Exposure of rhesus monkeys to cowpox virus Brighton Red by large-particle aerosol droplets results in an upper respiratory tract disease

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We previously demonstrated that small-particle (0.5–3.0 µm) aerosol infection of rhesus monkeys (Macaca mulatta) with cowpox virus (CPXV)-Brighton Red (BR) results in fulminant respiratory tract disease characterized by severe lung parenchymal pathology but only limited systemic virus dissemination and limited classic epidermal pox-like lesion development (Johnson et al., 2015). Based on these results, and to further develop CPXV as an improved model of human smallpox, we evaluated a novel large-particle aerosol (7.0–9.0 µm) exposure of rhesus monkeys to CPXV-BR and monitored for respiratory tract disease by serial computed tomography (CT). As expected, the upper respiratory tract and large airways were the major sites of virus-induced pathology following large-particle aerosol exposure. Large-particle aerosol CPXV exposure of rhesus macaques resulted in severe upper airway and large airway pathology with limited systemic dissemination.

INTRODUCTION

Variola virus (VARV) and its close relative monkeypox virus (MPXV) are considered potential bioterrorism weapons that require biosafety level 4 (VARV) and biosafety level 3 (MPXV) laboratories for study. Direct experimentation with VARV requires World Health Organization approval and can only be conducted in one laboratory in the USA and one laboratory in Russia. These restrictions limit widespread study of their pathogenesis and medical countermeasure development. The future of VARV research is also in question, as debates concerning the destruction of the known VARV stocks continue (Damon et al., 2014; Jahrling & Tomori, 2014; Tucker, 2011). Hence, there is a need to develop safe, accessible, alternative models of human orthopoxvirus infection that meet the United States’ Food and Drug Administration (FDA) ‘Animal Rule’ requirements (Aebersold, 2012). Cowpox virus (CPXV) may provide such an alternative model (Parker et al., 2007a, b; Vorou et al., 2008). CPXV does not require Select Agent permits, as it is a biosafety level (BSL)-2 pathogen, and mouse models are available for further experimentation.
Large-particle aerosol exposure of rhesus monkeys to CPXV

RESULTS

Experimental design

Four groups of four rhesus monkeys (n=16) were exposed to serial tenfold reductions from 5×10^7 p.f.u. to 5×10^4 p.f.u. of CPXV-BR by large-particle aerosol exposure. Large-particle aerosols were generated as described in Bohannon et al. (2015). Aerosol parameters by subject and outcome are shown in Table S1 (available in the online Supplementary Material). Pre- and post-exposure physical exams, computed tomography (CT), and sample collection was performed on study days –10, 3, 6, 9, 12, 15, 18 and 21, and on the day of necropsy. Subjects were considered survivors past day 21. Necropsies were performed between days 22 and 28.

Large-particle CPXV exposure results in a lethal respiratory disease

Three of four subjects receiving 5×10^7 p.f.u. met study endpoint criteria between days 11 and 12 post-exposure. Fifty percent of the monkeys receiving 5×10^6 p.f.u. met study endpoint criteria on day 12 post-exposure while the remaining survived to study end (day 21 post-exposure) (Fig. 1a). Subjects receiving 5×10^5 and 5×10^4 p.f.u. survived to study end but did experience disease. The subjects that succumbed demonstrated laboured breathing and slightly decreased peripheral oxygen saturation (Table S2). Respiratory rate peaks coincided with endpoint criteria for two of the five subjects. The lowest observed peripheral oxygenation coincided with endpoint criteria for one of those five monkeys. Laboured breathing, increased respiratory rate and decreased peripheral oxygen saturation are consistent with the histopathologic findings of severe respiratory tract disease.

The hallmark epithelial smallpox lesions were not a common observation in large-particle aerosol CPXV exposed monkeys (Table S2) and were only observed in the lowest doses (5×10^3 and 5×10^4) groups. The subjects in the 5×10^3 p.f.u. group developed few lesions (2–31 at peak of lesions), distributed on the head and limbs, that were first observed at a mean of 8.25 days post-exposure (range: 6–12 days). Other clinical signs included: serous nasal discharge, tussis, inappetence and dyspnea. Clinically, white blood cell (WBC) count increases and albumin decreases were observed and were consistent with disease progression (Fig. 1b, c). PBMCs were analysed by TruCount to evaluate concentrations of circulating leucocytes and were consistent with the total WBC. The 5×10^4 p.f.u. group demonstrated a lag in expansion of monocytes, NK cells and B-cells when compared to the other groups (Fig. 1d).

In keeping with our previous CPXV experiments, pro-inflammatory cytokines/chemokines were evaluated for changes associated with disease progression and are summarized in Table S3. Vascular endothelial growth factor (VEGF) and sCD40L demonstrated less than twofold

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changes from baseline for any subject in any group and were excluded from further analysis. Granulocyte-colony stimulating factor (G-CSF) changes were greatest in the $5 \times 10^7$ p.f.u. group with little change observed in the other groups. IFN-γ demonstrated greater than twofold elevations for each group with the peaks occurring between days 6 and 9 for the $5 \times 10^6$ to $5 \times 10^7$ groups. The $5 \times 10^4$ p.f.u. group had a peak of IFN-γ concentration which was consistent with changes in the PBMC population for this group. IL-6 increases were associated with early time points and did not parse with survival within the $5 \times 10^6$ p.f.u. group. IL-12 demonstrated peak mean fold concentration changes that were reciprocal to virus dose.

**Gross pathology and histopathology indicate severe upper respiratory tract pathology**

Full necropsies were performed on all subjects. Two subjects (nos 13 and 16) from the $5 \times 10^7$ p.f.u. group that met endpoint criteria first (day 11 post-exposure) exhibited

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**Fig. 1.** (a) Kaplan–Meier analysis of the large-particle aerosol exposure of rhesus monkeys. Three out of four (75%) NHPs receiving $5 \times 10^7$ p.f.u. of CPXV succumbed by day 12 post-exposure, and 50% of NHPs receiving $5 \times 10^6$ p.f.u. of CPXV succumbed by day 12 post-exposure. Groups receiving $5 \times 10^5$ and $5 \times 10^4$ p.f.u. survived to study end. (b) WBC concentrations during the course of the experiment. Dashed lines indicate normal value ranges. Subjects did not consistently demonstrate leucocytosis. (c) Serum chemistry value of albumin indicates a decrease across all groups, suggesting a response to exposure. (d) The major PBMC populations increased in response to infection. Various PBMC cell populations were assayed periodically by TruCount analysis as described in the Methods.
subacute, severe, necroulcerative pharyngitis and tracheitis (Fig. 2a) and fibrinosuppurative bronchopneumonia (Fig. 2b). This pneumonia was consolidating in the right middle, caudal part of the left cranial and accessory lung lobes. The remaining subjects that succumbed to CPXV, two from the 5\times10^6 p.f.u. group (subjects 9 and 11) and one from the 5\times10^7 p.f.u. dose group (subject 15), exhibited severe, diffuse, necrosuppurative and ulcerative pharyngitis, tracheitis (Fig. 2a), and bronchitis. However, the pulmonary parenchyma from these three subjects exhibited few signs of severe disease. In the two 5\times10^7 p.f.u. dose survivors (subjects 10 and 12), a mild, chronic, multifocal, organizing lymphoplasmacytic bronchopneumonia (Fig. 2c) was seen in alveoli but limited to the alveoli immediately adjacent to the tertiary bronchioles. Two non-survivors, one from the 5\times10^7 p.f.u. dose group (subject 15) and one from the 5\times10^6 p.f.u. dose group (subject 9), also exhibited a severe, necrosuppurative and ulcerative epiglottitis (Fig. 2d). No evidence of pneumonia was seen in the survivor from the 5\times10^7 p.f.u. dose group (subject 14). Histopathologically, the upper airways and bronchi in the three survivors from the higher dose groups exhibited mild chronic, lymphoplasmacytic infiltrates. Together, these data suggest that the upper respiratory tract and major airways were the focal point of disease with only limited dissemination into the alveoli.

**Immunohistochemistry and immunofluorescence indicate infection of macrophages and epithelial cells**

We evaluated the location of CPXV antigen (Fig. 3a–d) and the cell type infected (Fig. 3e, f) in both the lungs and trachea. As described above, sections of trachea exhibited severe inflammation and necrosis of the lining epithelium, and similar inflammation was present in the underlying submucosal stroma and within submucosal glands (Fig. 3a). Immunohistochemical evaluation of the similar regions showed abundant CPXV antigen staining in these aforementioned regions (Fig. 3b). Similarly, the lung exhibited regions of severe inflammation, fibrin and necrosis (Fig. 3c), which, immunohistochemically, were also consistent with areas of abundant virus antigen staining (Fig. 3d). Furthermore, in both the lung and trachea, virus-antigen-positive cells were morphologically suggestive of macrophage lineage and in order to confirm this, we evaluated the previously described sections with immunofluorescence to determine the identity of the infected cells (Fig. 3e, f). Using a pan-macrophage marker (IBA1) and a CPXV antibody, we found that in both the lungs and the trachea, positive for both IBA1 (macrophages) and CPXV antigen co-localized in the previously described regions of severe inflammation and virus antigen deposition (Fig. 3e–iv, f–vi). These data suggest that within the areas of severe inflammation and necrosis (trachea and lung), macrophages were the cells targeted for virus infection.

**CT indicates moderate lung pathology**

We evaluated real-time progression of lung disease by CT in all four groups of animals to determine if there was agreement to other parameters and our previous experiments (Fig. 4). CT scans were performed pre-exposure for baseline evaluation, and then every 3 days until study end (day 21 post-exposure) wherein a final necropsy day CT was performed (days 22–28 post-exposure). Day 3 scans performed on two animals from the high dose group had to be excluded due to poor positioning of the endotracheal tube (ET) and concomitant poor aeration of one lung and hyperinflation of the contralateral lung. There were no significant changes noted on day 3 scans in the other two animals from the same group.

Qualitatively, subjects from the 5\times10^7 and 5\times10^6 p.f.u. groups started demonstrating CT abnormalities by day 6 and day 9 post-exposure, respectively. For the 5\times10^5 and 5\times10^4 p.f.u. groups, minimal changes were first noticed on days 12 and 15, respectively. As expected, the severity of lung disease was generally proportional to the dose, with the 5\times10^7 p.f.u. and 5\times10^6 p.f.u. groups showing the most significant changes. Those changes were most severe on day 12 post-exposure in seven out of eight animals (one animal from the 5\times10^5 p.f.u. group met endpoint criteria prior to day 12) and included bronchial wall thickening, peribronchovascular infiltrates, patchy ground glass opacities as well as lobar and semilobar consolidations in two subjects. Interestingly, on day 12, only one out of three subjects from the 5\times10^5 p.f.u. group developed more severe respiratory disease than the four subjects in the 5\times10^6 p.f.u. group, suggesting a comparable disease burden between these two dose groups (Fig. 4). Changes in the lower dose groups (5\times10^4 and 5\times10^5 p.f.u.) were less marked and included peribronchial thickening/infiltrates, patchy ground glass opacities, with only one monkey (5\times10^5 p.f.u. group) showing consolidative changes in the lower lung fields (data not shown).

For the surviving subjects (one from the 5\times10^7 p.f.u. group, two from the 5\times10^6 p.f.u. group, and all monkeys from the lower dose groups), some lesions did not completely resolve by study end and were evident on CT and histopathologically.

CT abnormalities were quantified by percentage change in lung hyperdensity (PCLH) (Fig. 5). Two of four animals from the 5\times10^7 group had slight increases in PCLH observed at day 6 post-exposure and all four developed changes by day 9. The single survivor in this group demonstrated peak PCLH changes on day 12, which started decreasing on day 15 and almost completely resolved by day 27 (necropsy) (Fig. 2a). Subjects in the 5\times10^6 group demonstrated peak PCLH changes on days 11–12 that subsequently continued to resolve until study end (day 26 necropsy) (Fig. 4d). The 5\times10^5 group demonstrated peak changes in PCLH on day 12 post-exposure that quickly began to resolve and returned to near baseline values by day 21. Only one
subject from the $5 \times 10^4$ group demonstrated increased PCLH values that occurred on day 15 and resolved by necropsy on day 28.

Peripheral oxygen saturation correlated with increased PCLH for the $5 \times 10^7$ ($r=-0.8336, R^2=0.6949, P=0.0198$) and $5 \times 10^6$ ($r=0.7848, R^2=0.6159, P=0.0122$) p.f.u. groups, but no

Fig. 2. Histopathology of CPXV large-particle aerosol-exposed NHPs. (a) Histological evaluation of the upper respiratory tract. Cross-sections of the trachea are shown and demonstrate the severe inflammatory response of the non-survivors when compared to the survivors. Placement of the endotracheal (ET) tube displaced significant intraluminal exudates from subjects 13, 15, 9 and 11 (an ET tube was not placed in subject 16 prior to necropsy). Bars, 1 mm. (b) Severe, consolidating fibrinosuppurative and necrotizing bronchopneumonia for subject 13. Inset: Ulcerated bronchus (note supporting hyaline cartilage at left) with smooth muscle. The bronchial lumen is filled with a necrotic fibrinosuppurative exudate. Bars (including inset), 100 µm. (c) Right caudal lung lobe. The bronchus and surrounding pulmonary parenchyma exhibit mild, locally extensive, peribronchial and peribronchiolar organizing pneumonia. Inset: Focal bronchiolization and squamous metaplasia of affected alveoli. Bars (including inset), 100 µm. (d) Non-survivor epiglottis demonstrating severe necrosuppurative and ulcerative epiglottitis observed in subject 15. The junction of intact epithelium exhibited hydropic degeneration and necrotic epithelium with myriad, intracytoplasmic, intraepithelial inclusion bodies (black arrow). Inset: subgross cross-section of the epiglottis. Bars, 10 µm and 1 mm (inset). (e) Epiglottis from surviving subject (subject 10) which appears normal. Inset: subgross cross-section of the epiglottis. Bars, 100 µm and 1 mm (inset).
Fig. 3. (a) Haematoxylin and eosin (H&E)-stained sections of trachea show severe inflammation and necrosis involving the mucosal epithelial cells and the submucosal glands. (b) Immunohistochemistry for CPXV virus shows abundant virus antigen staining (brown) in both attached and free (necrotic) mucosal epithelial cells, and within the subjacent submucosal glands (inset). (c) H&E-stained sections of lung show severe inflammation and necrosis involving bronchioles and the surrounding alveolar interstitium. Alveolar spaces are filled with fibrin, inflammatory cells and karyorrhectic cellular debris (inset). (d) Immunohistochemistry for CPXV shows abundant virus antigen labelling (brown) within sloughed epithelial cells and within the cytoplasm of cells (macrophages) associated with damaged and necrotic alveolar walls. Magnification (a–d), main image ×4; inset ×40. Bars
consistent with the gross and histopathology data. Viraemia could be detected by day 9 in three of five groups. Viraemia could be detected from the lungs and from the trachea, however a wide variation in the peak viral load from the subject that succumbed to the lethal dose group subjects. We were unable to collect a sample to determine the peak viral load from the subject that succumbed to the lethal dose group, but was detected in the two surviving subjects. Due to wide variation in the peak viral load for the 5×10^7 (r=0.9580, R^2=0.9178, P<0.0001) and 5×10^5 (r=0.7121, R^2=0.5071, P=0.0209) groups.

Virology supports viremia and limited dissemination

Quantitative PCR (qPCR) was used to determine viral genomic load in peripheral blood and swabs (nasal and oral, Table S4). Using a Tukey’s multiple comparisons test from Graphpad Prism 6.0, comparison of peak viral load in the nasal swabs indicated statistically significant differences (P<0.05) were observed between the 5×10^6 and 5×10^5 groups and the 5×10^6 and 5×10^4 groups. Due to wide variation in viral genomic DNA could not be detected in peripheral blood from subjects in the 5×10^6 and 5×10^4 groups. Viraemia could be detected by day 9 in three of five non-survivors from the higher dose groups which was consistent with the gross and histopathology data. Viraemia was not detected in the single survivor from the 5×10^5 p.f.u. group, but was detected in the two surviving 5×10^6 p.f.u. group subjects. We were unable to collect a sample to determine the peak viral load from the subject that succumbed to the 5×10^7 p.f.u. group. At necropsy of non-survivors, virus was readily detected from the lungs and from the tracheobronchial lymph node, however a wide variation in the titres from each subject resulted in no statistically significant difference between the lethal doses (data not shown). Other evaluated tissues including adrenal gland (n=1; 4500 p.f.u. mg^-1), kidney (n=1; 750 p.f.u. mg^-1) and heart (n=2; 5600 p.f.u. mg^-1) contained infectious virus at concentrations that would be consistent with blood contamination of the tissue. Livers were also evaluated by plaque assay and no infectious virus could be detected.

**DISCUSSION**

We sought to determine if large-particle aerosol exposure of rhesus macaques with CPXV would provide an improved model of human smallpox. We demonstrated that increasing the aerosol particle size changed the anatomic location of virus deposition from the pulmonary parenchyma to the large conducting airways and, consequently, altered disease presentation. CPXV was chosen because it is a more accessible model of human smallpox than MPXV and VARV, is a BSL-2 pathogen that does not require Select Agent permits, and mouse models are available. Recently, we demonstrated that small-particle aerosol (0.5–3.0 µm) exposure of rhesus monkeys with 5×10^7 p.f.u. CPXV resulted in a severe, uniformly lethal, respiratory disease characterized by: (1) lung consolidation and pathology; (2) viraemia at endpoint only; (3) decreases in peripheral oxygen saturation; and (4) monocytosis. In this study, we observed: (1) an increased mean day to endpoint criteria (day 9 for small-particle aerosol, day 12 for large-particle); (2) viraemia prior to endpoint; and (3) severe pathology of the upper respiratory tract. A comparison of large-particle CPXV aerosol exposure to small-particle CPXV exposure can be found in Table 1. However, we did not observe uniformly lethal disease at any of the doses evaluated in this experiment. This may be due to the differing microanatomy of the upper respiratory tract where the large-particles deposit when compared with the lower respiratory tract where the small-particles deposit.

Further comparison of large-particle exposure of CPXV to rhesus monkeys to other NHP models of human smallpox, and smallpox itself suggests some similarities and notable differences as summarized in Fig. 6 (Fenner, 1988; Huggins et al., 2009; Jahrling et al., 2004; Johnson et al., 2011a, 2011b, 2015; Kramski et al., 2010; Nalca et al., 2010; Reynolds et al., 2006). Most notably, CPXV-infected macaques develop few skin lesions when compared with MPXV-infected macaques and VARV-infected humans. The incubation period (time from exposure to development of clinical signs) of the intranasal CPXV marmoset model more closely matches smallpox than the other models. However, reagent availability, subject availability and subject size limit the utility of the common marmoset model. The MPXV IV exposure models result in rapid disease with onset of clinical signs between days 4 and 6 and IV exposure is not a natural route of transmission, which impacts the utility of the model in terms of the FDA’s ‘Animal Rule’. Airway exposure models are...
more variable, but provide the best avenue for development of an NHP model of smallpox due to increased incubation periods, and more accurately reflect a natural exposure route than IV exposure.

Large-particle aerosol exposure of rhesus monkeys to CPXV resulted in a predictable distribution of lesions within the upper respiratory tract and major airways resulting in severe pharyngitis, tracheitis and bronchitis/bronchiolitis. Consolidating pulmonary

Fig. 4. CT indicates lung pathology and resolution. The selected images are representative of non-survivors or survivors from the two groups in which NHPs met endpoint criteria. The data shown demonstrates the CT image at the observed peak lung pathology for that subject: day 11 for panel (a), day 12 for panel (b), day 12 for panel (c), and day 12 for panel (d). Corresponding CT images from previous or subsequent days were selected for comparison of disease progression and regression within the same anatomical location. Subjects that succumbed or met endpoint criteria demonstrated similar CT findings (a–c). Subjects that survived demonstrated pathology followed by resolution (d). (a) Subject 16, 5×10⁷ p.f.u. group, succumbed at day 11. (b) Subject 15, 5×10⁷ p.f.u. group, euthanized on day 12. (c) Subject 9, 5×10⁶ p.f.u. group, euthanized on day 12. (d) Subject 10, 5×10⁶ p.f.u. group, survivor.
Parenchymal or alveolar lesions were seen in only two subjects in the high dose group. The pulmonary parenchymal lesions seen in the remainder of the subjects extended only a short distance from the tertiary bronchioles and were not as severe as those observed following small-particle aerosol exposure. Additionally, we showed that within these areas of severe lung and tracheal pathology, macrophages were commonly CPXV-antigen-positive, suggestive of direct infection. Independent of dose, lung pathology observed by CT in monkeys that met endpoint criteria was similar and corresponded with gross pathology and histopathology. The CT changes included peribronchial thickening/infiltrates, patchy foci of ground glass (alveolar) opacification, with occasional consolidations mainly seen in the high dose group subjects. No pleural effusions were seen. Quantification of disease severity using PCLH significantly correlated with various clinical parameters including peripheral oxygen saturation, respiratory rate and circulating monocyte numbers, especially for the $5 \times 10^7$ p.f.u. group. This suggests reliable assessment of disease severity using CT, and the potential to use imaging as surrogate endpoint of disease progression/treatment in future therapeutic intervention studies. Unfortunately, quantitative evaluation of the pharynx, epiglottis and proximal trachea using CT is limited, and was obscured by the presence of the ET tube in these anaesthetized monkeys.

Characterization of the immune response to large-particle exposure demonstrated many differences when compared with small-particle aerosol exposure to CPXV. Most notably, a dominant pro-inflammatory systemic cytokine response was not observed. This is in contrast to the systemic inflammatory response as has been suggested after IV CPXV, MPXV and small-particle aerosol exposures (Jahrling et al., 2004; Johnson et al., 2011a, b, 2015). Changes in the PBMCs were consistent with the dosing, for example, monocytes increased substantially and more rapidly for the $5 \times 10^5$ and $5 \times 10^6$ p.f.u. group subjects than for the $5 \times 10^4$ and $5 \times 10^5$ p.f.u. groups.

Fig. 5. Quantitative analysis of the CT data indicates progressive and resolving lung pathology that corresponds to dose. CT data was analysed as described in Methods. Individual subjects are shown by group. (a) $5 \times 10^7$ p.f.u., (b) $5 \times 10^6$ p.f.u., (c) $5 \times 10^5$ p.f.u., (d) $5 \times 10^4$ p.f.u. Asterisk indicates that the day 3 data points were dropped due to incorrect placement of the ET tube.
NK cells increased the most for the 5×10^5 and 5×10^3 p.f.u. group, underscoring their importance for resolution of poxvirus-induced disease (Americo et al., 2010; Bai et al., 2005; Born et al., 2000; Earl et al., 2012; Harris et al., 1995). Predictably, T-cell and immature B-cells increased in surviving subjects independent of dose. The specific function of these cells and cytokines in response to CPXV infection is beyond the scope of this work, but establishing the dynamics of these cell populations and cytokines provides many opportunities for future hypothesis-driven research. The current experiment was designed to establish the large-particle infection model. Future experiments would include a more detailed investigation of the tissue-specific response to infection. Such experiments would include serial euthanasia to directly compare CT and pathology during disease development. Since bronchoalveolar lavage can induce an inflammatory response that can be observed by CT (Haley et al., 1989; Von Essen et al., 1991), such procedures would be best performed as terminal procedures with control groups to provide an accurate basis of comparison.

A model that accurately mimics human smallpox should result in systemic disease. In this study, viraemia was detected 3 days prior to euthanasia for three of five subjects with a peak of 5.23 log_{10} genome copies ml^{-1}. Viral load in the tissue as measured by plaque assay indicates that many tissues were positive, but at levels that indicate blood contamination of the tissue rather than production of infectious virus in those tissues. However, viral load in the lung (6.23 log_{10} p.f.u. mg^{-1}) indicates that these tissues were actively infected and that the observed tissue damage is virus-induced. Regrettably, we did not collect trachea for evaluation of viral load, however, myriads of intraepithelial, eosinophilic, intracytoplasmic inclusion bodies consistent with orthopoxvirus were seen in the tracheal epithelium by histopathology.

The current study demonstrates that aerosol particle size impacts virus deposition within the respiratory tract and drives disease presentation and pathogenesis, and that systemic virus dissemination following large-particle aerosol exposure is possible. However, the observed severe respiratory disease may progress too quickly to allow for the development of secondary viraemia and systemic disease as described for smallpox. This may be overcome by large-particle aerosol exposure with other poxviruses such as MPXV or VAR as neither is as pathogenic as CPXV in Macaca species (Goff et al., 2011; Jahrling et al., 2004; Johnson et al., 2011a, b, 2015). In addition, CPXV strains other than Brighton Red, that possess differences in pathogenicity, may provide a model with greater fidelity to human smallpox.

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**METHODS**

**Virus and cells.** CPXV-BR was propagated as described previously (Johnson et al., 2015). Briefly, B-SC-1 cells (ATCC) were infected at a low multiplicity of infection until cytopathic effect reached 80% (roughly 2 days). B-SC-1 cells were maintained in modified Eagle’s medium (MEM) (HyClone) supplemented with 2% FBS (Sigma) and 1% penicillin/streptomycin at 37 °C with 5% CO_2. Virus was prepared for exposure by rapid thaw of the virus stock and sonication.

**Exposure and monitoring of NHPs.** All animal procedures were approved by the National Institute of Allergy and Infectious Diseases (NIAID) Division of Clinical Research Animal Care and Use Committee, and adhered to National Institutes of Health (NIH) policies. The experiments were carried out at the NIAID Integrated Research Facility, an AAALAC and AALAS accredited facility. Experiments followed the guidelines and basic principles of the United States Public Health Service Policy on Human Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals. Procedures were carried out under anaesthesia using ketamine and/or isoflurane by trained veterinary personnel. Subjects were housed in individual cages that allowed for social interactions in a temperature- and humidity-controlled environment. Food and water were available ad libitum and enrichment was provided twice daily. Endpoint criteria, as approved by the Division of Clinical Research, Animal Care and Use Committee (DCR ACUC), were used to determine when animals met humane endpoint criteria. At that time, they were euthanized under deep anaesthesia.

Sixteen rhesus monkeys (Macaca mulatta), that were panorthopox-negative and negative for simian retrovirus, simian T-lymphotrophic virus, and ranged in weight from 4.16 to 13.12 kg were included in this study. The groups consisted of 13 males and 3 females and ranged in age from to 3 to 8 years. Groups received a target dose of 5×10^7 p.f.u. – 5×10^6 p.f.u. in decreasing serial tenfold increments. Prior to and post-aerosol exposure, CT and physical exams, including temperature, weight and lesion counts, were performed, and blood draws and swabs from oral and nasal cavities were taken on physical exam days and necropsy. Subjects were maintained on inhalational isoflurane during CT procedures. Study subjects were monitored at least twice daily and euthanized when they met pre-established criteria; these criteria included: (1) overall clinical appearance, (2) laboured breathing, (3) activity and...
behaviour, (4) responsiveness, and (5) core body temperatures as evaluated by veterinary staff. At the time of necropsy, NHPs were humanely euthanized and additional blood, oral and nasal swabs, and select tissues were collected for further evaluation.

Large-particle aerosol exposure. Rhesus monkeys (n=16) were exposed to CPXV-BR using a 16 l, head only, aerosol exposure chamber and an aerosol management platform (AeroMP; Biaera Technologies) within a Class III biosafety cabinet (BSC) (Germfree). Characterization studies ensuring viral stability and viability were conducted prior to NHP aerosol exposures. Virus dilutions were prepared in Eagle’s minimum essential medium (Lonza) in 10 % glycerol (v/v) (Sigma Aldrich). The subjects were anaesthetized with ketamine and received a time-calculated aerosol exposure based on NHP minute ventilation. Aerosol
particles were generated by the Centered Flow Tangential Aerosol Generator (CenTAG; CH Technologies). A stainless steel filter sampler (In-Tox Products) using a gelatin membrane filter (Sartorius) operating at a continuous flow rate of 2 LPM was used to determine the aerosol concentration within the chamber. An air wash period of 5 min between exposures allowed the particles to decay. The exposure chamber (−0.1’ WC) and Class III BSC (−1.0’ WC) both maintained negative airflow throughout the challenge. Prior to an exposure, subject-specific minute volume values were obtained using plethysmography acquisition equipment (Buxco-DSI). An averaged respiratory minute volume (1 min⁻¹) for a 2 min collection period was obtained and used to calculate the presented dose. A presented dose was calculated using the simplified formula \( D = R \times C_p \times T \), where \( D \) is the presented or estimated inhaled dose (p.f.u.), \( R \) is the respiratory minute volume (1 min⁻¹), \( C_p \) is the aerosol concentration (p.f.u. 1⁻¹), and \( T \) is the duration of the exposure (min) (Hartings & Roy, 2004).

**Computed tomography (CT) and image analysis.** CT was acquired as described previously in Johnson et al., 2015. PCLH was determined as described previously (Solomon et al., May 2014). The quantitative analysis was compared with a blinded, qualitative evaluation of CT lung pathology over time, performed by a radiologist (D. A. H.).

**Haematology and serology.** Samples for complete blood cell differential count (CBC/diff) were collected in EDTA-coated blood tubes and analysed using a Sysmex XT2000iV (Sysmex America). Blood samples for serum chemistries evaluation were drawn using a serum separator tube and analysed on a COBAS INTEGRA 400Plus (Roche Diagnostics).

**PBMC dynamics were evaluated as previously described in Johnson et al. (2011b).** Briefly, EDTA whole blood samples were collected from NHPs and analysed using TrueCount tubes (BD Biosciences) using a commercial antibody cocktail containing markers for CD45, CD3, CD4, CD8, CD14, CD16, CD20 (BD Biosciences), and CD159 (NKG2a) (Beckman Coulter). Samples were analysed on the BD Fortessa flow cytometer. Data were analysed using BD FACSDiva software v6.1.3 (BD Biosciences). Cells were first gated on CD45, then CD3⁺ and CD3⁻ populations were selected against CD45. CD3⁺ populations were then gated against CD4 and CD8 subpopulations. CD3⁻ populations were gated against CD14, NKG2a and CD20 subpopulations.

**Quantification of viraemia by quantitative PCR and tissue viral load by plaque assay.** Swabs and whole blood samples were extracted with Trizol and evaluated for CPXV genomes using the HA gene assay (Johnson et al., 2014). The quantitative analysis was compared with previously described (Johnson et al., 2015). Viral load in lungs were the mean titres of samples from the six lobes for each NHP. Liver titres were the mean of left, right, medial and caudal liver lobe sections. Sections of the right kidney and right adrenal gland for each non-surviving subject were excised and titres determined.

**Cytokine and chemokine analysis.** The concentrations of the cytokines and chemokines MCP-1, IL-6, IL-8, IFN-γ, sCD40L, G-CSF, IL-12 and VEGF in plasma samples were measured by multiplexing the Millipore NHF Cytokine Panel (Millipore) following manufacturer’s recommendations and were assayed on the FlexMap 3D System (Lumines). 

**Histopathology, immunohistochemistry and immunofluorescence.** Forty-one tissues from all major organ systems were collected and fixed in 10% neutral buffered formalin. Samples were embedded in paraffin, sectioned at 4 µm, and stained with haematoxylin and eosin (H&E) according to established protocols. H&E sections were examined by light microscopy by a veterinary pathologist (D. L. P.). For immunohistochemical (IHC) and immunofluorescence (IF) analyses, lung sections were baked at 60°C for 1 h then deparaffinized with xylene and rehydrated with alcohol-gradated washes. Sections were microwaved with Antigen Unmasking Solution (Vector Laboratories). After exposure to protein block (Dako) for 30 min, rabbit anti-Vaccinia virus antibody was added at a dilution of 1:800 (IHC) or 1:1000 (IF), followed by biotinylated goat anti-rabbit immunoglobulin G (IgG; Vector Laboratories) at a dilution of 1:200 (IHC) or AlexaFluor 594 (Vaccinia) and AlexaFluor 488 (IgA; Macrophyge at a dilution of 1:100) (IF). Sections were incubated for 30 min with Vectorstain ABC reagent (Vector Laboratories) and for 25 min with 3,3’-diaminobenzidine (DAB) were counterstained with haematoxylin (IHC) or DAP (IF) and evaluated by a veterinary pathologist (I. N. M.).

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