutralizing, anti-vaccinia virus antibody titers were measured by plaque reduction neutralization (PRNT) assay. Heat-inactivated sera (56°C for 30 min) were serially diluted and incubated with ~50 PFU of wild-type Lister-Elstree vaccinia virus for 1 h at 37°C in 5% CO_2. The samples were then incubated with Vero E6 monolayers using the method described above. The neutralizing antibody titers were defined as the serum dilutions resulting in a 50% reduction relative to the total number of plaques counted without antibody, according to the Behrens-Karber formula (23). Titers were standardized to a standard preparation of human Vaccinia Immune Globulin CNJ-016 (BEI Research Repository Resource).

Virus detection by quantitative PCR. Tissue samples collected postchallenge and snap-frozen in liquid nitrogen were defrosted and homogenized in PBS using a Precellys24 tissue homogenizer. Viral DNA was isolated from homogenates by using a tissue kit (Qiagen, Crawley, West Sussex, United Kingdom) according to the manufacturer's instructions. Blood and throat swabs were processed using a Qiagen blood DNA minikit according to the manufacturer's instructions. Real-time PCR was performed using an Applied Biosystems 7500 Fast instrument (Life Technologies) with an in-house TaqMan assay targeted at the viral hemagglutinin (HA) gene and residues in the Z79 genome (GenBank accession no. HQ857562.1 [V79-I-005]; monkeypox virus, residues 158734 to 158798, inclusive).

Clinical and euthanasia observations. The clinical observations were scored, and routine in-house welfare assessments were made at regular intervals pre- and postchallenge. These included measurements of the rectal temperature and body weight. These parameters also fed into a euthanasia scoring scheme. Both clinical and euthanasia scoring schemes used a scale to indicate severity (0 = none, 1 = mild, 2 = substantial, and 3 = intense). Clinical observation score sheets were used to record anorexia, behavioral changes (depression/unresponsiveness/repetitive activity), nasal discharge, cough, dyspnea, and rash/skin swelling, whereas euthanasia score sheets were used to record appearance and provoked and natural behavior. The criteria for immediate euthanasia included signs of severe systemic infection, >20% loss in body weight, convulsions, hemorrhagic rash, and persistent prostration. Postchallenge detailed clinical and euthanasia assessments were made on all animals every four to 6 h until recovery, at which time the frequency was reduced to twice daily.

Euthanasia and necropsy procedures. Animals were sedated with ketamine hydrochloride (10 mg/ml, i.m.; Fort Dodge Animal Health, Ltd.). Anesthesia was deepened using intravenous pentobarbitone sodium at 30 mg/kg (Sagatal; Rhone Merieux), and exsanguination was effected via the heart, before termination by injection of an anesthetic overdose (Dolelethal, 140 mg/kg; Vetquinol UK, Ltd.). A full necropsy was performed immediately to provide tissues.

Pathological studies. At necropsy, gross observations, including skin lesions, were recorded, and samples were collected of all lung lobes, trachea, heart, liver, kidneys, spleen, tongue, tonsil, esophagus, stomach, ileum, descending colon, lymph nodes (tracheobronchial, axillary, mesenteric, mandibular, and inguinal), adrenal gland, ovary or testis, skin (with or without lesion), and brain. The samples were placed in 10% neutral buffered formalin; fixed tissues were then processed routinely to paraffin wax, and sections cut at 5 μm and stained with hematoxylin and eosin (H&E).

Statistical analysis. Flow cytometry data and antibody titers as measured by ELISA and PRNT assays were compared across treatments using one-way Mann-Whitney tests. A Pearson product-moment correlation was performed on the transformed (log_{10}) real-time PCR data set and the transformed (log_{10}) PFU/ml data set for the blood and throat samples. All statistical analyses were performed using Minitab version 15.1. Differences were considered significant at P values of <0.05.

RESULTS

Local effects at site of vaccination. Red patches were observed on all animals at the vaccination site 4 days postvaccination by scarification with Acam2000. These developed into raised scabbed sites ~10 mm in diameter by day 6 postvaccination. Dry scabs persisted for ~3 weeks postscarification. No reactive signs were detected at the vaccination sites of any Imvamune-vaccinated animals. Similarly no vaccination-specific marks were seen on the TBS negative control animals.

Vaccine-induced humoral immune responses. Sera from MVA vaccinated (Imvamune), vaccinia-virus vaccinated (Acam2000), and TBS negative control animals were tested with a PRNT assay against one antigen, wild-type Lister-Elstree vaccinia virus to determine the levels of vaccinia virus-specific neutralizing antibodies (Fig. 1a) prior to challenge. Antibodies were induced and continued to rise after vaccination with Acam2000 (Fig. 1a), with a maximum median titer of 132 U/ml 6 days prior to challenge. Significantly lower levels ($P < 0.01$) of neutralizing antibodies were detected by PRNT assay in animals vaccinated with a single dose of Imvamune (13 U/ml, 6 days prior to challenge). Animals that received a second boost of Imvamune showed a rise in neutralizing antibodies after the booster vaccination (Imvamune

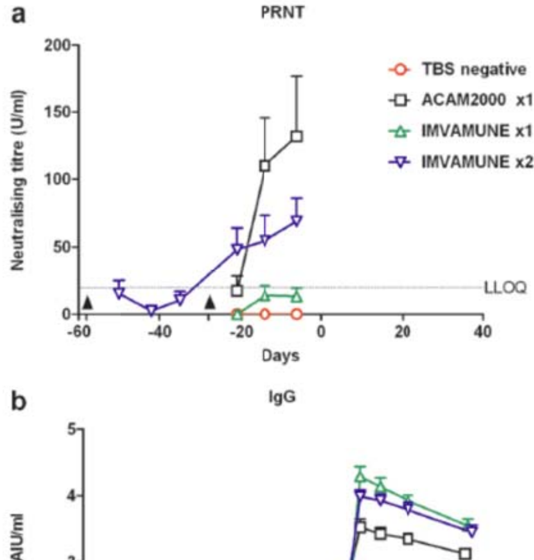

FIG 1 Vaccine-induced humoral immune responses. (a) Vaccinia virus-specific neutralizing serum antibody titres (median + 1 standard error [SE], $n = 6$) in samples collected on different days during the study, measured by PRNT assay. (b) Development of specific serum IgG responses in samples collected pre- and postchallenge, measured by ELISA (mean + 1 SE, $n = 6$), day 0, time of challenge with monkeypox. TBS negative, TBS negative control group; closed triangles, times of immunization; LOD, limit of detection; LLOQ, lower limit of quantification.

×2). This reached 69 U/ml 6 days prior to challenge and was significantly higher ($P < 0.05$) than the titer in single-dose Imvamune group. Although higher concentrations of neutralizing antibody were detected in the Acam2000 group, this was not significantly different from the amount of antibody detected in the two dose Imvamune group ($P > 0.05$) (Imvamune ×2) (Fig. 1a).

The levels of circulating IgG antibodies were detected by ELISA pre- and postchallenge using only one antigen, vaccinia virus. Prior to challenge, no rise in IgG antibody was seen in the TBS negative control or the single Imvamune dose group. In the Acam2000 group after vaccination, IgG rose to 2.4 $\log_{10}$ AIU/ml, 9 days prior to challenge (Fig. 1b). A similar rise in antibody was seen after the second dose of Imvamune in animals (Imvamune ×2) (2.3 $\log_{10}$ AIU/ml, 7 days prior to challenge) (Fig. 1b). After infection, the kinetics of the antibody responses for the Acam2000, Imvamune ×1, and Imvamune ×2 groups were similar; large increases in antibody titer from baseline levels were found which peaked at day 9 postchallenge and then decreased slowly to the end of the study. Vaccinia virus-specific IgG was not detected in the TBS negative control group until day 11 (2.0 $\log_{10}$ AIU/ml). Significantly ($P < 0.05$) higher levels of antibody were detected in the two-dose Imvamune group (4.0 $\log_{10}$ AIU/ml) on day 9 postchallenge than in the Acam2000 group, where a peak of 3.5 $\log_{10}$ AIU/ml was seen. Antibody concentrations in the single and two dose Imvamune groups remained significantly ($P < 0.05$) higher than the Acam2000 group on days 14 and 21 (Fig. 1b) postchallenge.

Vaccine-induced cell-mediated immune responses. The cell-mediated immune responses after vaccination and challenge were monitored by flow cytometry. Lymphocyte numbers rose in the TBS negative control, and Acam2000 groups after vaccination by scarification (Fig. 2a to c). These small peaks were probably caused by local irritation at the site of vaccination caused by scarification. Small rises in $CD3^+$, $CD4^+$, and $CD8^+$ cells were seen initially, following vaccination with one dose of Imvamune, but there was no marked increase following the second vaccination (Fig. 2). After challenge, however, there were noticeable rises in the different cell populations in animals that succumbed to disease in the TBS negative control group. For example, on day 9, there were significant differences ($P < 0.05$) between the TBS control and the Imvamune ×2 group. On closer inspection, these differences were caused by the significant rise in circulating NK cells in the TBS control ($P < 0.05$). By day 14, there were significantly higher ($P < 0.05$) numbers of B cells and $CD8^+$ T cells in the surviving animals (4/6) of the one dose Imvamune group compared to the Acam2000 group. Also, by day 14 significantly higher ($P < 0.05$) numbers of $CD4^+$ and $CD8^+$ cells were recorded in single Imvamune group than in the two-dose Imvamune group (Imvamune ×2).

Elevated concentrations of IFN-γ, as detected by Luminex assay, were seen in the TBS negative control group on day 6 after challenge (4630%) (Fig. 3a). In contrast, no rise in serum gamma-IFN was observed in animals in Acam2000 and two-dose Imvamune groups (Fig. 3a) as they were protected by vaccination. The single Imvamune dose group also had raised concentrations of serum gamma-IFN 6 days after challenge (Fig. 3a).

A significant rise in IL-6 cytokine was seen in the TBS negative control group following aerosol challenge, which continued to increase until the animals succumbed to infection on day 11 (4592% change from baseline) (Fig. 3b). Smaller increases in IL-6 were seen (day 6) in the single-dose Imvamune group (Fig. 3b).

Clinical signs of disease and mortality. Aerosol challenge with monkeypox virus at a mean presented dose of 2.6×10^5 PFU resulted in a severe or lethal infection in susceptible individuals. Most animals showed a decline in weight from their prechallenge weights (Fig. 4). This was most severe in the TBS negative control group, with a 10 to 18% loss in weight prior to euthanasia (Fig. 4a). All surviving animals in the vaccination groups—Acam2000, Imvamune ×1, and Imvamune ×2—had a consistent increase in body weight from day 14 postexposure, indicating recovery from the infection (Fig. 4b to d).

Signs of infection generally appeared from day 5 postchallenge. Animals in the TBS negative control displayed progressing depression, dyspnea, and nasal discharge. All six animals succumbed to infection between days 7 to 11 postchallenge. In the single-dose Imvamune group, two animals succumbed to infection on different days. Both animals displayed mild depression and dyspnea and were recumbent from day 6 postchallenge; animal M064F was found dead in cage on day 7 postchallenge, and animal I320I had

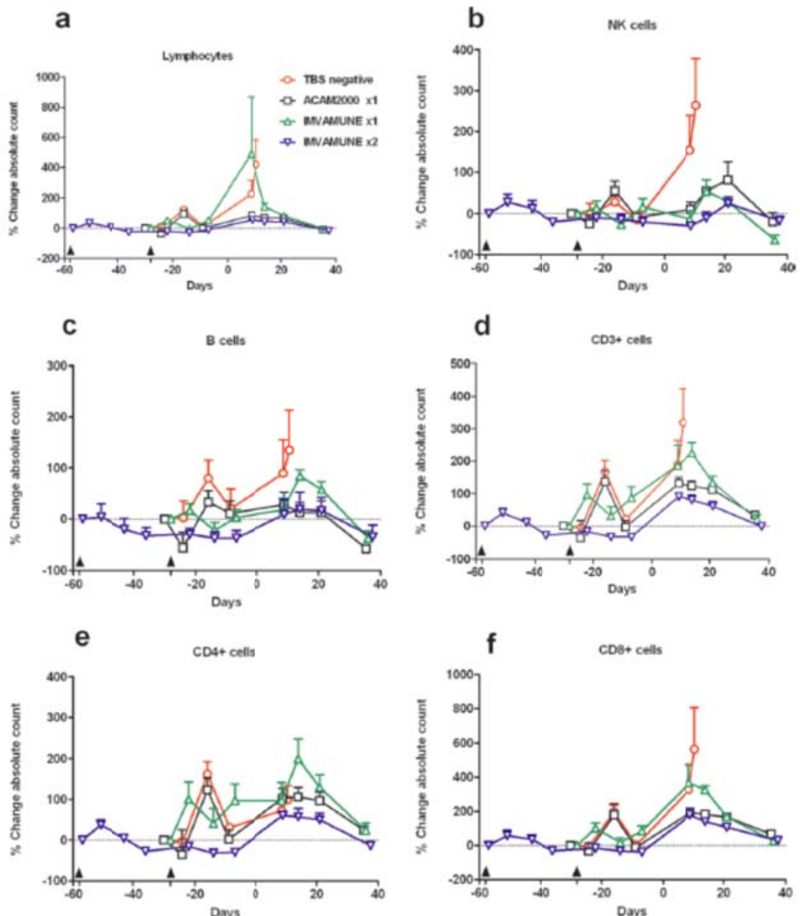

FIG 2 Cell-mediated immune responses, indicated as the mean percent (%) change compared to baseline levels (+ 1 SE, n = 6), in different cellular populations in whole blood on various days in the study. Different cellular populations were evaluated. (a) Lymphocytes; (b) natural killer (NK) cells; (c) B lymphocytes; (d) CD3+ T lymphocytes; (e) CD4+ T lymphocytes; (f) CD8+ T lymphocytes. Day 0, time of challenge with monkeypox; TBS negative, TBS negative control group; closed triangles, times of immunization.

clinical signs that progressed to severe and met the criteria for immediate euthanasia on day 9 postchallenge. The remaining four animals in the one-dose Imvamune group were generally free of clinical signs and survived to the end of the study (67% survival). All of the animals vaccinated with Acam2000 or two doses of Imvamune survived the monkeypox virus challenge. The animals appeared to be clinically normal, although there were differences in the level of protection afforded by these vaccines, as demonstrated in some of the test parameters, such as radiographs, lesion counts, and viral load (Table 1).

Skin lesions, as a result of monkeypox virus infection, first appeared at day 6 after challenge (Table 1). There was a peak in the mean number of lesions, across all vaccination and control groups at day 9. The greatest mean number of lesions was 51 per animal (range, 5 to 169) in the TBS negative control group (Table 1). Fewer lesions were detected in the vaccination groups. Vaccination with one or two doses of Imvamune led to fewer lesions on day 9, with means of 10 (range, 0 to 42) and 7 (range, 0 to 18) lesions per animal, respectively. The lowest mean number of lesions was 3 (range, 0 to 7) per animal in the Acam2000 treatment group.

Radiographs were taken postchallenge at the time when clinical signs were most severe (9 days). They were scored independently against a corresponding baseline image. Animals in the TBS negative control group generally displayed the most severe clinical signs (Table 1). Radiographs taken from animals in the Acam2000 vaccination group were normal. A wide spectrum of conditions was observed in the Imvamune single- and two-dose groups ranging from normal to severe edema (Table 1). One animal (Z385A) in the two-dose Imvamune group had moderate to severe pulmonary edema but recovered fully.

Whole blood, throat, and tissue viral loads of NHP exposed to

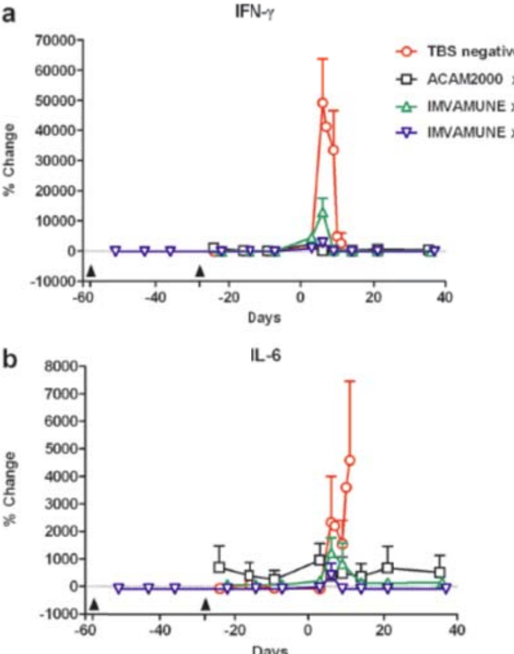

FIG 3 Cytokine profiles in the serum of cynomolgus macaques. The mean percent (%) change from baseline levels (+ 1 SE, $n = 6$) in cytokine levels, on various days during the study, was determined. The IFN-ã (a) and IL-6 (b) responses are shown. TBS negative, TBS negative control group; day 0, time of challenge with monkeypox; closed triangles, times of immunization.

aerosolized monkeypox virus: the monkeypox viral load of blood and throat swabs were assessed by plaque assay (PFU/ml) (Fig. 5a and b) and real-time quantitative PCR (genomes/ml) (Fig. 5c and d) (there was a strong correlation [$r = 0.746$; df = 26, $P < 0.001$, Pearson product-moment correlation] between plaque assay and real-time PCR data). The peak in the mean load of viral DNA (4×10^6 genomes/ml) in the blood of animals from the TBS negative control group (Fig. 5c) occurred on day 7 postchallenge. In contrast, no viral DNA was also detected in the group that received the Acam2000 vaccine on any day examined postchallenge. A peak in the mean level of viral DNA in the blood was detected in animals that had received one (6×10^7 genomes/ml; day 7 postchallenge) and two (1×10^4 genomes/ml; day 6 postchallenge) doses of Imvamune (Fig. 5c). Low levels of live virus were detected in the blood by day 3 postchallenge in all three vaccination groups and the TBS negative control group, ranging from 25 to 200 PFU/ml (Fig. 5a).

In the TBS negative control group, live virus (10^5 PFU/ml) and viral DNA (10^7 genome copies/ml) were detected in the throats of all animals challenged with monkeypox virus (Fig. 5b and d). Live virus (10^4 to 10^5 PFU/ml) was also detected in the throats of animals that had received a single dose of Imvamune (Fig. 5b and d). In the two-dose Imvamune group, four of six animals did not excrete virus in the throat (within the sensitivity of the plaque assay [<25 PFU/ml]). Live virus was detected at low levels (50 PFU/ml) in one of six animals, and one animal (Z385A) excreted high levels of virus that peaked (4.6×10^4 PFU/ml) on day 9 postchallenge. In contrast, live virus was not detected in the throats of animals (5/6 animals) vaccinated with Acam2000. Note that no plaque assay data were obtained for one remaining animal (M016D) in the Acam2000 group due to contamination of the cell monolayer.

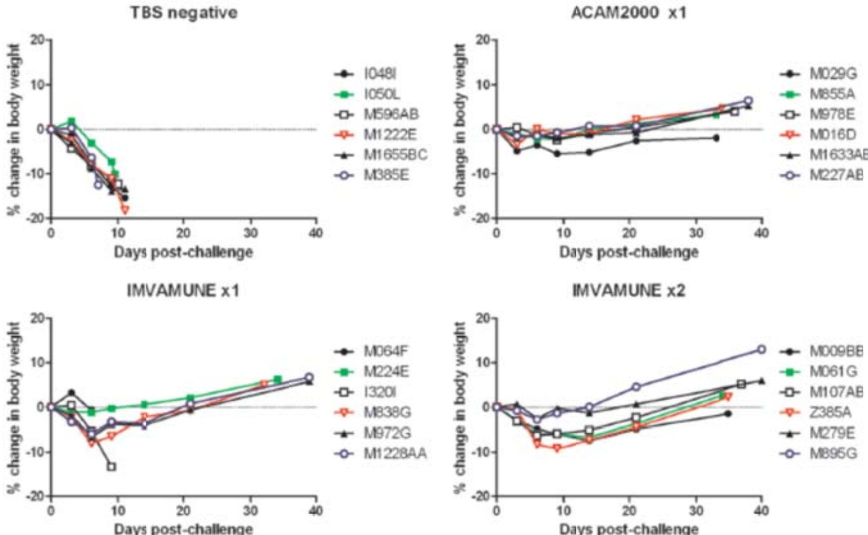

FIG 4 Change in the body weight of cynomolgus macaques. The percent (%) change in body weight compared to baseline levels in animals challenged with aerosolized monkeypox, over time, following vaccination, was determined. The results for individual animals are plotted. TBS negative, TBS negative control group.

TABLE 1 Clinical signs of monkeypox disease[a]

Measurement	Treatment group			
	TBS negative control	Acam2000 ×1	Imvamune ×1	Imvamune ×2
Temp	Normal	Normal	Normal	Normal
Clinical signs[b]	+++	+	++[c]	+
Mean no. of lesions (day 9)	51	3	10	7
Onset of lesions	Day 6	Day 9	Day 9	Day 6
Resolution of lesions	Not resolved	Within 5 days	Within 5 days	Within 5 days
Thoracic radiography[d]	+++	–	++	+
Survival (%)	0[e]	100	67[f]	100

[a] Clinical signs were monitored every 4 to 6 h until disease recovery and thereafter twice daily. Treatment groups are as described in Materials and Methods.
[b] Clinical signs: –, none; +, mild; ++, substantial; +++, intense signs.
[c] Two of the that animals succumbed to infection had intense clinical signs (+++).
[d] A thoracic radiograph was obtained 9 days postchallenge. Findings were scored as follows: –, normal; +, minor pulmonary edema; ++, mild pulmonary edema; +++, moderate pulmonary edema.
[e] All animals died between 7 and 11 days postchallenge and displayed high euthanasia scores.
[f] Two animals succumbed to infection on day 7 and day 9 postchallenge; the animal that died on day 7 had low euthanasia scores prior to death.

In addition to blood and throat swabs, tissues were collected postmortem and assayed by real-time quantitative PCR for viral load. The majority of tissues were positive for monkeypox virus in the TBS negative control group (Fig. 6a). The greatest viral loads were found in the tonsil and lung tissues, with between 10^6 and 10^7 genomes/mg. Two animals (M064F and I320I) vaccinated with a single dose of Imvamune also succumbed to monkeypox infection. These two animals also showed similar patterns of viral load, as seen in the TBS negative control group. Both tonsil and lung tissue reflected the greatest values of between 10^5 and 10^6 copies/mg (Fig. 6c). The remaining animals in the Imvamune ×1 group that survived to the end of the study (>30 days postchal-

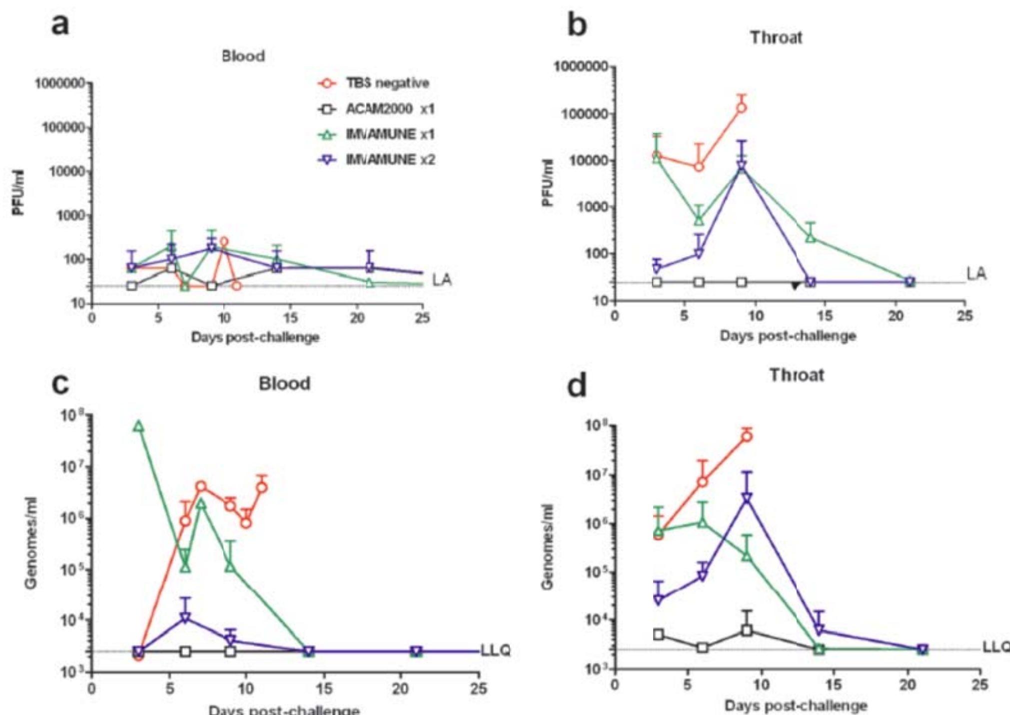

FIG 5 Mean detectable levels (+ 1 SD) of live monkeypox virus in the blood (a) and throats (b) of macaques after a challenge with aerosolized virus (the limit of plaque assay sensitivity was 25 PFU/ml). The numbers of viral genomes (HA gene), as determined from quantitative PCR analyses in the blood (c) and throats (d) in samples postchallenge, are shown. TBS negative, TBS negative control group; LLQ, the lower limit of quantification for quantitative PCR was 2,500 genomes/ml; LA, limit of plaque assay sensitivity. The plaque assay result for 1/4 animals in the Imvamune ×2 was lost due to monolayer contamination on day 14.

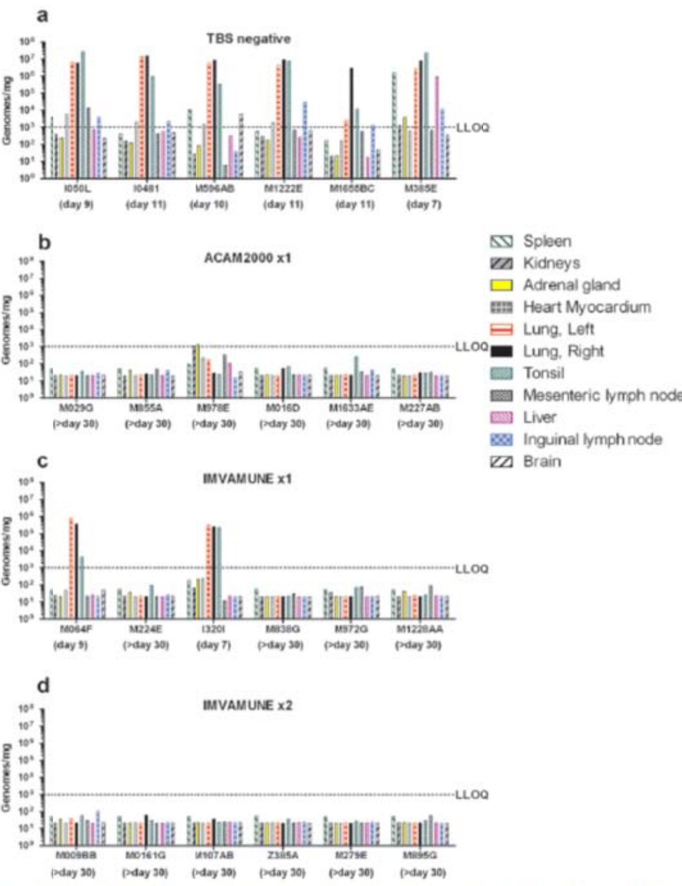

FIG 6 Viral load in tissues after challenge with monkeypox virus. The viral load, as determined by real-time PCR, in the tissues of different animals in the treatment groups. Individual animals in the TBS negative control (a), Acam2000 ×1 (b), Imvamune ×1 (c), and Imvamune ×2 (d) groups were evaluated. LLQ, lower limit of quantification was 1,000 genomes/mg (the time postmortem is given as the number of days postchallenge). TBS, TBS negative control group.

lenge) did not have any detectable viral loads by PCR in their tissue postmortem. No detectable viral loads were seen in the tissue of animals from the Acam2000 vaccination or the two doses of Imvamune (Fig. 6b and d). All of these animals also survived to the end of the study (between 30 and 40 days).

Pathological and histopathological findings. Gross findings on postmortem examination revealed that the main gross lesions associated with monkeypox infection consisted of lung consolidation in all animals in the TBS negative control and in two of the six animals in Imvamune ×1 group. An enlarged spleen was seen in five of six animals in the TBS negative control and in one of six animals in the Imvamune ×1 group.

On histological examination, changes consistent with acute monkeypox infection were observed in the TBS negative control group in the lungs, comprising (i) focal, acute necrotizing bronchitis and bronchopneumonia (Fig. 7a); (ii) focal, fibrinous, necrotizing alveolitis (Fig. 7b), often accompanied by edema; and (iii) focal acute vasculitis, sometimes together with thrombosis and perivascular edema. In addition, focal necrosis with or without neutrophil infiltration was observed in the trachea, larynx, and tracheobronchial lymph node. In the skin (with lesion), spleen, tonsils, the axillary, inguinal, and mandibular lymph nodes, and the descending colon, focal necrosis—with or without neutrophil infiltration—was observed.

In animals vaccinated with Acam2000, which were killed 33 to 38 days postchallenge, only mild, chronic lesions were observed. These comprised focal alveolar epithelialization and/or infiltration of alveolar walls by lymphocytes and macrophages, which were seen in two (M016D and M978E) of the six animals (Fig. 7c). Hyperplasia of bronchus-associated lymphoid tissue (BALT) was recorded in five of six animals.

Animals vaccinated with a single dose of Imvamune that died (M064F) or were killed for welfare reasons (I302I) (days 7 and 9 postchallenge, respectively) had lesions of acute disease in all lung lobes, similar to those described above in the TBS negative control group (Fig. 7d). In the trachea of one animal and in the larynx of another, focal necrosis, with or without neutrophil infiltration, was also observed. Hyperplasia of BALT was observed in one of

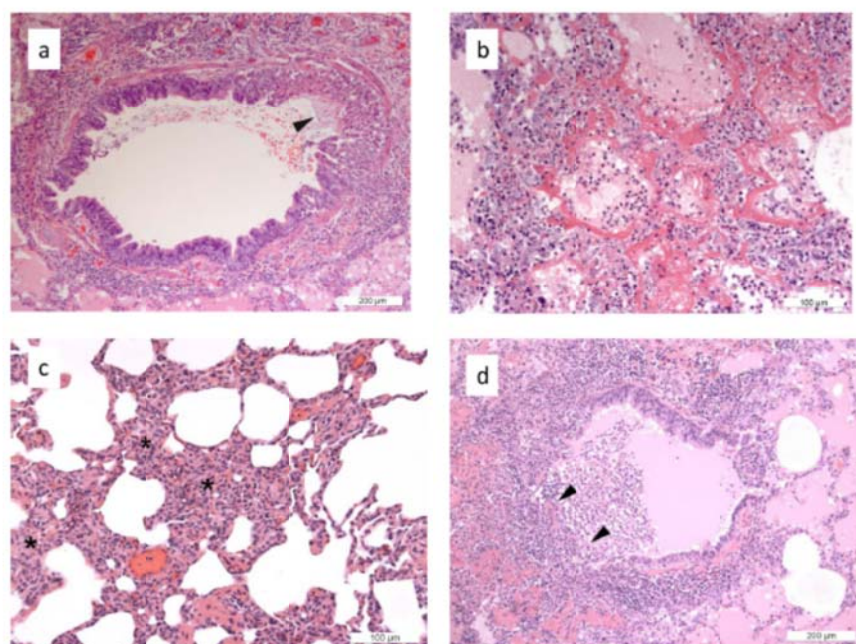

FIG 7 Histological lesions associated with monkeypox infection in the lung. (a) TBS negative control, animal M596AB (lung; focal, acute, and necrotising bronchiolitis [arrowhead]; H&E staining). (b) TBS negative control, animal M595AB (lung; focal, fibrinous, and necrotising alveolitis; H&E staining). (c) Acam2000 vaccination group, animal M016D (lung; patchy infiltration of alveolar walls by lymphocytes and macrophages [asterisks]; H&E staining). (d) Single Imvamune dose, animal I320I (lung; focal, acute, and necrotising bronchiolitis [arrowheads]; H&E staining).

four animals that were euthanized as scheduled 32 to 39 days after challenge. In all animals in the single-dose Imvamune group, mild changes of focal alveolar epithelialization and/or infiltration of alveolar walls by lymphocytes and macrophages, a finding consistent with chronic or resolving lesions, were recorded.

In animals that received two doses of Imvamune and were euthanized as scheduled 35 to 40 days after challenge, only mild lesions of chronic disease, comprising focal alveolar epithelialization and/or infiltration of alveolar walls by lymphocytes and macrophages were seen in two of the six animals. Hyperplasia of BALT was recorded in four of six animals.

Lesions attributable to monkeypox infection were not detected in the liver, kidney, heart, tongue, esophagus, stomach, ileum, mesenteric lymph node, adrenal gland, ovary, testis, or brain of any animal.

DISCUSSION

Since smallpox has been eradicated, the future licensing of a new generation of smallpox vaccines relies, in part, on the demonstration of efficacy in animal models of monkeypox (19). When monkeypox virus infects people as an epizootic pathogen, it presents a clinical disease similar to smallpox in that the time course and manifestation of disease is similar to that seen with human smallpox, particularly the rash which progresses through the macular, papular, vesicular, and pustular phases (24). Thus, a well-defined animal model using monkeypox virus should mimic the natural course of smallpox disease. Macaques have been used at various stages of smallpox vaccine and antiviral research (16, 25–36), and in each case the route of infection, dose, and choice of challenge strain have been key factors in determining whether the macaque model of monkeypox resembles human clinical variola virus infection.

Imvamune is a more recent smallpox vaccine and is being fast-tracked by the FDA for use in humans (37). This vaccine is currently stockpiled in the United States for use during an emergency, such as an imminent bioterrorist attack, to protect individuals who are at risk of developing side effects from older vaccines (37). Studies have been published demonstrating the safety, immunogenicity (4), and protective efficacy against vaccinia virus scarification in humans (38) of Imvamune delivered in a single- or two-dose regime. Several studies have also shown its protective potency in animals (33, 39, 40). In the present study, for the first time, the efficacy of both one and two doses of Imvamune vaccine was assessed in cynomolgus macaques following challenge with an aerosolized dose of 2.6×10^5 PFU of monkeypox Zaire Z9. Inhalation of aerosolized virus more closely resembles the natural route of infection of smallpox in humans (41) and therefore initiates the onset of clinical signs that are similar to human clinical disease (20, 42).

When aerosolized monkeypox virus was used at a dose (2.6×10^5 PFU) to produce severe or lethal disease in naive cynomolgus macaques, animals in the TBS negative control group succumbed to infection within 7 to 11 days. Pock lesions began to appear on day 6 postchallenge, and there was a peak in the number of lesions by day 9 (a mean of 51 lesions per animal). These data are in sharp

alignment with other natural history and pathology studies conducted at our laboratories, as well as with work performed by Nalca et al. (36). In contrast, in other vaccine trials where control animals have been challenged by a different route, such as the intravenous route with a dose 5×10^7 PFU (16) or 2×10^7 PFU (32), pock lesions ranging from 250 to >500 per animal appeared from days 3 to 6. These differences highlight the importance of the challenge route and the dose.

All of the animals that received the second-generation vaccine (Acam2000) survived the monkeypox virus challenge, although some signs of viral infection were observed, such as lesions (mean number of three per animal) on day 9 and low levels of viremia. The animals were generally well and lost very little weight. Both humoral and cell-mediated immune responses were primed, and high concentrations of neutralizing antibody and IgG antibody were detected after vaccination.

The optimal and intended vaccination regime for Imvamune is a prime-boost approach, and results from the present study highlight the importance of this vaccination strategy. The use of a prime-boost regime with Imvamune protected all of the animals challenged (100% survival). Both antibody and cell-mediated immune responses were stimulated, and high titers of neutralizing and IgG antibody were detected following the second dose of Imvamune. There was still some evidence of monkeypox virus infection in the group, as indicated by the presence of pock lesions on day 9 and minor pulmonary edema; nevertheless, this is comparable to the results from the Acam2000-vaccinated animals. In addition, however, there was evidence of virus excretion in the throats of two of six animals. Viral excretion in the throat after MVA vaccination has previously been shown when using the intratracheal (33) and intravenous (32) challenge routes (a different source of MVA and a different route of vaccination was used in the intravenous challenge study).

In the present study, a single dose of Imvamune did not protect all of the animals in the group, and two animals succumbed to infection. Postmortem, the virus was isolated from the lungs and tonsils of both animals. The titer of vaccinia virus-specific IgG antibody and neutralizing antibody prior to challenge was very low, and this may have contributed to the poorer outcome in this group. It should be noted that a single dose is not the optimal regime for Imvamune vaccination; however, one dose does give partial protection and thus may potentially be useful as a primer vaccine, in certain groups of people, which are then subsequently boosted during an emergency. Further work is clearly needed in this area.

Our data not only provide supportive information for the use of Imvamune as a vaccine against variola virus but also show that it could be useful as a vaccine to protect against human infections with monkeypox virus. Recent epidemiologic studies suggest that human monkeypox is currently exhibiting a robust emergence in the Democratic Republic of the Congo (7, 43, 44). Cessation of smallpox vaccination worldwide has resulted in diminished vaccine-induced orthopoxvirus immunity, creating a new "immunologic niche" for the emergence of human monkeypox (45). The use of next-generation smallpox vaccines for the prevention of human monkeypox is currently being discussed (45, 46).

Overall, we have demonstrated here that a prime-boost vaccination regime with Imvamune provides complete protection, as does the comparator vaccine Acam2000. Two doses of Imvamune should be used rather a single dose which only offers partial protection. This evaluation of different human smallpox vaccines in cynomolgus macaques helps to address questions about optimal vaccine strategies, in the absence of human challenge studies, during a time when the efficacy of Imvamune is being established under the "Animal Rule."

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Original article

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Original article

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Sequence of Pathogenic Events in Cynomolgus Macaques Infected with Aerosolized Monkeypox Virus

J. A. Tree, G. Hall, G. Pearson, E. Rayner, V. A. Graham, K. Steeds, K. R. Bewley, G. J. Hatch, M. Dennis, I. Taylor, A. D. Roberts, S. G. P. Funnell, J. Vipond

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ABSTRACT

To evaluate new vaccines when human efficacy studies are not possible, the FDA's "Animal Rule" requires well-characterized models of infection. Thus, in the present study, the early pathogenic events of monkeypox infection in nonhuman primates, a surrogate for variola virus infection, were characterized. Cynomolgus macaques were exposed to aerosolized monkeypox virus (10^5 PFU). Clinical observations, viral loads, immune responses, and pathological changes were examined on days 2, 4, 6, 8, 10, and 12 postchallenge. Viral DNA (vDNA) was detected in the lungs on day 2 postchallenge, and viral antigen was detected, by immunostaining, in the epithelium of bronchi, bronchioles, and alveolar walls. Lesions comprised rare foci of dysplastic and sloughed cells in respiratory bronchioles. By day 4, vDNA was detected in the throat, tonsil, and spleen, and monkeypox antigen was detected in the lung, hilar and submandibular lymph nodes, spleen, and colon. Lung lesions comprised focal epithelial necrosis and inflammation. Body temperature peaked on day 6, pox lesions appeared on the skin, and lesions, with positive immunostaining, were present in the lung, tonsil, spleen, lymph nodes, and colon. By day 8, vDNA was present in 9/13 tissues. Blood concentrations of interleukin 1ra (IL-1ra), IL-6, and gamma interferon (IFN-γ) increased markedly. By day 10, circulating IgG antibody concentrations increased, and on day 12, animals showed early signs of recovery. These results define early events occurring in an inhalational macaque monkeypox infection model, supporting its use as a surrogate model for human smallpox.

IMPORTANCE

Bioterrorism poses a major threat to public health, as the deliberate release of infectious agents, such smallpox or a related virus, monkeypox, would have catastrophic consequences. The development and testing of new medical countermeasures, e.g., vaccines, are thus priorities; however, tests for efficacy in humans cannot be performed because it would be unethical and field trials are not feasible. To overcome this, the FDA may grant marketing approval of a new product based upon the "Animal Rule," in which interventions are tested for efficacy in well-characterized animal models. Monkeypox virus infection of nonhuman primates (NHPs) presents a potential surrogate disease model for smallpox. Previously, the later stages of monkeypox infection were defined, but the early course of infection remains unstudied. Here, the early pathogenic events of inhalational monkeypox infection in NHPs were characterized, and the results support the use of this surrogate model for testing human smallpox interventions.

Since smallpox was declared as being eradicated by the World Health Organization in 1980 (1), laboratory investigations of variola virus have been restricted, leaving a significant gap in the understanding of the immune responses and pathogenesis of this infection (2). Recently, the majority of the human population has not been vaccinated; consequently, a proportion of the population lacks protective immunity (3). Concerns over the use of variola virus or monkeypox virus (a closely related orthopoxvirus) as a biological weapon remain high, as a deliberate release would have catastrophic consequences on global health (4).

The efficacy of therapeutics and vaccines against smallpox cannot be tested in phase III clinical trials in humans, as this is neither ethical nor feasible. Therefore, testing new medical countermeasures requires FDA marketing approval according to the "Animal Rule" (5). Monkeypox virus infection of nonhuman primates (NHPs) presents a potential surrogate disease model for testing intervention strategies for smallpox. Monkeypox virus is related to variola virus and causes a lethal systemic infection in primates. It can also infect humans and presents clinical symptoms similar to those of classic smallpox (6, 7).

Several studies have reported the development of an NHP model of monkeypox virus infection. A variety of challenge routes have been used, including intrabronchial (8), intravenous (8–14), intratracheal (15, 16), intratracheal with MicroSprayer (17), and subcutaneous (18, 19). Natural infection of smallpox usually occurs as a result of close contact with an infected person, via the oropharynx or nasopharynx (20). A deliberate release of variola or monkeypox virus, however, would probably be in aerosol form for rapid dispersion over large areas (21). A limited number of studies have used the aerosol route, characterizing the pathogenic events following aerosol monkeypox virus infection (22–24). Zaucha and

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colleagues described the systemic dissemination of the monkeypox virus in cynomolgus macaques through a monocytic-cell-associated viremia, similar to that of variola in human beings (23). More recently, two studies described the clinical progression of disease in NHPs following exposure to different doses of aerosolized monkeypox virus (22, 24). These three studies described disease progression from 8 to 17 days after exposure. Pathogenic events earlier than 8 days postinfection have not been reported.

The purpose of this study was to gain a better understanding of the early pathogenic events of monkeypox virus infection following aerosol challenge with a target dose of 10^5 PFU. This study also further characterizes the use of this challenge dose, as used previously, for testing smallpox vaccines (25). In this work, clinical signs of disease, immune cell and antibody responses, viral spread through the body, and pathological changes were examined from days 2 to 12 postchallenge.

MATERIALS AND METHODS

Experimental animals. Twenty-one captive-bred, healthy, male cynomolgus macaques (*Macaca fascicularis*) of Mauritian origin were obtained from a United Kingdom breeding colony. They weighed >2.5 kg and were >2 years of age. All animals were negative for neutralizing antibodies to orthopoxvirus prior to the start of the study. The macaques were housed as required by the United Kingdom Home Office *Code of Practice for the Housing and Care of Animals Used in Scientific Procedures* (26) and the National Committee for Refinement, Reduction, and Replacement (NC3Rs) *Guidelines on Primate Accommodation, Care, and Use* (27). When a procedure required the removal of a primate from a cage, it was sedated by intramuscular (i.m.) injection with ketamine hydrochloride (10 mg/kg of body weight) (Ketaset; Fort Dodge Animal Health Ltd., Southampton, United Kingdom). All procedures were conducted under a project license approved by the local Ethical Review Process of Public Health England, Salisbury, United Kingdom, and the United Kingdom Home Office.

Pathogenesis study. The animals were divided into seven groups of 3 animals and were exposed to aerosolized monkeypox virus on one of two separate occasions with a target dose of 1×10^5 PFU. The mean presented dose was 7.3×10^4 PFU. Animals were scheduled to be euthanized on days 2, 4, 6, 8, 10, and 12 postchallenge. Three animals were designated the reserve group and were used to replace animals that met humane endpoints earlier than their planned necropsy, thus ensuring that 3 animals were available for necropsy at each scheduled time point.

On the day of necropsy, animals were sedated and whole blood and throat swabs collected. For the latter, a flocked swab (Copan Diagnostics, California, USA) was gently stroked 6 times across the back of the throat in the tonsillar area. Viral loads were determined in the blood and throat samples by real-time PCR and plaque assay (see below). The concentration of IgG circulating in the blood was determined by enzyme-linked immunosorbent assay (ELISA; see below). The concentrations of cytokines and the phenotypes of cellular immune populations were determined by Luminex and flow cytometry, respectively (see below). At necropsy, tissues were sampled for histological examination and the determination of viral load by real-time PCR (see below).

Monkeypox virus challenge strain. Monkeypox virus, strain Zaire 79, NR-2324, was obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources, Virginia, USA). On the day of challenge, stocks of virus were thawed and diluted appropriately in minimum essential medium (MEM) containing Earl's salts (Sigma, Poole, United Kingdom), 2 mM L-glutamine (Sigma), and 2% (vol/vol) fetal calf serum (FCS) (Sigma).

Aerosol exposure. Animals were challenged with monkeypox virus using the AeroMP-Henderson apparatus, in which the challenge aerosol was generated using a six-jet Collison nebulizer (BGI, Waltham, MA, USA). This apparatus was designed to deliver a particle size with a mass median diameter of 2.5 μm and a geometric standard deviation of approximately 1.8 (28, 29). The aerosol was mixed with conditioned air in the spray tube (30) and delivered to the nose of each animal via a modified veterinary anesthesia mask. Samples of the aerosol were taken using an SKC BioSampler (SKC Ltd., Dorset, United Kingdom) and an aerodynamic particle sizer (TSI Instruments Ltd., Bucks, United Kingdom); these processes were controlled and monitored using the AeroMP management platform (Biaera Technologies LLC, Maryland, USA). To enable delivery of consistent doses to individuals, each animal was sedated and placed in a "head-out" plethysmograph (Buxco, North Carolina, USA). The aerosol was delivered simultaneously with measurement of the respired volume. A back titration of the aerosol samples taken at the time of challenge was performed to calculate the presented/inhaled dose.

ELISA. Samples of blood were collected at time points throughout the study; serum was separated and assayed for IgG antibodies to vaccinia virus using an ELISA. MaxiSorp 96-well plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with a preparation of commercially prepared psoralen/UV-inactivated, sucrose density gradient-purified vaccinia virus (Lister strain) (Autogen Bioclear United Kingdom Ltd., Wiltshire, United Kingdom) in calcium carbonate buffer at 2.5 $\mu g\ ml^{-1}$. Unbound antigen was removed by washing the plates 3 times. The plates were treated with blocking buffer (phosphate-buffered saline [PBS], 5% milk powder [Sigma], 0.1% Tween 20 [Sigma]) for 1 h at room temperature with shaking. Unbound blocking solution was removed by washing 3 times with Tris-buffered saline (TBS). Fourfold serially diluted serum samples (starting at 1:50) were added to the plate for 2 h at room temperature, with shaking. Unbound antibodies were removed from the plate by washing 3 times with TBS. The plates were incubated for 2 h, with shaking, with horseradish peroxidase-labeled anti-monkey IgG (Kirkegaard and Perry Laboratories, Maryland, USA). Unbound detection antibody was removed by washing 5 times with TBS and developed using the 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate (6)] (ABTS) peroxidase substrate system (Kirkegaard and Perry Laboratories). The development of the ELISA was stopped by using ABTS stop solution (Kirkegaard and Perry Laboratories). ELISA titers were calculated and compared with a vaccinia immune globulin standard (BEI Research Repository Resource, USA), which was used to convert the titer into arbitrary international units (AIU) per milliliter.

Flow cytometry. Whole blood was collected on days 2, 4, 6, 8, 10, and 12 by using heparin as the anticoagulant. Antibodies to CD3e, CD4, CD20, CD16 (BD Biosciences, Oxford, United Kingdom), and CD8a (Invitrogen, United Kingdom) conjugated to R-phycoerythrin (R-PE)-cyanine dye (Cy7), allophycocyanin (APC), PE, fluorescein isothiocyanate (FITC), and PE-Texas red (TR), respectively, were incubated with the blood for 30 min at room temperature. The red blood cells were removed from the whole blood by lysis with Uti-Lyse reagent (Dako, Cambridgeshire, United Kingdom) and decontaminated overnight with a 4% formaldehyde final concentration solution. Flow count beads (Beckman Coulter, High Wycombe, United Kingdom) were added to provide a standard to enable cell counts per microliter of blood, before being acquired on the flow cytometer. Data were collected on an FC500 flow cytometer (Beckman Coulter) and analyzed with CXP Analysis software version 2.1.

Luminex analysis of cytokines. The concentrations of cytokines were determined in serum samples using an NHP 23-plex kit (Merck Millipore, Massachusetts, USA) according to the manufacturer's instructions. Samples were acquired using a Luminex 200 system (Luminex, Austin, TX, USA), and the data were analyzed using the Xponent software, version 3.0. The concentration of each cytokine in the serum was calculated based on a comparison with the corresponding standard curve generated using purified cytokines from the kit.

Monkeypox virus plaque assay. During the course of the study, EDTA-treated blood and throat swabs were collected and frozen at −80°C. At necropsy, tissues were collected and snap-frozen in liquid nitrogen. Before being tested, tissue was thawed and homogenized in PBS with 1.8-mm ceramic beads in a Precellys24 tissue homogenizer (Ber-

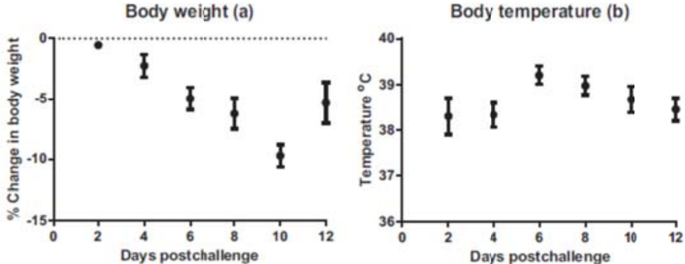

FIG 1 Clinical parameters. (a) Percentage change in body weight; (b) change in body temperature of cynomolgus macaques following aerosol challenge with monkeypox virus (mean ± 1 SE, $n = 3$ to 5 macaques). Body temperature was taken upon euthanasia.

tin Technologies, Villeurbanne, France). The titers of the infectious virus in the tissues, blood, and throat swabs were determined by plaque assay. Samples were incubated in 24-well plates (Nunc; Thermo Fisher Scientific, Loughborough, United Kingdom) with Vero E6 (ATCC CRL-1586; American Type Culture Collection, USA) cell monolayers under MEM (Life Technologies, California, USA) containing 1.5% carboxymethylcellulose (Sigma), 5% (vol/vol) fetal calf serum (Life Technologies), and 25 mM HEPES buffer (Sigma). After incubation at 37°C for 72 h, they were fixed overnight with 20% (wt/vol) formalin-PBS, washed with tap water, and stained with methyl crystal violet solution (0.2% [vol/vol]) (Sigma).

Virus detection by quantitative PCR (qPCR). Tissue samples collected and snap-frozen in liquid nitrogen were defrosted and homogenized in PBS using a Precellys24 tissue homogenizer. Viral DNA was isolated from homogenates using a Qiagen tissue kit (Qiagen, Crawley, West Sussex, United Kingdom) by following the manufacturer's instructions. Blood and throat swabs were processed using a Qiagen blood DNA minikit (Qiagen) by following the manufacturer's instructions. Real-time PCR was performed using an Applied Biosystems 7500 Fast instrument (Life Technologies) with an in-house TaqMan assay targeted at the viral hemagglutinin (HA) gene, residues 158734 to 158798, inclusive, in the Z79 genome (GenBank accession no. HQ857562.1, strain V79-I-005).

Clinical and euthanasia observations. Clinical observations were made and scored every 4 to 6 h postchallenge. A scale was used to define disease severity (0 = none, 1 = mild, 2 = substantial, 3 = intense), based on observations that included rectal temperature, body weight, behavioral changes (depression/unresponsiveness/repetitive activity), nasal discharge, cough, dyspnea, and rash/skin swelling. In order to meet the requirement of the project license to limit suffering of animals and identify an endpoint for euthanasia, the clinical well-being of each subject was assessed using a euthanasia scoring scheme which included observations of >20% loss in body weight, convulsions, hemorrhagic rash, and persistent prostration.

Necropsy procedures. Animals were anesthetized with ketamine hydrochloride (20 mg ml^{-1}, i.m.) (Fort Dodge Animal Health Ltd.), and exsanguination was effected via cardiac puncture, followed by injection of an anesthetic overdose (Dolelethal, 140 mg/kg; Vetquinol United Kingdom Ltd.) to ensure euthanasia. A necropsy was performed immediately after confirmation of death.

Pathological studies. At necropsy, abnormalities were recorded and samples were collected from lung lobes, trachea, heart, liver, kidneys, spleen, tongue, tonsil, esophagus, stomach, ileum, descending colon, lymph nodes (tracheobronchial, axillary, mesenteric, mandibular, and inguinal), adrenal gland, ovary or testis, skin (with and without lesion), and brain and placed in 10% neutral buffered formalin. Fixed tissues were processed to paraffin wax, and 5-μm sections were cut and stained with hematoxylin and eosin (H&E). For immunohistochemistry, formalin-fixed, paraffin-embedded tissue sections were mounted on positively charged X-tra adhesive slides (Leica Biosystems, United Kingdom), deparaffinized, and rehydrated. Immunohistochemical staining was achieved using a BOND-MAX immunostainer (Leica Microsystems, United Kingdom) and a Leica Bond Polymer Refine detection kit (Leica Biosystems, United Kingdom). A heat-induced epitope retrieval cycle with buffer ER2 (Leica Biosystems, United Kingdom) was applied for 20 min, followed by a peroxide block for 5 min (Leica Biosystems). Incubation was then performed with a mouse monoclonal antivaccinia antibody (ViroStat, USA) at a dilution of 1:1,800, followed by the post-primary antibody for 8 min (Leica Biosystems). Hematoxylin was used as the counterstain. Positive- and negative-control slides were included. Immunolabeled slides were evaluated using light microscopy.

RESULTS

Clinical signs. Clinical observations from the animals necropsied on days 2 ($n = 3$) and 4 ($n = 3$) scored 0, similar to preexposure levels. Euthanasia scores and body weights (Fig. 1a) remained similar to preexposure levels. On day 4, three animals (from other groups) began to cough. On day 6, signs of acute disease were noted that included increased weight loss, dyspnea, anorexia, and a peak in body temperature (mean, 39.2°C; $n = 3$ animals) (Fig. 1b). On day 8, animals showed signs of severe illness; additionally, two animals from the "reserve group" met the euthanasia criteria on day 8, thus the group size for necropsy on day 8 was $n = 5$ animals. Day 8 was the peak time for clinical signs; coughing was reported for 10 out of 12 animals, and the mean loss in body weight was −9.7% (Fig. 1a). By day 12, surviving animals were showing signs of recovery, with only one animal still coughing. The remaining animal from the "reserve group" was euthanized on day 12; hence, the sample size was $n = 4$ animals.

Enlargement of the lymph nodes (lymphadenopathy) was observed at necropsy in all animals from day 2 onward (see the detailed description in the "Pathology" section below).

The skin of the animals on days 2 and 4 were normal. Pox lesions appeared on day 6 (mean lesion count = 5, range = 0 to 16, $n = 3$ animals). The peak number of lesions occurred on day 8 (mean lesion count = 52, range = 0 to 189, $n = 5$ animals). The mean number of lesions decreased by day 10 (mean lesion count = 45, range = 17 to 70, $n = 3$ animals) and remained similar on day 12 (mean lesion count = 47, range = 4 to 92, $n = 4$ animals).

Immunophenotyping. The changes in absolute counts of different populations of immune cells, in the whole blood, are shown in Table 1. Compared to baseline levels, by day 2 postinfection, there were reductions in lymphocytes ($-2 \times 10^6 ml^{-1}$), $CD4^+$ T cells ($-4 \times 10^5 ml^{-1}$), $CD8^+$ T cells ($-2 \times 10^5 ml^{-1}$), $CD3^+$ T

TABLE 1 Changes in immune populations

Day postchallenge	Cellular population change (cells × 10^3 ml^{-1})[a]													
	Lymphocytes		$CD4^+$ T cells		$CD8^+$ T cells		$CD3^+$ T cells		NK cells		B cells		Monocytes	
	Mean	±1 SE	Mean	±1 SE	Mean	±1 SE	Mean	±1 SE	Mean	±1 SE	Mean	±1 SE	Mean	±1 SE
2	−1,607	606	−398	184	−160	63	−626	267	−132	40	−508	315	−141	7
4	−794	169	−64	75	−60	15	−197	84	−13	17	−278	78	75	83
6	−4,782	3,210	−851	240	−370	156	−1,397	444	−254	121	−625	170	−25	76
8	427	471	−136	142	46	131	−417	279	−60	65	−186	152	262	121
10	2,329	842	186	589	796	181	−77	1348	−66	67	−448	316	556	364
12	1,298	1,225	201	354	645	293	1,636	864	−34	84	−450	293	102	105

[a] Mean absolute cell counts (cells × 10^3 ml^{-1}) in different populations (+ 1 SE; n = 3 to 5 macaques) compared to baseline levels (average from two bleeds, taken on different days, prior to challenge) in whole blood, following challenge with aerosolized monkeypox virus.

cells (−6 × $10^5 ml^{-1}$), NK cells (−1 × $10^5 ml^{-1}$), B cells (−5 × $10^5 ml^{-1}$), and monocytes (−1 × $10^5 ml^{-1}$). By day 4, cell counts started to rise but remained below baseline levels, except for monocytes, which rose above prechallenge counts (+7.5 × 10^4 ml^{-1}) (Table 1). A second reduction in most immune cells was noted on day 6, followed by a steady rise until day 10 (Table 1). A positive increase was seen in lymphocytes (notably $CD8^+$ T cells) and monocytes on days 8, 10, and 12 ($CD4^+$ T cells rose above baseline from day 10 onward).

Cytokine responses. On day 2, a rise in interleukin 8 (IL-8) (increase of 567 pg ml^{-1}) was seen compared to prechallenge levels, and other biomarkers either decreased (e.g., monocyte chemoattractant protein 1 [MCP-1], IL-12/23, and IL-2) or remained unchanged (Table 2). On day 4, there were increases in the concentrations of gamma interferon (IFN-γ), IL-1ra, IL-6, IL-8, MCP-1, vascular endothelial growth factor (VEGF), IL-18, and IL-2. The concentrations of some cytokines, e.g., IFN-γ (1,322 pg ml^{-1}), IL-1ra (3,335 pg ml^{-1}), IL-6 (570 pg ml^{-1}), IL-8 (2,546 pg ml^{-1}), and MCP-1 (726 pg ml^{-1}), peaked on day 8. Small peaks in the concentrations of other biomarkers, e.g., tumor necrosis factor alpha (TNF-α), IL-1β, macrophage inflammatory protein 1β (MIP-1β), and IL-12/23, occurred on day 10. In contrast, small rises in granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-10 occurred later, by day 12 (Table 2).

Humoral immune responses. The levels of IgG serum antibodies in the blood to vaccinia virus, determined by ELISA, were increased from day 10 onward (Fig. 2).

Viral load. Viral DNA was first detected in the throat (3.4 × 10^4 copies ml^{-1}) on day 4. The concentration increased to 9.5 × 10^5 copies ml^{-1} on day 8 and remained high until the last study day (day 12) (Fig. 3). In contrast, viral DNA (vDNA) was first detected in the blood (8.3 × 10^3 copies ml^{-1}) on day 8, and its concentration rose to 4.5 × 10^5 copies ml^{-1} on day 10 and returned to 1.1 × 10^4 copies ml^{-1} by day 12 (Fig. 3). Viral PFU were detected in throat swabs (above the limit of detection = 25 PFU ml^{-1}) from day 4 to day 12, and a peak in the infectious virus concentration was detected on day 8 (1.4 × 10^3 PFU ml^{-1}). No

TABLE 2 Serum cytokine profiles of cynomolgus macaques taken during the pathogenesis study

Biomarker	Change in concn of biomarker (pg ml^{-1}) by day postchallenge[a]											
	Day 2		Day 4		Day 6		Day 8		Day 10		Day 12	
	Mean	±1 SE	Mean	±1 SE	Mean	±1 SE	Mean	±1 SE	Mean	±1 SE	Mean	±1 SE
IFN-γ	−2	1	212	55	842	250	1,322	210	380	168	32	13
IL-1ra	−2	4	52	8	313	173	3,335	3,200	18	15	21	7
IL-6	−1	0.3	43	22	213	88	570	339	70	29	17	3
TNF-α	0	0	0	0	2	2	1	1	15	11	1	1
IL-8	567	754	1,186	1,067	193	499	2,546	1,112	1,458	1,511	671	373
IL-1β	0	0	0	0	0	0	1	1	7	6	1	1
MCP-1	−131	64	383	78	651	413	726	93	201	105	132	103
MIP-1α	0	0	0	0	0	0	0	0	1	1	0	0
VEGF	−6	3	6	3	27	11	59	12	20	8	15	9
GM-CSF	0	0	0	0	0	0	1	0.4	1	1	7	7
MIP-1β	−2	1	−1	0	0.3	1	2	1	13	8	3	3
Il-5	−2	1	1	1	−0.3	0.3	1	1	6	3	1	0.5
IL-13	0	0	0	0	0	0	0.2	0.2	2	2	0.3	0.3
IL-10	−1	0	−0.3	0.3	−1	0	0	0.8	3	3	4	4
IL-12/23	−22	11	−5	10	−37	8	−68	13	69	80	46	44
IL-15	−2	1	−1	0.3	3	1	0	1	2	3	−1	1
IL-17	0	0	0	0	0	0	0.2	0.2	0	0	0	0
IL-18	−4	1	4	1	2	3	6	2	3	2	2	0.4
IL-2	−18	2	16	4	17	7	26	5	6	9	9	6

[a] Mean concentration (pg ml^{-1}) (+1 SE; n = 3 to 5 macaques) change in cytokine levels compared to baseline levels (average from two bleeds, taken on different days, prior to challenge) on various days following challenge with aerosolized monkeypox virus.

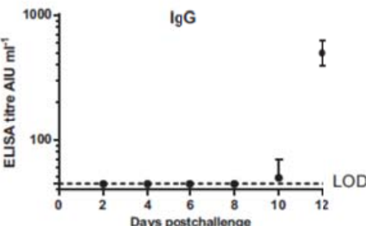

FIG 2 Serum IgG responses following challenge, measured by ELISA (mean + 1 SE, $n = 3$ to 5 macaques). Day 0, time of challenge with monkeypox virus. LOD, limit of detection (44.1 AIU ml^{-1}).

TABLE 3 Mean number of PFU of monkeypox virus in whole blood and throat swabs taken on different days postchallenge

Day postchallenge	Live virus PFU ml^{-1} (±SE)	
	Whole blood	Throat swab
2	<LOD[a]	25 ± 0
4	<LOD	$4.5 \times 10^2 \pm 4.3 \times 10^2$
6	<LOD	$1.9 \times 10^2 \pm 85$
8	<LOD	$1.4 \times 10^3 \pm 1.2 \times 10^3$
10	<LOD	$2.2 \times 10^2 \pm 1.9 \times 10^2$
12	<LOD	$6.4 \times 10^2 \pm 6.2 \times 10^2$

[a] <LOD, less than the limit of detection (250 PFU ml^{-1}).

infectious virus (PFU) above the limit of detection was detected in the blood on any of the days tested (Table 3).

Body tissues were assayed by real-time PCR, and on day 2, positive PCR results were restricted to the lungs (2.5×10^4 to 2.7×10^4 copies mg^{-1}); by day 4, this increased to 5.1×10^5 to 2.9×10^6 copies mg^{-1} (Fig. 4). On day 4, vDNA was also detected in the spleen and tonsils. By day 6, at least seven of the 13 tissues contained vDNA. The level of vDNA in the lungs remained high (3.7×10^6 to 5.7×10^6 copies mg^{-1}), while at least approximately 1×10^5 copies mg^{-1} of vDNA were detected in the spleen, tonsil, and tongue. By day 8, 9/13 tissues were positive for vDNA, including the kidney, heart, tongue, cerebrospinal fluid, spleen, and mediastinal lymph nodes. The concentration of virus in the tonsils (6×10^6 copies mg^{-1}) exceeded that in the lungs (3×10^6 copies mg^{-1}). Two animals in the "reserve group" were euthanized on humane grounds on day 8, and there was no difference in virological data between these two animals and the scheduled three animals. By day 10, the PCR values detected in the lungs decreased to 6.8×10^5 to 1.3×10^6 copies mg^{-1} (Fig. 4), although the concentration of vDNA in the tonsil was still high at 1.8×10^7 copies mg^{-1}. A decrease in viral load was apparent in all the animals on day 12, with values down to 1.1×10^4 to 3.8×10^5 copies mg^{-1} in the lungs (Fig. 4). This was consistent with clinical observations indicating that these animals were recovering.

Pathology. Histopathological changes and results of immunostaining are summarized in Table 4. On day 2 postchallenge, histological changes were restricted to respiratory bronchioles (Fig. 5a) in the lungs of two of three animals. They comprised occasional, small foci of dysplastic and sloughed cells. In immunostained sections, monkeypox antigen was present focally in all three animals, most frequently in the bronchioles and alveoli but occasionally in the epithelium of bronchi. Positive immunostaining was not observed in any other tissue on day 2.

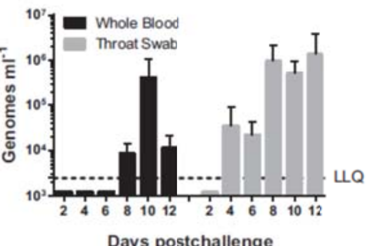

FIG 3 Mean number (+1 SD) of viral genomes (HA gene) as determined from qPCR in the blood and throat in samples taken following challenge with aerosolized monkeypox virus. The lower limit of quantification for qPCR (LLQ) was 2,500 genomes ml^{-1}.

On day 4 postchallenge, histological abnormalities were observed in the lungs of 3 animals. They were more numerous and developed than on day 2 postchallenge, comprising foci of epithelial necrosis and inflammation. In bronchioles, focal epithelial dysplasia, epithelial necrosis, and sloughing (Fig. 5b), with infiltration by neutrophils and occasionally eosinophils, were observed. Thickening of alveolar walls was seen diffusely in one animal and focally in another; a small number of alveoli were dilated. In the tracheobronchial lymph node of 1/3 animals, scattered foci of inflammatory cells, primarily histiocytes, were detected (Fig. 5c). Pathological changes were not observed in any other organ. Monkeypox antigen was detected by immunostaining in all lung lobes in all animals, more prominently than on day 2, located in the epithelium of bronchi (Fig. 5b inset), bronchioles, and alveolar walls. In the hilar (tracheobronchial) lymph node, positive staining was located in the outer cortex adjacent to the subcapsular sinus (Fig. 5c, inset). In addition, in the absence of histological changes, a few scattered positive cells were detected in the spleen (Fig. 5d) and submandibular lymph nodes of one animal and the lamina propria of the colon of another animal.

On day 6 postchallenge, severe lesions were noted in the lungs, comprising focal necrosis of airway epithelium and neutrophil infiltration, with lower airways affected more severely than upper airways (Fig. 5e). Diffuse and focal thickening of alveolar walls by macrophages and neutrophils was common, and some alveolar dilation was present. Peribronchial edema and periarterial edema, sometimes with infiltration by macrophages and neutrophils, were seen less frequently. Vascular lesions, comprising endothelial margination of macrophages and neutrophils, were seen occasionally. Focal necrosis with neutrophil infiltration was noted in the tracheobronchial lymph node, occurring in the outer cortex and subcapsular sinuses and infrequently in the medulla. In addition, lesions were detected in all animals in the tonsil and spleen and in the trachea/larynx of one of three animals. Lesions in the tonsil comprised focal necrosis of the crypt epithelium and/or lymphoid follicles, with neutrophil infiltration. In the spleen, focal necrosis with neutrophil infiltration was noted, most frequently in periarteriolar lymphoid sheaths. Immunostaining was positive, with a distribution similar to that for the lesions described above, including the spleen (Fig. 5f). In addition, scattered, positive staining was found in the colon and axillary lymph node in 1/3 animals, in the absence of histological changes.

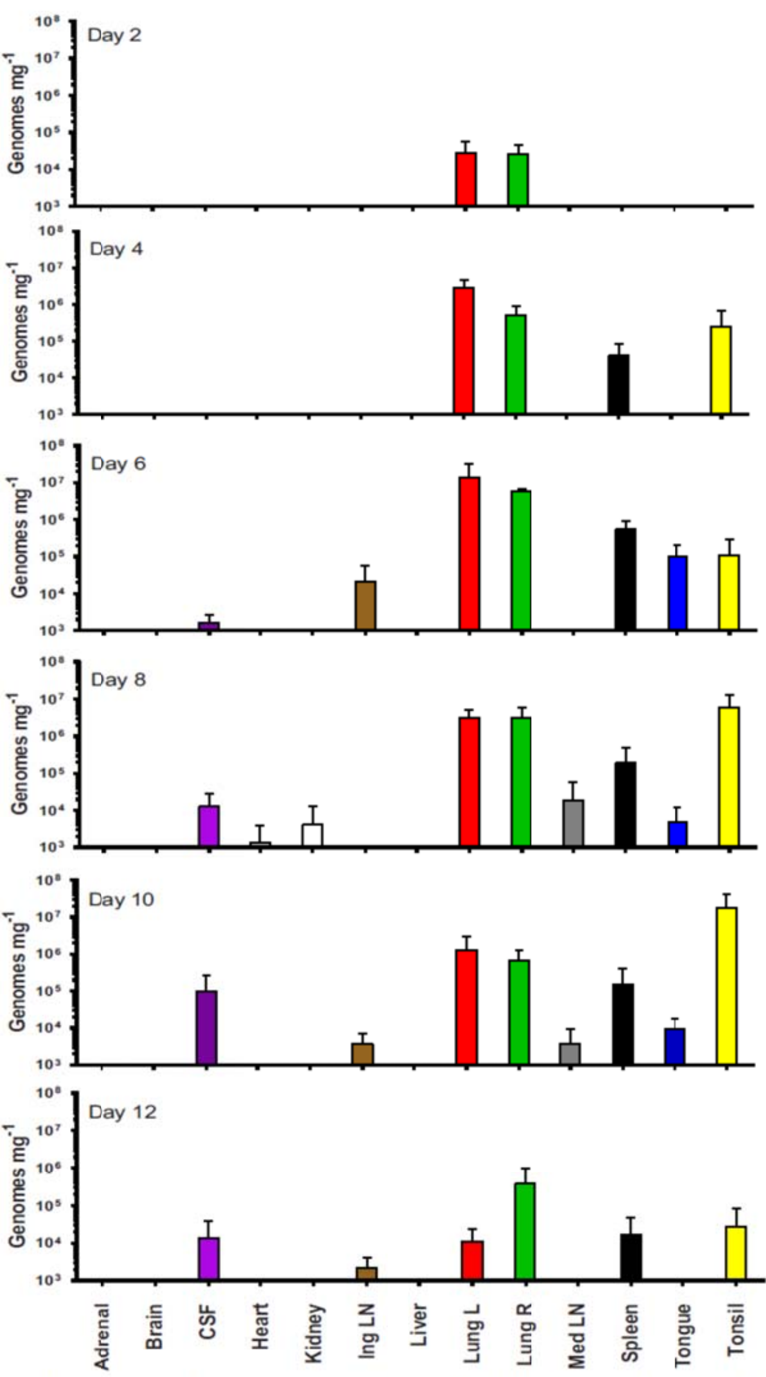

FIG 4 Viral load in tissues following challenge with monkeypox virus. The mean viral load (+1 SD), as determined by real-time PCR, in the tissues of animals taken at postmortem on different days of the study. For graphical purposes, the LLQ has been assigned 1,000 genomes mg^{-1} due to variability in the weights of tissue recovered at necropsy.

TABLE 4 Summary of the occurrence of monkeypox-related lesions and positive immunohistochemistry results in cynomolgus macaques following challenge with monkeypox virus[a]

	No. of samples with positive H&E or IHC results/total no. of samples											
	Day 2		Day 4		Day 6		Day 8		Day 10		Day 12	
Organ	H&E	IHC	H&E	IHC	H&E	IHC	H&E	IHC	H&E	IHC	H&E	IHC
Trachea/larynx	0/3	0/3	0/3	0/3	1/3	1/3	1/5	1/5	0/3	0/3	1/4	1/4
Lung RU	0/3	3/3	3/3	3/3	3/3	3/3	5/5	5/5	3/3	3/3	4/4	2/4
Lung RM	0/3	3/3	3/3	3/3	3/3	3/3	5/5	5/5	3/3	3/3	4/4	2/4
Lung RL	1/3	3/3	3/3	3/3	3/3	3/3	5/5	5/5	3/3	3/3	4/4	2/4
Lung LU	1/3	3/3	2/3	3/3	3/3	3/3	5/5	5/5	3/3	3/3	4/4	3/4
Lung LL	2/3	3/3	3/3	3/3	3/3	3/3	5/5	5/5	3/3	3/3	4/4	3/4
Hilar LN	0/2	0/2	1/3	2/3	3/3	3/3	4/5	4/5	1/3	2/3	2/3	1/4
Spleen	0/3	0/3	0/3	1/3	3/3	3/3	3/5	4/5	2/3	1/3	2/4	0/4
Tongue	0/3	0/3	0/3	0/3	0/3	0/3	2/5	2/5	0/3	0/3	0/3	0/3
Tonsil	0/3	0/3	0/3	0/3	3/3	3/3	3/3	3/3	2/2	2/2	0/2	0/2
Mandibular LN	0/3	0/3	0/2	1/2	2/2	2/2	2/5	3/5	1/3	1/3	0/3	0/3
Stomach	0/3	0/3	0/3	0/3	0/3	0/3	0/5	1/5	0/3	0/3	0/3	0/3
Ileum	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Colon	0/3	0/3	0/3	1/3	0/3	1/3	1/5	2/5	1/3	1/3	0/3	0/3
Mesenteric LN	0/3	0/3	0/3	0/3	0/3	0/3	1/5	1/5	0/3	0/3	0/3	0/3
Skin	0/3	0/3	0/3	0/3	0/3	0/3	1/5	1/4	0/3	0/2	1/4	1/3
Inguinal LN	0/3	0/3	0/3	0/3	0/3	0/3	0/5	3/5	0/3	0/3	0/3	0/1
Axillary LN	0/3	0/3	0/3	0/3	0/3	1/3	0/3	0/2	0/3	0/3	0/3	0/2

[a] Lung RU, right upper lobe; lung RM, right middle lobe; lung RL, right lower lobe; lung LU, left upper lobe; lung LL, left lower lobe; LN, lymph node; H&E, hematoxylin and eosin; IHC, immunohistochemistry (to identify monkeypox virus antigens).

From days 8 to 10 postchallenge, lesions in the lung and other tissues attributed to monkeypox infection were severe and extensive. Distribution of positive immunostaining was widespread (Table 4).

In all three animals remaining on day 12 after challenge, occasional acute lesions were observed, but there was some evidence of early resolution that comprised cuboidal, nonciliated epithelial cells. Immunostained monkeypox antigen was still present in the respiratory tract, including in the hilar lymph node, but was not observed in the remaining tissues examined (Table 4).

DISCUSSION

When human efficacy studies are neither ethical nor feasible, the FDA may grant marketing approval of a new product, such as a vaccine, based upon the Animal Rule. Well-characterized animal models, however, are a requirement of this rule. This study characterized the early stages of monkeypox infection in cynomolgus macaques, using a dose of 10^5 PFU, delivered by the aerosol route. These early events have not been previously investigated in detail. Here, special emphasis was placed on examining the virological, immunological, and pathological changes in early infection.

Previous experiments examining monkeypox disease progression have monitored the clinical signs of disease until animals have reached a humane endpoint or have died in the study. This invariably happened 6 to 9 days after challenge, depending on the dose and route of challenge. In this work, a different approach was taken; animals were divided into groups and euthanized on designated days unless individuals were observed to have reached a humane endpoint. Thus, a mix of animals was studied; some had developed severe disease at the time of euthanasia, while some developed only mild disease. This approach has allowed the detailed study of the early events following challenge that were missing from studies based on animals with severe disease (22, 23).

Few studies have examined the immune response of cynomolgus macaques following exposure to aerosolized monkeypox virus. In this work, trends in the data showed a decrease in different immune cell populations early on in the course of the infection. Larger animal group sizes, however, are needed in order to confirm this statistically. Nevertheless, this could be indicative of immunosuppression, as the immunomodulatory effect of monkeypox virus has been previously observed by others (2, 31, 32). The rises in lymphocytes, in particular $CD8^+$ T cells, observed later in monkeypox infection, are likely to be the result of the host raising an adaptive immune response to the viral infection. Monocytes also increased above baseline levels on day 4 postinfection and again on days 8, 10, and 12. In this work, individual, circulating monocytes were not examined for infection with monkeypox virus; however, Zaucha and colleagues showed in their NHP studies that monocytic cells were immunopositive for poxviral antigen in a variety of organs and proposed that monkeypox virus spread initially via the lymph nodes and the mononuclear phagocytic/dendritic cell system (23).

Changes in the concentrations of circulating cytokines in the blood have been recorded in NHPs following challenge with different doses of monkeypox virus (5×10^4 to 5×10^7 PFU), via the intravenous and intrabronchial routes (8). Maximum mean peak fold changes in IFN-γ (8,028), IL-1ra (4,854), and IL-6 (3,207) were recorded on a variety of days (8). In this study, the mean peak changes in the biomarkers IFN-γ (1,322 pg ml^{-1}), IL-1ra (3,335 pg ml^{-1}), and IL-6 (570 pg ml^{-1}) occurred on day 8 postchallenge. The results of different studies are difficult to compare, because of the different ways data sets are presented and because different challenge doses and inoculation routes are used. However, it is clear IFN-γ, IL-1ra, and IL-6 play a role in the host response to monkeypox infection.

In this study, day 8 was the time when vDNA was detected in

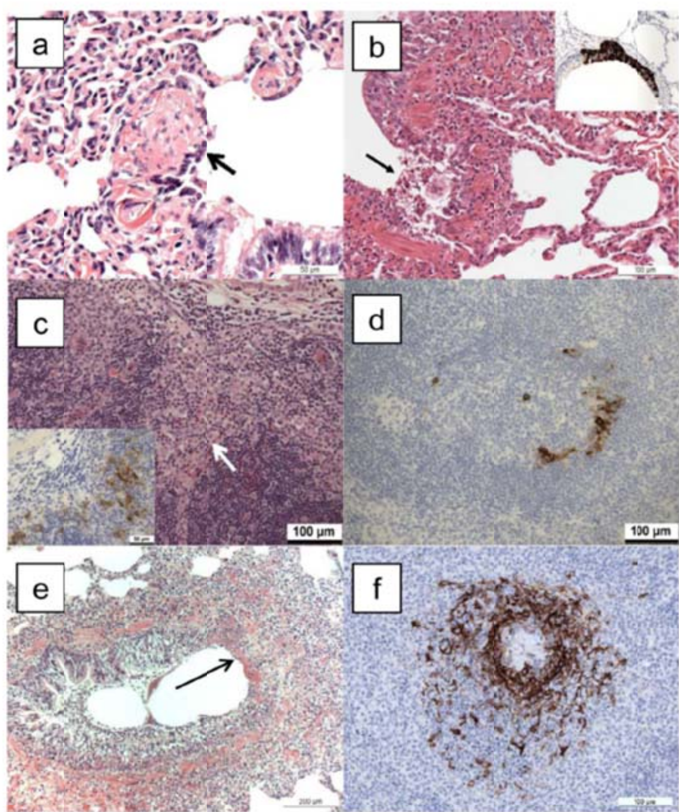

FIG 5 Histopathological changes associated with infection with monkeypox virus. (a) Lung, animal M965A, euthanized on day 2 postchallenge. Sloughing of respiratory bronchiolar epithelial cells (arrow) close to the junction with a bronchus. H&E. (b) Lung, animal M029F, euthanized on day 4 postchallenge. Focal epithelial cell necrosis (arrow), with sloughed cells and neutrophil infiltration at the junction of a bronchus and bronchiole. H&E. Inset, lung, animal I089K, euthanized on day 4 postchallenge. Focal, positive immunostaining of bronchial epithelium for monkeypox viral antigen. IHC. (c) Tracheobronchial lymph node, animal M029F, euthanized on day 4 postchallenge. Infiltration of superficial cortex by macrophages (arrow). HE. Inset, scattered, positively stained cells for monkeypox viral antigen. IHC. (d) Spleen, animal M293E, euthanized on day 4 postchallenge. Scattered, positively stained cells for monkeypox viral antigen in the white pulp. IHC. (e) Lung, animal M865B, euthanized 6 days postchallenge. Focal, bronchial epithelial necrosis (arrow). HE. (f) Spleen, animal I430G, euthanized on day 6 postchallenge. Positive immunostaining for monkeypox viral antigen in a periarteriolar lymphoid sheath (PALS) within the white pulp. IHC.

most tissues (9/13) and for death to most likely occur. It was also apparent that there was considerable animal-to-animal variation, and while the majority of the animals reached humane endpoints at approximately days 8 to 10, some survived the acute disease, as evidenced by the decline in the viral load in the blood and lung and tonsil tissues on day 12. The results of this experiment are similar to those reported by Nalca et al., in which, following an inhaled dose of 1.4×10^5 PFU, the mean time to death in cynomolgus macaques was 9 days (22). They also reported the peak viral load in blood (10^6 to 10^7 genomes ml^{-1}) was seen on day 10 postexposure; this was also noted on day 10 in this study (5×10^5 genomes ml^{-1}). Barnewall et al. also exposed two macaques to an aerosolized target dose of 1×10^5 PFU monkeypox virus; one died on day 8 and the other on day 9, and 2.7×10^5 to 3.6×10^5 genomes ml^{-1} were recorded in the blood on day 8 (24).

The histopathological and immunohistochemical findings in this study from animals examined on days 2, 4, and 6 postchallenge confirm earlier findings (23) and have identified features of pathogenesis not reported previously, to the authors' knowledge. Zaucha and colleagues studied cynomolgus macaques exposed to an aerosol of monkeypox virus that died or were euthanized 9 to 17 days after exposure (23). They concluded that the lower airway epithelium was the principal target, and the present study supports their proposition and additionally illustrates infection and lesions in these epithelia as early as 2 days after challenge. They proposed that tonsil, mandibular nodes, and mediastinal nodes were infected early. The findings of this study support their proposition; on day 4, 2/3 hilar nodes immunostained positively and 3/3 on day 6. On day 4, one of the two mandibular lymph nodes immunostained positively, and 2/2 were positive on day 6 when 3/3 tonsils were positive. Zaucha and colleagues (23) concluded that the spleen and gastrointestinal tract became infected later, but this was not supported by the findings of this study, which recorded immunostaining in 1/3 spleens on day 4 and 3/3 on day 6.

Positive immunostaining was present in the colon of 1/3 animals on day 4. The findings from animals euthanized on days 8, 10, and 12 after challenge were consistent with those of a previous study (23).

This work describes, for the first time, the sequence of early pathological events that occur following inhalation of 10^5 PFU monkeypox virus in cynomolgus macaques. Monkeypox virus vDNA was detected in the lungs 2 days postinfection, and by day 4 the virus was detected in the throat, tonsil, and spleen. Viral antigen was also detected by immunohistochemistry in the lungs, spleen, colon, and mandibular and hilar lymph nodes on day 4. From day 6, signs of acute disease were obvious and pox lesions appeared. In 1988, Fenner and colleagues inferred, from limited available information, that the clinical manifestations of naturally acquired smallpox in humans began after an incubation period of 7 to 17 days following exposure (33). The first febrile phase lasted for 3 to 4 days, followed by the appearance of a rash. Our model partially resembles some of the features of the human condition, which helps to bridge the gap in our understanding of variola virus infection and improves our understanding of monkeypox viral disease while at the same time further characterizing this important smallpox animal model, as required by the FDA's Animal Rule.

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RESEARCH PAPER

OPEN ACCESS

Repeated high-dose (5×10^8 $TCID_{50}$) toxicity study of a third generation smallpox vaccine (IMVAMUNE) in New Zealand white rabbits

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ABSTRACT
Concern over the release of variola virus as an agent of bioterrorism remains high and a rapid vaccination regimen is desirable for use in the event of a confirmed release of virus. A single, high-dose (5×10^8 $TCID_{50}$) of Bavarian Nordic's IMVAMUNE was tested in a Phase-II clinical trial, in humans, as a substitute for the standard (1×10^8 $TCID_{50}$), using a 2-dose, 28-days apart regimen. Prior to this clinical trial taking place a Good Laboratory Practice, repeated high-dose, toxicology study was performed using IMVAMUNE, in New Zealand white rabbits and the results are reported here. Male and female rabbits were dosed twice, subcutaneously, with 5×10^8 $TCID_{50}$ of IMVAMUNE (test) or saline (control), 7-days apart. The clinical condition, body-weight, food consumption, haematology, blood chemistry, immunogenicity, organ-weight, and macroscopic and microscopic pathology were investigated. Haematological investigations indicated changes within the white blood cell profile that were attributed to treatment with IMVAMUNE; these comprised slight increases in neutrophil and monocyte numbers, on study days 1-3 and a marginal increase in lymphocyte numbers on day 10. Macroscopic pathology revealed reddening at the sites of administration and thickened skin in IMVAMUNE, treated animals. After the second dose of IMVAMUNE 9/10 rabbits seroconverted, as detected by antibody ELISA on day 10, by day 21, 10/10 rabbits seroconverted. Treatment-related changes were not detected in other parameters. In conclusion, the subcutaneous injection of 2 high-doses of IMVAMUNE, to rabbits, was well tolerated producing only minor changes at the site of administration. Vaccinia-specific antibodies were raised in IMVAMUNE-vaccinated rabbits only.

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IMVAMUNE; MVA; rabbits; smallpox; toxicology; vaccine

Introduction

Routine smallpox vaccination declined throughout the world after the causative agent, variola virus, was declared eradicated by the WHO in 1980.[1] Only 20% of the global population has some immunity to smallpox, due to previous vaccination,[2,3] hence a deliberate release of this virus would have catastrophic consequences. Stockpiles of first and second generation vaccines based on replicating-vaccinia virus (eg. Dryvax and ACAM2000) are maintained in some countries, for instance, the United States, to help counter the threat of re-emerging smallpox following a bioterrorist attack.[4,5] First and second generation (conventional) smallpox vaccines have been shown to be highly efficacious, however, they have also been associated with rare but severe adverse events, especially in populations with compromised immune systems.[6,7] These adverse events include progressive vaccinia eczema vaccinatum, myo/pericarditis, Stevens-Johnson syndrome, fetal vaccinia encephalititis and occasionally death.[5] In 2002, Kemper *et al* estimated that 25% of the US population would be excluded from vaccination with conventional smallpox vaccines because they are, or have close contact with, individuals who have eczema or are immunocompromised.[8] Safer alternative smallpox vaccines are thus desirable and interest in attenuated viruses, such as modified-vaccinia Ankara virus (MVA), has led to the development of new third generation smallpox vaccines.

MVA was generated by passaging vaccinia virus more than 500 times through chicken embryo fibroblasts. During this time the virus acquired multiple deletions and mutations and lost its capacity to replicate efficiently in people and most mammalian cell lines.[9] MVA was used during the smallpox eradication campaign during the 1970s, in Germany, as a priming vaccine prior to the administration of conventional smallpox vaccine, to mitigate potential reactogenicity.[10,11] More than 120,000 people took part in this program. Several high-risk groups were vaccinated, including young children with skin conditions.[10-12] There were no reports of serious adverse events using this 2-step inoculation process.[12]

IMVAMUNE, a vaccine based on a strain of the modified-vaccinia Ankara (MVA) virus, is currently being developed as a safe and effective vaccine, by Bavarian-Nordic (BN), Denmark.[12] Early studies determined whether IMVAMUNE was safe, prior to the initiation of human trials. These studies included repeat administrations (subcutaneous and intramuscular) in animal models and the results showed reversible non-dosing-limiting injection site reactions and lymphoid changes.[12] Tetraology studies in rats and rabbits did not demonstrate teratogenic or intrauterine toxicity, and peri- and postnatal studies did not reveal toxicity to embryos or developing offspring at doses up to

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1×10^8 $TCID_{50}$.[4] IMVAMUNE was tested in humans for safety and immunogenicity using subcutaneous and intramuscular, administration of a range of doses (10^6-10^8 $TCID_{50}$) and different vaccination regimes; 1 or 2 doses at various intervals sometimes followed by a conventional smallpox vaccine.[4,12-15] The protective efficacy of IMVAMUNE was also demonstrated in animal models[16-20] with promising results against rabbitpox and monkeypox viruses (surrogates for variola virus).

Overall, the safety and efficacy data generated for IMVAMUNE, so far, have been very promising. Currently, the optimal vaccination schedule is a prime-boost regimen with 2 doses (1×10^8 $TCID_{50}$) administered with a 28-day interval.[13] If, however, this vaccine were to be used to protect the population following a bioterrorist attack or response scenario, the use of a single dose would be highly advantageous as this could potentially help to limit casualties quickly.[13] An investigation into the suitability of a single high-dose of IMVAMUNE (5×10^8 $TCID_{50}$) was thus initiated. Since early safety studies utilised different doses up to and including 1×10^8 $TCID_{50}$ it was necessary to perform a repeat-dose toxicology experiment using the higher proposed dose of 5×10^8 $TCID_{50}$ to ensure this vaccine dose was safe prior to it being given to humans. Vaccination doses were given to rabbits on day 0 and day 7, this allowed sufficient time for recovery from local reactogenicity to the first vaccination before the second vaccination was given. This work was conducted according to Good Laboratory Practice (GLP). After this toxicology study was performed this high-dose of virus was used in the randomized Phase II clinical trial (http://clinicaltrials.gov/show/NCT00879762) reported by Frey et al.[21]

Results

Clinical signs: Neither abnormal systemic nor local clinical signs were observed, and no deaths were recorded prior to the pre-determined necropsy times. There were changes in the skin at the vaccination sites, observed at necropsy on days 10 and 21 (Table 2). Changes were not observed at day 35. On day 10, when compared with controls, an increased number of dark red areas were noted after injection of IMVAMUNE at site 1 and site 2. Thickened areas were seen in one male and one female animal 3 d after the second treatment with IMVAMUNE. On day 21, when compared with controls, an increased incidence of dark red areas at the vaccination sites with IMVAMUNE (site 1) were seen; a similar response was seen (14 d after the second vaccination) at site 2. Thickened areas were seen in one male and one female animal after treatment with IMVAMUNE at site 2. At the end of the study (day 35) macroscopic, treatment related findings were not apparent.

Body weight and food consumption: Body weights and food consumption were unaffected by treatment (data not shown).

Clinical chemistry and haematology: The biochemistry of the plasma was unaffected by treatment. Minimal changes in the white blood cell profile were noted following treatment with IMVAMUNE (Table 3). A slight increase in neutrophil numbers was apparent in males on days 1 ($P<0.05$) and 10 ($P<0.05$) and a slight increase in monocyte numbers was evident in females on days 1 and 2 ($P<0.05$) and in males on day 2 ($P<0.01$). A marginal increase in lymphocyte numbers was evident 3 d after the last dose (day 10) in females but this was not statistically significant.

Table 1. Vaccination sites examined on rabbits vaccinated with IMVAMUNE or Saline Control. No macroscopic change related to treatment was apparent at necropsy on day 35 (n = 3).

	Treatment Group and Sex			
	Male		Female	
Parameters	Saline Control n=5	IMVAMUNE n = 5	Saline Control n=5	IMVAMUNE n=5
Animals euthanised on Day 10				
Treated site 1				
Dark area(s)	3	5	1	2
Treated Site 2				
Dark area(s)	2	5	2	4
Thickened / Oedematous	0	1	0	1
Animals euthanised on Day 21				
Treated Site 1				
Dark area(s)	0	2	1	3
Treated Site 2				
Dark area(s)	1	2	1	3
Thickened / Oedematous	0	1	0	1

Immunogenicity (BN Nordic ELISA): Animals treated with saline (control) were negative for vaccinia-specific IgG at all time-points. Animals treated with IMVAMUNE were negative for vaccinia-specific IgG before the first administration (day 0) (Fig. 1) and on day 7 prior to the second administration of IMVAMUNE. Vaccinia-specific IgG could be detected in the majority of IMVAMUNE immunized rabbits 10 d after the first and 3 d after the second administration in both males and females. Out of 5 males, 3 had high titres and 2 were close to the limit of detection. Out of 5 females, one did not have antibodies and 4 were at the limit of detection. Titres increased until day 21, the first day when all animals had seroconverted, and were maintained on day 35. Males tended to have slightly higher titres than females although this was not significantly different ($P>0.05$) on days 21 and 35.

Organ weights: Mean adrenal weights that were higher than control values were apparent among IMVAMUNE treated males on day 35 ($P<0.05$) (Table 4). No difference in mean adrenal weights in IMVAMUNE treated females were observed ($P>0.05$) compared with controls. In males, on day 10 and 21, mean prostate weight was lower than controls, however, on day 35, mean prostate weight was higher than controls (none of these observations were significant ($P>0.05$)). Pathological change was not observed in the adrenal glands and prostate, therefore, the relationship between treatments and organ weight was uncertain and they were considered not to be adverse.

Microscopic findings: Changes were observed at the vaccination sites and are summarised in Supplementary Data Tables 1-3. On day 10, the dermis and subcutaneous tissues were infiltrated by mixed inflammatory cells. Dermal and subcutaneous hemorrhage, subcutaneous fibrosis and myofibre necrosis of the panniculus muscle, were noted at both sites.

Table 2. Summary of treatment-related changes in the average number of Leucocytes.

Cell Types (mean)		Male Saline Control	Male IMVAMUNE	Female Saline Control	Female IMVAMUNE
Total white blood cells (x10^9/L)	Pretreatment	8.71	7.94	7.77	8.37
	Day 1	8.95	8.49	9.05	9.03
	Day 2	8.22	8.56	8.18	8.92
	Day 3	8.56	8.60	8.08	8.58
	Day 10	7.96	8.89	7.71	9.25
	Day 21	8.69	8.14	7.11	8.02
	Day 35	6.95	6.49	6.30	8.45*
Mean Neutrophils (x10^9/L)	Pretreatment	1.62	1.81	1.72	1.62
	Day 1	1.83	2.37*	1.86	1.74
	Day 2	1.54	2.03	1.46	1.56
	Day 3	1.78	1.87	1.64	1.61
	Day 10	1.44	1.87*	1.51	1.91
	Day 21	1.62	1.74	1.32	1.33
	Day 35	0.79	0.85	1.13	1.22
Monocytes (x10^9/L)	Pretreatment	0.12	0.17*	0.10	0.08
	Day 1	0.08	0.09	0.08	0.13**
	Day 2	0.07	0.16**	0.11	0.26*
	Day 3	0.08	0.12	0.13	0.17
	Day 10	0.17	0.17	0.06	0.08*
	Day 21	0.10	0.10	0.11	0.09
	Day 35	0.10	0.17	0.04	0.09
Lymphocytes (x10^9/L)	Pretreatment	6.30	5.31**	5.42	5.93
	Day 1	6.34	5.42*	6.61	6.46
	Day 2	5.98	5.78	6.07	6.41
	Day 3	6.04	6.02	5.76	6.10
	Day 10	5.75	6.27	5.56	6.83
	Day 21	6.31	5.75	5.22	6.08
	Day 35	5.62	5.12	4.67	6.55

Statistically significant when compared with the Control (Group 1): *-$p<0.05$; **-$p<0.01$.

On day 21, at sites 1 and 2, dermal and/or subcutaneous inflammatory cell infiltrates, subcutaneous fibrosis and subcutaneous hemorrhage were evident. On day 35, dermal and/or subcutaneous mixed inflammatory cell infiltrates and/or subcutaneous hemorrhage were apparent.

In the axillary lymph nodes, on days 10, 21 and 35, germinal centers appeared to be increased (minimal or slight) (Table 5) in IMVAMUNE treated animals. Changes were not observed in the controls.

Discussion

The optimal dosing regimen for IMVAMUNE, a third generation smallpox vaccine, is 2 doses of 1×10^8 $TCID_{50}$ of virus, 28-days apart. However, should a deliberate release of *Variola major* virus occur, a post-exposure vaccination program with a single dosing schedule would be desirable to limit casualties. To address this issue, a single high-dose (5×10^8 $TCID_{50}$) of MVA was tested in vaccinia-naïve individuals in a phase II

Table 3. Summary of treatment-related changes in adrenal and prostate weights (g). Absolute values and difference from control (xn).

Organ Day euthanised	No. of animals	Male Saline Control	Male IMVAMUNE	Female Saline Control	Female IMVAMUNE
Adrenals					
Day 10	n = 5	0.211	0.170 (x0.81)	0.249	0.256 (x1.03)
Day 21	n = 5	0.234	0.233 (x1.00)	0.237	0.282 (x1.19)
Day 35	n = 3	0.167	0.285* (x1.71)	0.287	0.370 (x1.29)
Prostate					
Day 10	n = 5	0.797	0.603 (x0.76)	–	–
Day 21	n = 5	0.683	0.503 (x0.74)	–	–
Day 35	n = 3	0.597	1.115 (x1.87)	–	–

Statistically significant when compared with the Saline Control (Group 1): *-$p<0.05$

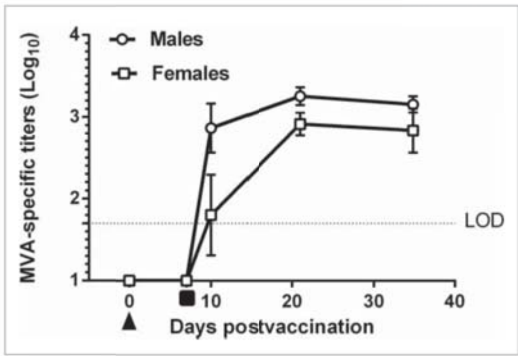

Figure 1. Mean vaccinia-specific IgG titer ($Log_{10} \pm 1$ SE) of New Zealand white rabbits vaccinated with MVA-BN (IMVAMUNE) vaccine (Group 2). Animals were bled on various days (Prebleed Day 0, Day 7 after the first immunization, 3 d (Day 10), 14 d (Day 21) and 28 d (Day 35) after the second immunisation. Mean values are from 13 (Day 0 and Day 7), 5 (Day 10 and Day 21) or 3 (Day 35) animals each. LOD = Limit of Detection (1.7 log_{10}). First immunization (▲), Second immunisation (■). For graphical purposes values below the limit of detection (LOD) were assigned a value of $1Log_{10}$.

randomized clinical trial[21] [21] and, prior to this being carried out, this GLP, repeated-dose, toxicology study was performmed, to assess the safety profile of this new dose, before it was given to humans. This is the first time a toxicology study has been reported using a high-dose (5×10^8 $TCID_{50}$) of IMVAMUNE in rabbits, this contributes information toward the safety assessment for the clinical use of this vaccine in humans.

Differences were not observed between rabbits treated with IMVAMUNE or saline in respect of clinical observations, blood clinical chemistry, food consumption or body weight. However, a local skin reaction to treatment with IMVAMUNE was evident *post mortem* comprising dark red areas and thickened vaccination sites. Microscopically, inflammatory cell infiltrates, both mononuclear and polymorphonuclear, and hemorrhage were evident in the dermis and subcutis of the administration sites. Subcutaneous fibrosis and inflammation with myofibre necrosis of the panniculus muscle were also apparent at the inoculation sites. A higher incidence and longer persistence of these changes were apparent in animals receiving IMVAMUNE

Table 4. Summary of treatment-related changes in the axillary lymph nodes on days 10, 21 and 35. Number of animals with treatment related changes/Total number of animals.

Prominent germinal centers	Treatment group and sex			
	Male		Female	
	Saline Control	IMVAMUNE	Saline Control	IMVAMUNE
Day 10				
Minimal	0/5	0/5	0/4	3/5
Slight	0/5	2/5	0/5	1/5
Day 21				
Minimal	0/5	4/5	0/5	4/5
Day 35				
Minimal	0/3	3/3	0/3	3/3

compared with the controls, indicating that IMVAMUNE exerted a local response. Overall, the incidence was low and the severity minimal or slight. This low severity was confirmed by the absence of an overt behavioral change. The incidence was highest at the dose sites 3 d after the second administration (day 10) and diminished subsequently. However, recovery was not complete at the sites 21 and 35 d after treatment. Local skin reactions in humans following the administration of IMVAMUNE have also been reported and this was, therefore, not an unexpected finding.[13-15,21] Collectively the data from this study and others suggest that skin reactions at the site of injection should be monitored during clinical trials with IMVAMUNE.

Marginal increases in peripheral neutrophil and monocyte numbers after the first dose were seen and may be related to the role of these cells in the immunological response to IMVAMUNE virus particles following immunisation on day 0. The marginal increase in lymphocyte numbers in females after the second dose may represent an immune response to the second insult of the vaccine. These effects were not considered adverse.

The prominent germinal centers of the axillary lymph nodes, seen following vaccination with IMVAMUNE, were considered to be part of an immune response to the virus. This change decreased in severity on days 21 or 35, when compared to those at day 10, indicating germinal center involution had occurred following the initial immune response. These observations are consistent with reports of lymphoid changes and reversible non-dose-limiting injection site reactions reported in early safety tests on IMVAMUNE in animal models, as reviewed by Kennedy and Greenberg (2009).[12]

In this study, both male and female test rabbits raised vaccinia-specific antibodies when vaccinated with IMVAMUNE. Initially, titres were higher in males than females although by days 21 and 35 there was no significant difference between groups. Interestingly, Troy *et al* (2015) report on gender differences in the immune response to vaccination with IMVAMUNE in humans, males tended to have higher levels of antibody than females.[22] In this work, none of the rabbits that received saline mounted a detectable vaccinia-specific antibody response, on any of the days tested. Other immune parameters for example, plaque reduction neutralisation assay [PRNT], cell mediated responses and vaccine efficacy (challenge against rabbitpox) could have been analyzed in this study, however, they were excluded because detailed studies addressing these issues had already been performed elsewhere.[16-19]

Repeated-dose and embryofetal toxicity studies in animals for the standard dose (1×10^8 $TCID_{50}$) of IMVAMUNE have already been performed and severe adverse events were not reported.[12] Similarly, in the present study in rabbits, a repeated-dose of IMVAMUNE at a high concentration (5×10^8 $TCID_{50}$), was tolerated, producing only minor changes at the site of administration. This good safety profile, in rabbits supported the use of a high-dose of this vaccine in humans and, as a result, a phase-II clinical trial was conducted.[21]

Materials and methods

Animals: Male (n = 26) and female (n = 26) New Zealand White rabbits were acclimatised for at least 12 d before treatment. At the start of treatment, animals were 12-17 weeks old

Table 5. Study Plan: Animals were vaccinated subcutaneously twice (7-days apart) with either MVA-BN (IMVAMUNE) (Group 2), or with saline control (Group 1). Animals were monitored for 27 d (upto day 35) after the last vaccination. Animals were euthanised at days 10, 21 and 35.

			Number of animals					
			Euthanised Day 10		Euthanised Day 21		Euthanised Day 35	
Group No.	Treatment	Dose volume (ml)	Male	Female	Male	Female	Male	Female
1	Saline Control	2×1ml	5	5	5	5	3	3
2	IMVAMUNE	2×1ml[a]	5	5	5	5	3	3

[a]One dose of IMVAMUNE consisted of 2 × 0.5ml equivalent to 4.9×10^{8} $TCID_{50}$.

and males weighed between 2.9-3.3 kg (inclusive) and females between 2.9-3.4 kg (inclusive). Rabbits were housed individually in stainless steel cages and environmental controls were set to maintain the following conditions: temperature range 16-20°C, relative humidity 40-70%, 12-hr light/12-hr dark cycle. The animals were offered 150 g of a standard laboratory diet each day throughout the study. This diet contained no added antibiotic or other chemotherapeutic or prophylactic agent; water (from the public water supply) was given via water bottle, *ad libitum*.

Experimental studies were conducted at Envigo CRS Limited (Cambridge, UK). The general procedures were in compliance with the "Code of practice for the housing and care of animals used in scientific procedures," published by the UK Home Office which forms part of the Animal (Scientific Procedures) Act (1986). This study was also performed in compliance with GLP.

Vaccine formulation and study design: The duration of this study was 35 d. The test vaccine, IMVAMUNE, supplied by Bavarian Nordic (Batch No 0061205), had a viral concentration of 4.9×10^{8} $TCID_{50}$/ml. Animals were weighed and assigned randomly to groups; group 1, (control) (n=26), group 2 (test) (n=26) (Table 1). The dorsum was shorn using electric clippers; 2 injection sites (site 1 and site 2) were identified, on the back of the neck. The animals received 2 doses (2 × 0.5 ml each) of the vaccine (test) or physiological saline (control) by subcutaneous injection on day 0 at site 1 (dose 1) and on day 7 at site 2 (dose 2). Thus, the animals received 4.9×10^{8} $TCID_{50}$ of virus on each day of administration.

Necropsy procedures were undertaken on days 10, 21 and 35 (Table 1).

Observations and clinical signs: During the study periods, each animal was examined twice daily for evidence of ill-health or reaction to treatment. On each day of administration, 5 observations were recorded; 1) immediately before, 2) immediately after dosing, 3) on completion of dosing each group, 4) between 1-2 hours after completion of dosing, and 5) as late as possible in the working day. In addition, a more detailed weekly physical examination was performed on each animal to monitor general health. Injection sites were assessed daily for 3 d after each injection and weekly throughout the study. Injection sites were scored according to the numerical scoring system of Draize.[23]

Body-weight: The weight of each rabbit was recorded one week before treatment commenced (day -7), on the day treatment commenced (day 0), weekly throughout the study and before necropsy. Group mean weight changes were calculated from the weight changes of individual animals.

Food and water consumption: The weight of food supplied to each animal, food remaining and an estimate of any spilled, was recorded for the week before treatment started (week -1), and each week throughout the study. From these data the estimated weekly consumption per animal (g/rabbit/week) was calculated.

Haematological studies: Before the commencement of treatment (day 0) and on days 1, 2, 3, 10, 21 and 35 of the study, blood samples (0.5 ml) were collected into EDTA anticoagulant (TekLab, County Durham, UK, Catalogue No: K1230) from the central auricular artery. Blood samples were examined using a Bayer Advia 120 haematology analyzer (Siemens, Surrey, UK). The following characteristics were determined; haematocrit (L/L), hemoglobin concentration (g/dL), erythrocyte count (RBC), reticulocyte count (%), mean cell hemoglobin (pg), mean cell hemoglobin concentration (g/dL), mean cell volume (fL), total leucocyte count (cells/L), differential leucocyte count (including neutrophils, lymphocytes, eosinophils, basophils, monocytes, large unstained cells and platelet count) (cells/L).

Additional blood samples were collected into citrate anticoagulant (TekLab, Catalogue No: C1130) and examined for Prothrombin time (sec) using an ACL 3000 Plus analyzer (Instrument Laboratory, Cheshire, UK) with IL PT-Fibrinogen reagent (Instrument Laboratory; Catalogue No: 0008469810). Also, activated partial thromboplastin time (sec) was measured using an ACL 3000 Plus Analyser (Instrument Laboratory) and IL APTT reagent (Instrument Laboratory; Catalogue No: 0020006800).

Clinical biochemistry: When blood was obtained for haematology, additional samples (0.7 ml) were collected into lithium heparin anticoagulant (TekLab, Catalogue No: H2130). Plasma was separated and concentrations of alkaline phosphatase (U/L), alanine amino-transferase (U/L), aspartate amino-transferase (U/L), total bilirubin (μmol/L), urea (mmol/L), creatinine (μmol/L), glucose (mmol/L), total cholesterol (mmol/L), triglycerides (mmol/L), sodium (mmol/L), potassium (mmol/L), chloride (mmol/L), calcium (mmol/L), inorganic phosphorus (mmol/L) and total protein (g/L) were determined, using a Hitachi 917 Clinical Chemistry Analyser (Roche, Sussex, UK).

Electrophoretic protein fractions; albumin (g/L), $\alpha1$ globulin (g/L), $\alpha2$ globulin (g/L), β-globulin (g/L) and γ-globulin (g/L) were analyzed with agarose gel and scanning with a densitometer. Albumin/globulin ratio (A/G ratio) was

calculated from total protein concentration and analyzed albumin concentration.

Immunogenicity: Before dosing on days 0 and 7 and on days of euthanasia (days 10, 21, and 35), blood samples (1.5 ml) were taken from the central auricular artery. Serum was isolated and frozen (−20°C). Samples were assayed for immunoglobulin G (IgG) serum antibodies to vaccinia virus using an enzyme-linked immunosorbent assay (ELISA) by Bavarian Nordic (the ELISA was not performed under GLP conditions).

Necropsy: Animals were killed humanely on days 10, 21 and 35 by an intravenous overdose (2.5 ml/animal) of pentobarbitone (Pharmasol, Hampshire, UK; Catalogue No: 80640). All external features and orifices were examined visually, including the parenteral site. The cranial roof was removed to allow observation of the brain, pituitary gland and cranial nerves. After ventral mid-line incision, the neck and associated tissues and the thoracic, abdominal and pelvic cavities and their viscera were exposed and examined *in-situ*.

Organ weights: Adrenals, brain, heart, kidneys, liver, lungs, pituitary gland, salivary glands, spleen, thyroid with parathyroids and thymus were weighed in all animals. Additionally from males, epididymides, prostate, seminal vesicles and testes were weighed and from females, uterus with cervis and ovaries; bilateral organs were weighed together.

Histopathological examination: Testes and epididymides were fixed in Bouin's solution prior to transfer to 70% methylated spirit and eyes were fixed in Davidson's fluid prior to transfer to 70% methylated spirit. Other tissues comprising adrenals, aorta-thoracic, brain, caecum, colon, duodenum, femur, gall bladder, Harderian glands, heart, ileum, jejunum, kidneys, lachrymal glands, larynx, liver, lungs, lymph nodes (mandibular, axillary, inguinal, mesenteric, draining and distal nodes), mammary area (caudal), esophagus, optic nerves, ovaries, pancreas, pituitary, prostate, rectum, salivary glands (submandibular, parotid, sublingual), sciatic nerves, seminal vesicles, skeletal muscle, skin (treated site), skin (untreated site), spinal cord, spleen, sternum, stomach, thymus, thyroid with parathyroids, tongue, trachea, ureters, urinary bladder, uterus and cervix and vagina were fixed in 10% neutral buffered formalin. All tissues were processed to paraffin wax, sections were cut at 5 μm, and then stained with haematoxylin and eosin.

Statistical analysis: Startox version 3.2 was used for the statistical analysis of the haematological and blood chemistry data. Quasar version 1.1 was used to analyze the body weight, organ weight and pathological data. All analyses were carried out using the individual animal as the basic experimental unit. The following data types were analyzed separately at each timepoint: bodyweight, using gains over appropriate study periods; blood chemistry and haematology; organ weights, both absolute and adjusted for terminal bodyweight.

The following sequence of statistical tests was used if 75% of the data (across all groups) were the same value, for example c, then a frequency analysis was applied. Groups were compared using pairwise Fisher's Exact tests (FE) both for i) values $< c$ versus values $> c$, and for ii) values $\leq c$ vs. values $> c$, as applicable.

If Bartlett's test for variance homogeneity was not significant at the 1% level, then parametric analysis was applied. Groups were compared using *t*-tests (Tt). If Bartlett's test was significant at the 1% level, then logarithmic and square-root transformations were tried. If Bartlett's test was still significant, then non-parametric tests were applied. Groups were compared using the Wilcoxon rank sum tests (Wc).

For organ weight data, analysis of covariance was initially performed using terminal bodyweight as covariate. If the within group relationship between organ weight and bodyweight was significant at the 10% level,[24] then the treatment comparisons were made on adjusted group means (calculated using analysis of covariance, where the factor is group and the covariate is the terminal bodyweight) in order to allow for differences in bodyweight which might influence the organ weights.

Abbreviations

GLP	Good Laboratory practice
ELISA	Enzyme-linked-immunoassay
EDTA	Ethylenediaminetetraacetic acid
Kg	kilogram
hr	hour
g	gram
UK	United Kingdom
Pg	picograms
%	percentage
sec	second
cells/L	cells per liter
No	number
n	number
ml	milliliter
Wc	Wilcoxon
Tt	t-tests
FE	fisher's exact
PHE	Public Health England
L	liter
A	α
mmol	millimolar
A/G	albumin/globulin
BN	Bavarian Nordic
IgG	Immunoglobulin
ml	milliliter
PT	Prothrombin Time
RBC	Red Blood Cells
NIAID	National Institute of Allergy and Infectious Diseases
ACL	Automated Coagulation Analyzer
Sec	second
U/L	units per liter
APPT	Activated Partial Thromboplastin Time
β	beta
γ	gamma
MVA	modified vaccinia Ankara
$TCID_{50}$	50% Tissue Culture Infective Dose
°C	degrees Celsius
d	day

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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The effect of a non-denaturing detergent and a guanidinium-based inactivation agent on the viability of Ebola virus in mock clinical serum samples

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ABSTRACT

The 2014 Ebola outbreak in West Africa required the rapid testing of clinical material for the presence of potentially high titre Ebola virus (EBOV). Safe, fast and effective methods for the inactivation of such clinical samples are required so that rapid diagnostic tests including downstream analysis by RT-qPCR or nucleotide sequencing can be carried out. One of the most commonly used guanidinium – based denaturing agents, AVL (Qiagen) has been shown to fully inactivate EBOV once ethanol is added, however this is not compatible with the use of automated nucleic acid extraction systems. Additional inactivation agents need to be identified that can be used in automated systems. A candidate inactivation agent is Triton X-100, a non-denaturing detergent that is frequently used in clinical nucleic acid extraction procedures and has previously been used for inactivation of EBOV. In this study the effect of 0.1% and 1.0% Triton X-100 (final concentration 0.08% and 0.8% respectively) alone and in combination with AVL on the viability of EBOV (10^6 $TCID_{50}$/ml) spiked into commercially available pooled negative human serum was tested. The presence of viable EBOV in the treated samples was assessed by carrying out three serial passages of the samples in Vero E6 cells (37 °C, 5% CO_2, 1 week for each passage). At the end of each passage the cells were observed for evidence of cytopathic effect and samples were taken for rRT-PCR analysis for the presence of EBOV RNA. Before cell culture cytotoxic components of AVL and Triton X-100 were removed from the samples using size exclusion spin column technology or a hydrophobic adsorbent resin.

The results of this study showed that EBOV spiked into human serum was not fully inactivated when treated with either 0.1% (v/v) Triton X-100 for 10 mins or 1.0% (v/v) Triton X-100 for 20 mins (final concentrations 0.08% and 0.8% Triton X-100 respectively). AVL alone also did not consistently provide complete inactivation. Samples treated with both AVL and 0.1% Triton X-100 for 10 or 20 mins were shown to be completely inactivated. This treatment is compatible with downstream analysis by RT-qPCR and next generation sequencing.

1. Introduction

The Ebola outbreak in West Africa required the testing of unprecedentedly high numbers of high titre clinical samples for the presence of Zaire ebolavirus (EBOV, family Filoviridae) (Carroll et al., 2015; Shiwani et al., 2017). After inactivation of the clinical samples within high containment facilities (Containment Level4/CL3 or a flexible film isolator in field laboratories) downstream sample processing was performed at a lower level of containment (CL2) (Logue et al., 2017; Bailey et al., 2016). For processing the samples at CL2 there is a requirement to demonstrate that procedures used to inactivate the virus potentially present within the clinical sample are effective. In addition, inactivated material from high consequence pathogens such as EBOV is a valuable resource for the development of new diagnostic assays or therapeutic reagents. Recent failures in inactivation have caused significant concern, particularly where ACDP 3 and 4 pathogens are involved ('CDC Report on the Potential Exposure to Anthrax' 2014; Weiss et al., 2015). For these reasons proof of effective inactivation of virus is an increasingly important component of study design.

The effectiveness of inactivation is dependent on factors including the nature and concentration of the pathogen, the sample matrix and the concentration and contact time of the inactivation agent with the sample. For PCR-based techniques, guanidine-based denaturing agents are commonly used, although each buffer uses different concentrations of active ingredients and ratios of inactivation agent to sample. The precise formulations of inactivation buffers are generally proprietary

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and the efficiency of a particular buffer against a specific pathogen cannot be predicted with complete confidence.

During the Ebola outbreak in West Africa many laboratories employed manual nucleic acid extractions using the QIAamp viral nucleic acid extraction kit (Qiagen, 52904) which uses guanidinium-based AVL buffer as the inactivation agent (Reusken et al., 2015). Data have shown that buffer AVL will inactivate high titre EBOV in cell culture medium (Blow et al., 2004). When EBOV is present in clinical samples, however, complete inactivation is not guaranteed (Smither et al., 2015; Haddock et al., 2016) and ethanol also needs to be added before full inactivation is achieved. For manual extraction methods ethanol can be added to the AVL treated samples before removal from primary containment. For high sample throughput using the AVL compatible automated nucleic acid extraction system (Qiagen EZ1) ethanol cannot be added before the samples are removed from containment and loaded onto the machine. In PHE laboratories in Sierra Leone samples were removed from containment and inactivation was completed using heat (60 °C for 15 min) (Bhagat et al., 2000; Bailey et al., 2016). The potential problem with using heat inactivation in such a high throughput situation is that constant temperature monitoring of the samples is required to ensure that the temperature is held and full inactivation is achieved. The addition of a defined volume of a chemical inactivation agent such as Triton X-100 allows consistent treatment of the samples.

Triton X-100, a non-denaturing detergent that solubilises lipid membranes, is a commonly used inactivation agent that has been shown to reduce infectivity of samples without affecting blood chemistry results (Tempestilli et al., 2015; Lau et al., 2015). Triton X-100 was recommended by CDC as an inactivation agent which reduces the titre of haemorrhagic fever viruses. The Guidelines, however, state that 100% inactivation should not be assumed. https://www.cdc.gov/hai/pdfs/bbp/vhfinterimguidance05_19_05.pdf. Triton-X 100 is also included in many commercial nucleic acid extraction buffers at various concentrations such as the Roche Magnapure 96 external lysis buffer (Rosenstierne et al., 2016). The addition of 1.0% (v/v, final concentration) Triton X-100 to mock clinical serum samples prior to RNA extraction has been shown to have no effect on downstream processing for RT-qPCR and PCR amplification of longer DNA fragments (Lewandowski et al., 2016). The generation of larger amplicons (up to 2874 bp) shows that samples treated with 1.0% (v/v) Triton X-100 produces high quality nucleic acid material that is suitable for next generation whole genome sequencing applications, an approach that is increasingly used in viral diagnostics (Kwong et al., 2015). The addition of Triton X-100 to the inactivation buffer before downstream processing of a sample on an automated extraction platform could provide an additional inactivation stage. The use of two different inactivation agents is recommended by the World Health Organisation (WHO); Ideally two different methods of inactivation should be used (e.g. one physical method and one chemical method) although two chemical methods may be just as effective www.who.int/bloodproducts/publications/WHO_TRS_924_A4.pdf. It has recently been shown that 0.1% Triton X-100 effectively reduced EBOV infectivity, although complete inactivation was not achieved (Colavita et al., 2017).

The aim of this study was to determine the effectiveness of Triton X-100 for inactivation of EBOV in serum samples, alone or in combination with guanidinium thiocyanate containing AVL buffer. The addition of Triton X-100 could be used as a well defined additional inactivation treatment before nucleic acid extraction.

2. Materials and Methods

2.1. Virus

Work with live virus was carried out in the CL4 laboratories at PHE Porton Down. The virus stock used was Ebola virus/H.sapiens-tc/COD/1976/Yambuku-Ecran, obtained from the first Ebola haemorrhagic fever outbreak in Zaire in 1976, passaged through guinea pigs. To generate mock positive patient serum the EBOV stock virus was diluted (1: 10) in negative human serum (Sigma H4522) to a concentration of 10^6 $TCID_{50}$/ml.

2.2. Effect of Triton X-100 alone and in combination with AVL on the viability of EBOV in serum

EBOV in serum (140 µl) was treated with 560 µl volumes of the following inactivation agents 1) 0.1% Triton X-100; 2) 1.0% Triton X-100; 3) AVL; 4) 0.1% Triton X–100 + AVL, each with a 10 min contact time and 5) 1.0% Triton X-100 with a 20 min contact time. As a positive control, EBOV in serum was added to 560 µl of tissue culture medium and passed through size exclusion columns or treated with SM2 adsorbent resin in parallel with inactivated samples. For comparison the same volume of EBOV in serum was added directly to cells. The concentration of Triton X-100 alone was increased to 1.0% with a contact time of 20 min to determine whether it could be used as an inactivation agent in the absence of AVL. Each treatment was set up in duplicate or triplicate.

2.2.1. Removal of AVL or Triton X-100 before cell culture

AVL and Triton X-100 are toxic to cells (Colavita et al., 2017; Kumar et al., 2015) and the addition of 100–200 µl volumes to Vero E6 cells in 25 cm^2 flasks with 5 ml medium was sufficient to cause cell death. Work up experiments carried out at CL2 showed that AVL could be removed from samples using size exclusion spin columns. Initially Vivaspin 2.0 columns (100 000 MW CO PES VS0201) that were able to hold 2 ml of liquid were used, however the protocol that worked successfully at CL2 did not translate well to use within the CL4 cabinet line, so after the first experiment AVL was removed using Amicon 50 000 MWO, UFC50500 Ultra-0.5 centrifugal filter devices (Kumar et al., 2015) in a microcentrifuge. These held a smaller volume of liquid (0.5 ml) but were easier to handle within the cabinet line. Triton X-100 was most effectively removed from samples using SM2 resin. This was used alone and in combination with the size exclusion spin columns as required.

2.2.2. Removal of AVL and 0.1% Triton X-100 using Sartorius Vivaspin 2 columns (100 000 MW CO PES VS0201)

AVL and 0.1% Triton X-100 were removed from the samples using Sartorius Vivaspin columns. The entire sample (700 µl) was added to the spin columns and centrifuged at 2000 x g for 20 min. The eluate (approximately 500 µl) was removed and phosphate buffered saline (PBS, Gibco 10010-015) added to the column to a final volume of 2 ml. The samples were centrifuged a further three times at 2000 x g for 15 min and after each spin the eluate removed and PBS added to the column to a final volume of 2 ml. The liquid from the column reservoir after the final spin was approximately 50 µl. PBS (200 µl) was added to the concentrated virus to allow the liquid to be efficiently removed from the column.

2.2.3. Removal of 1.0% Triton X-100 using SM2 resin

To remove 1.0% Triton X-100 the samples were treated with a hydrophobic resin SM2 beads (Bio Rad, 152–8920) using a modification of a batch method (Holloway 1973). SM2 resin (0.15 g) was added to each sample containing 1.0% Triton X-100. The tubes were mixed by inversion for 1 min followed by 5 min static incubation at room temperature. This was repeated 10 times (total of 10 mins of mixing and 1 h of static incubation). The resin was allowed to settle to the bottom of the tube and the sample carefully removed using a pipette.

2.2.4. Removal of AVL buffer using Amicon ultra-0.5 centrifugal filter devices (50 000 MWO, UFC505008)

To prepare the column 500 µl 70% ethanol was added to the column, centrifuged and the eluate removed. This was repeated using 500 µl sterile water. All centrifugation steps were 10 mins at 11 000 x g

in a microcentrifuge. Columns were used within 20 min of preparation. Sample (500 µl) was added to the column and centrifuged. The eluate was removed and the remainder of the sample (200 µl) and 300 µl PBS was added to the column and centrifuged. To remove residual traces of AVL the columns were washed twice with 500 µl PBS. Finally the sample was diluted in 500 µl Leibovitz's L-15 Medium (Gibco, 11415-064) and removed from the column.

2.2.5. Cell culture

Each treated sample was used to infect two 25 cm^2 flasks of 80% confluent Vero E6 cells. For treatment with 0.1% Triton X-100, AVL and AVL + 0.1% Triton X-100, 100 µl of sample was added to each flask, allowed to adsorb for 30 mins at 37 °C then 5 ml of L15 medium + 2% Fetal Calf Serum (Gibco, 26140-079) was added. For samples treated with 1.0% Triton X-100 for 10 min, 200 µl of sample was added directly to 5 ml fresh medium. When samples were treated with 1.0% Triton X-100 with a 20 min incubation period, 350 µl of sample was added to each flask so that the whole sample could be tested. Positive controls (untreated EBOV in serum subjected to the same AVL/Triton X-100 removal treatment as test samples and EBOV in serum inoculated directly onto cells) and negative controls (culture medium) were also performed. Baseline samples were taken from the original flasks after adsorption and addition of medium. These samples were used for nucleic acid extraction and rRT-PCR (see Section 2.3).

2.2.6. Serial passage of inactivated EBOV infected serum samples

Samples were passaged three times (37 °C, 5% CO_2 for 1 week). At each passage 100 µl of cell supernatant was added to a fresh 25 cm^2 flask containing 80% confluent Vero E6 cells, allowed to adsorb for 30 min then 5 ml L15 + 2% FCS medium was added. At the time of each passage the cells were observed for signs of EBOV cytopathic effect (cpe) by viewing under a low magnification microscope and the results recorded. Samples were considered to show cpe when cells were rounded and detaching causing disruption to the cell sheet. Cells in the control flask remained attached in a uniform layer. At the beginning and end of each passage a sample of the cell supernatant (140 µl) was taken into AVL (560 µl) for nucleic acid extraction and rRT-PCR analysis as described in Section 2.3. For the samples purified with the Amicon spin columns due to servicing requirements of the laboratory it was necessary to freeze the cell culture supernatants between the second and third passages.

For samples where no cpe was observed at the end of the first passage, cells were transferred into a fresh flask to increase the probability of detecting any live virus. Cells were detached from the original flask by incubation with trypsin-EDTA solution (Gibco 25200-056) for 10 min. One third of the cells were replaced into a new 25 cm^2 flask containing 5 ml fresh L15 medium. After one week 140 µl of cell supernatant was added to 560 µl AVL and processed as described in Section 2.3.

2.3. Real-Time PCR

Nucleic acids were extracted using QIAamp viral RNA extraction kit (Qiagen 52904) according to the manufacturer's instructions. Ethanol was added to the samples and tube transfers performed at CL3. The remainder of the nucleic acid extraction was carried out at CL2 and nucleic acids eluted into 60 µl AVE buffer. The presence of EBOV RNA was determined using a TaqMan rRT-PCR assay (Trombley et al., 2010) using the ABI7500 Fast PCR platform. The master mix comprised EBOV F and EBOV R primers and EBOV TM-P (650 nM, 650 nM and 250 nM final concentration respectively), 4 x TaqMan® Fast Virus 1-Step Master Mix (ABI, Cat No. 4444432) made up with molecular-grade nuclease-free water (Ambion, Cat No. AM9916) to a final volume of 15 µl. The amount of template material added was 5 µl. Cycling conditions were 50 °C for 5 min, followed by 95 °C for 20 s then 40 cycles of 95 °C for 3 s and 60 °C for 30 s. For each run positive (EBOV RNA with a known Ct value) and no template control (NTC) samples were included. All PCRs were performed at least in duplicate. All samples with a detectable Ct were considered to contain EBOV RNA. Samples were considered to contain viable EBOV RNA when a reduction in Ct value was observed from subsequent passage samples.

2.4. Data analysis

The aim of this work was to determine whether complete inactivation of EBOV was attained rather than a reduction in titre. Inactivation of the EBOV infected serum samples was considered to be complete if no cpe was observed during any of the passages and no decrease in Ct value occurred after three passages by rRT-PCR. After each treatment and removal of the AVL/Triton X-100 the sample was divided between two flasks of cells. Where samples showed the same trend at the end of the three passages the Ct values from duplicate RT-PCR from each flask of cells were combined into a single bar. Where the same treatment gave a different result at the end of the three passages results were grouped together according to outcome. Bars represent the mean Ct value of all the rRT-PCRs. Error bars represent 2 x the standard deviation of the mean Ct value of all rRT-PCRs. The rRT-PCR assays (Trombley et al., 2010) were performed at least in duplicate on each nucleic acid sample. Where rRT-PCR results gave the Ct value as undetermined a representative Ct value of 40 was assigned.

3. Results

3.1. Control virus growth

For each method used to remove AVL and Triton X-100 from samples an EBOV in medium only control was run at the same time as the treated samples. For all three AVL/Triton X-100 removal methods the growth profile of the recovered EBOV over the three passages was comparable to that of EBOV in serum added directly to cells. The mean Ct values of samples taken after each removal method at each sample point are shown (Fig. 1a blue bars). For comparison the mean Ct value of control virus added directly to cells set up in parallel for each experiment are shown (Fig. 1a red bars). The Ct values from the virus controls subject to treatment and the virus controls added directly to cells were comparable for P1 D0 (Ct 23.8 compared to 23.3) and P1 D7 samples (Ct 12.9 compared to 13.1). Passage of the controls added directly to cells was not continued after the first passage. During the second passage the mean Ct decreased over 7 days (an initial increase in Ct was observed when the sample was diluted in fresh culture medium in a new 25 cm^2 flask). In the third passage the mean Ct value also decreased. Cytopathic effect was observed in the cells at the end of the first passage. A summary of the initial and final Ct values and the observation of the presence of cpe at the end of each passage is shown in Table 1 (lines 27–29).

3.2. Treatment with Triton X-100

Samples treated with 0.1% Triton X-100 for 10 min showed no reduction in Ct value during the first passage (Fig. 1b). During the second passage the mean Ct value was reduced and in the third passage the mean Ct dropped to 12.7. In samples treated with 1.0% Triton X-100 for 10 min two of six samples showed a reduction in Ct value by the second passage and the remaining four samples showed a reduction in Ct value by the third passage (Fig. 1c). For this reason the error bars (which represent 2 x standard deviations of the mean Ct value) on this graph are wide. In samples treated with 1.0% Triton X-100 for 20 min two of six samples showed a reduction in Ct after the second passage, whereas for the other four samples no reduction was observed (Fig. 1d). The rRT– PCR results from the split flasks were in agreement with the rRT-PCR results obtained at the end of the three serial passages (Table 1 column 8, lines 9–14).

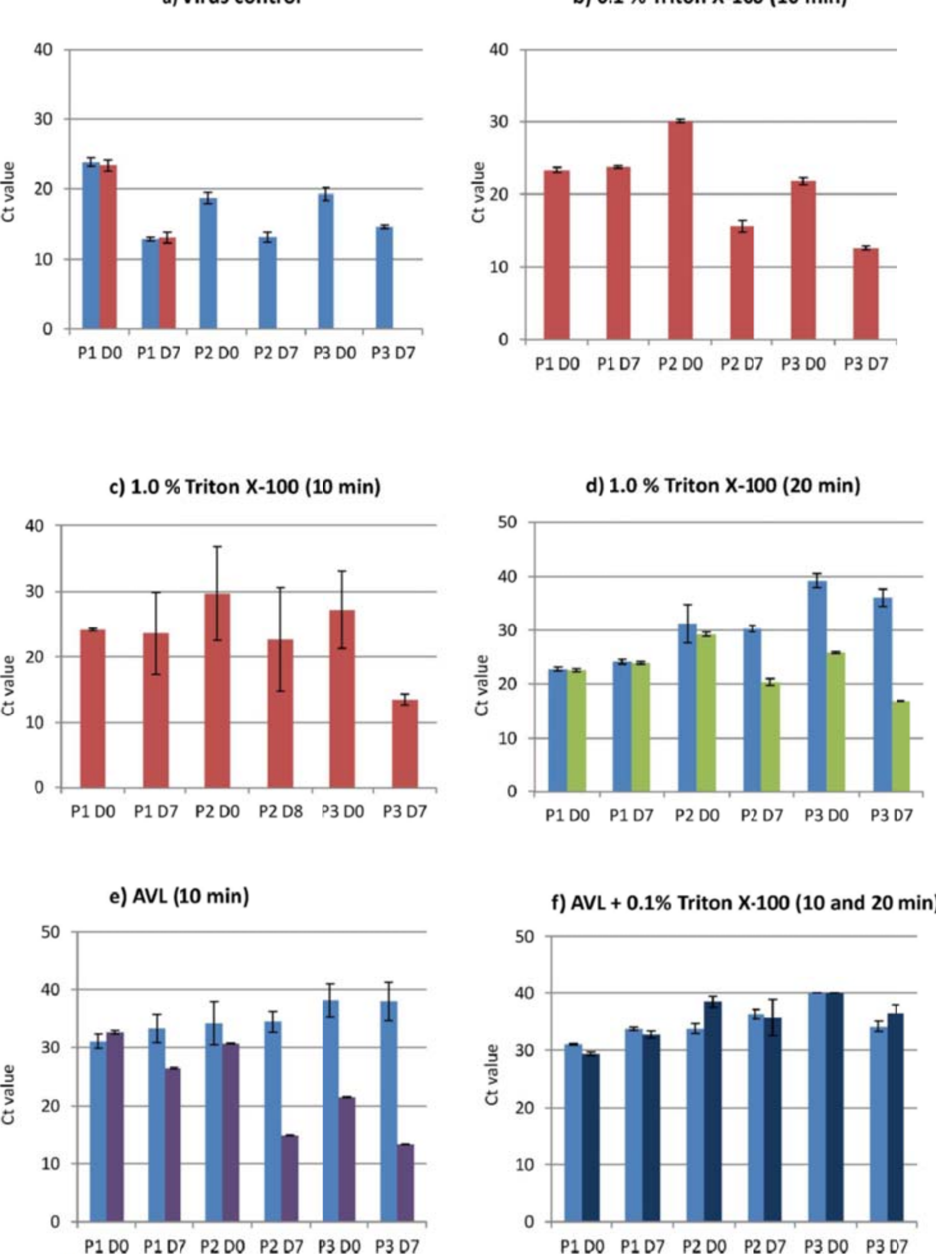

Fig. 1. 1a) Control: The blue bars show the mean Ct value determined from three separate experiments each consisting of three serial passages of untreated EBOV in serum diluted in cell culture medium a) passed through 2.0 ml size exclusion spin column (Sartorius) b) treated with SM2 adsorbent resin c) passed through 0.5 ml size exclusion spin columns (Amicon). The red bar shows mean Ct values from control flasks (the same amount of EBOV in serum added directly to the cells instead of being treated the same way as the test samples). When EBOV was added directly to cells culture was not continued after the first passage. 1b) Mean Ct values of duplicate samples treated with 0.1% Triton X-100 for 10 min 1c) Mean Ct values of six samples treated with 1.0% Triton X-100 for 10 min. All samples showed a reduction in Ct value over the course of the serial passage. Three of the six samples showed a reduction in Ct after the first passage, and three samples showed a reduction in Ct after the second passage, leading to the increase in the standard deviation of the samples. 1d) Mean Ct values of six samples treated with 1.0% Triton X-100 for 20 mins. For four of the samples no reduction of Ct was observed (blue bars) and two samples showed a reduction in Ct after the first passage (green bars). 1e) Bars show Ct values of six samples treated with AVL for 10 mins. Five of the samples showed no growth after 3 serial passages (blue bars) and one sample showed a reduction in Ct indicating virus growth purple (bars). 1f) Blue bars show Ct values of two samples treated with AVL + 0.1% Triton X-100 for 10 min and dark blue bars show Ct values of four samples treated with AVL + 0.1% Triton X-100 for 20 min (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
The effect of AVL and Triton X-100 (0.1% and 1.0%) individually and in combination on the viability of EBOV in serum.

	Treatment: Percentage of Triton X-100 /± AVL	Inactivation time (min)	Passage number when cpe first observed	Passage number Ct value decreased	Initial Ct value (P1 D0)	Final Ct value	Ct value after sample split (P1)	Sample inactivated
1	0.1%	10	2	2	23.0	12.9	ND	No
2	0.1%	10	2	2	23.6	12.9	ND	No
3	1.0%	10	3	1	24.2	12.0	ND	No
4	1.0%	10	3	1	23.4	12.5	ND	No
5	1.0%	10	3	3	24.4	13.7	ND	No
6	1.0%	10	3	3	23.9	14.2	ND	No
7	1.0%	10	3	1	24.0	12.9	ND	No
8	1.0%	10	3	3	24.2	14.2	ND	No
9	1.0%	20	No cpe	No decrease	ND	35.4	26.4	Yes
10	1.0%	20	No cpe	No decrease	22.7	38.5	26.8	Yes
11	1.0%	20	2	2	22.4	ND	12.4	No
12	1.0%	20	2	2	22.6	16.7	13.5	No
13	1.0%	20	No cpe	No decrease	22.7	Undetermined	26.3	Yes
14	1.0%	20	No cpe	No decrease	22.7	35.0	26.5	Yes
15	0.1% + AVL	10	No cpe	No decrease	31.1	Undetermined	35.5	Yes
16	0.1% + AVL	10	No cpe	No decrease	30.8	Undetermined	35.8	Yes
17	0.1% + AVL	20	No cpe	No decrease	29.6	36.0	34.8	Yes
18	0.1% + AVL	20	No cpe	No decrease	29.1	35.1	34.2	Yes
19	0.1% + AVL	20	No cpe	No decrease	29.6	ND	ND	Yes
20	0.1% + AVL	20	No cpe	No decrease	29.0	38.5	32.5	Yes
21	AVL	10	No cpe	No decrease	32.1	Undetermined	Undetermined	Yes
22	AVL	10	No cpe	1	32.6	14.9	11.5	No
23	AVL	10	No cpe	No decrease	30.5	Undetermined	36.4	Yes
24	AVL	10	No cpe	No decrease	29.4	36.2	33.7	Yes
25	AVL	10	No cpe	No decrease	29.3	ND	33.2	Yes
26	AVL	10	No cpe	No decrease	29.7	38.5	33.2	Yes
27	Control 1	10	1	1	23.1	13.3	ND	No
28	Control 2	10	1	1	23.7	14.9	ND	No
29	Control 3	10	1	1	23.6	12.6	ND	No

ND – Sample not tested
Ratio of clinical sample to inactivation agent (1 + 4). Inactivation agent contact times of 10 and 20 min were tested, followed by three serial passages in E6 Vero cells. Different methods of removing cytotoxic AVL/Triton X-100: ☐ Sartorius Spin column (2 ml); ☐ SM2 Adsorbent resin; ☐ Amicon Spin column (0.5 ml) and SM2 Adsorbent resin. For samples purified using Amicon Spin columns the tissue culture supernatant was frozen between the second and third passages.

3.3. Treatment with AVL

Of the six flasks inoculated with AVL-treated EBOV (Fig. 1e) one showed a reduction in Ct during the first passage and five showed no reduction in Ct. The rRT– PCR results from the split flasks were again in agreement with the rRT- PCR results obtained at the end of the three serial passages (Table 1 column 8 lines 21–26).

3.4. Treatment with AVL and 0.1% Triton X-100

Full inactivation was observed in all six samples when both 0.1% Triton X-100 and AVL were added to the EBOV infected serum sample and incubated for 10 min (for two samples) or 20 min (for four samples). No decrease in Ct value was observed over the course of the three passages. (Fig. 1f). In samples where no amplification of EBOV RNA was observed low levels of RNA remained leading to a high Ct value (Ct > 30) being determined. This was considered to be amplification of low levels of non-viable EBOV RNA carried over from the initial inoculum.

4. Discussion

The significant finding of the work reported in this study is that AVL and 0.1% Triton X-100 in combination showed consistent inactivation of EBOV in serum. This is consistent with the observation that Roche Magna Pure lysis buffer which contains much higher concentrations of Triton X-100 (20–25%) and Guanidine thiocyanate (25–50%; MSDS data) fully inactivates EBOV in whole blood, allowing automated extraction of RNA using the Magna Pure automated platform (Rosenstierne et al., 2016). The results of this work are also consistent with the findings of Smither et al. (2015) in that AVL alone did not completely inactivate EBOV in all experiments, although the delayed growth kinetics suggest that it caused a significant reduction in titre. The sample matrix used in this study was negative human serum spiked with EBOV, whereas Smither et al. (2015) used serum from an infected marmoset and spiked naïve murine blood. As reported by Lewandowski et al. (2016) samples treated with a low percentage of Triton X-100 are suitable for downstream RT-qPCR and next generation sequencing applications.

When EBOV infected serum samples were added to 1.0% Triton X-100 and incubated for 20 min (final concentration 0.8%) EBOV was not fully inactivated. The contact time was not increased to greater than 20 min as this would make the method less attractive for use in diagnostic labs where a fast sample processing time is required. These results are consistent with those of Colavita et al. (2017) who demonstrated that 0.1% Triton X-100 strongly reduced the EBOV infectivity, although complete inactivation was not observed.

The cytotoxic effect of AVL and Triton X-100 on cells makes it difficult to test inactivated samples for residual viable EBOV. For successful passage all traces of Triton X-100 and AVL needed to be removed from the samples before cell culture. Two methods were used to remove the Triton X-100 and AVL from the treated sample; size exclusion spin columns and a hydrophobic absorbent resin. When the inactivated and purified samples were left to adsorb into the cells some localised cytotoxic effects were observed on the cell sheet. To reduce this effect treated samples were subsequently added directly to the fresh medium in the flask. Similar cytotoxicity effects have been encountered when using $TCID_{50}$ end point dilution tests (Colavita et al., 2017).

In samples treated with 0.1% Triton X-100 for 10 min a reduction in virus growth occurred, although some viable virus was still present. In samples treated with 1.0% Triton X-100, despite the ten-fold increase in concentration of Triton X-100 viable virus remained present. When baseline samples were taken from cells to which AVL treated samples were added the Ct value was consistently higher than baseline samples from cells to which samples treated with Triton X-100 alone was added (30.2 ± 1.09 compared to 23.3 ± 0.65, $p < 0.001$, student's *t*-test), see Table 1 Column 6, lines 21 − 26 and 1 − 14. This could be due to the greater inactivating effect of AVL.

In summary, AVL and 0.1% Triton X-100 in combination with a 10 min contact time (1:4 sample to AVL + 0.1% Triton X-100) could be used as a pre-treatment for clinical serum samples potentially infected with EBOV prior to removal from high containment for lower containment procedures. This would allow subsequent nucleic acid extraction on an automated extraction platform at Containment Level 2. This can be done without significantly increasing the time taken to inactivate the sample and has no effect on downstream processing of the samples by RT-qPCR or next generation whole genome sequencing.

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Additional supporting information

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**University of the West of England
Bristol**

Allen Douglas Glen Roberts

has been awarded the degree of

MASTER OF PHILOSOPHY

having followed an approved programme of research study in

THE DEVELOPMENT OF A SYSTEM FOR THE DETECTION OF ESCHERICHIA COLI SEROTYPE 0157 BASED UPON THE SPECIFIC ADSORPTION OF BACTERIOPHAGE

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Department:	Faculty of Science	**First Supervisor:**	Professor Graham Mills
Start Date: (or progression date for Prof Doc students)	01 October 2017		

Study Mode and Route:	Part-time	☒	MPhil	☐	MD	☐
	Full-time	☐	PhD	☒	Professional Doctorate	☐

Title of Thesis:	Approaches to Handling High Consequence Pathogens
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