Intrabronchial inoculation of cynomolgus macaques with cowpox virus

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The public health threat of orthopoxviruses from bioterrorist attacks has prompted researchers to develop suitable animal models for increasing our understanding of viral pathogenesis and evaluation of medical countermeasures (MCMs) in compliance with the FDA Animal Efficacy Rule. We present an accessible intrabronchial cowpox virus (CPXV) model that can be evaluated under biosafety level-2 laboratory conditions. In this dose-ranging study, utilizing cynomolgus macaques, signs of typical orthopoxvirus disease were observed with the lymphoid organs, liver, skin (generally mild) and respiratory tract as target tissues. Clinical and histopathological evaluation suggests that intrabronchial CPXV recapitulated many of the features of monkeypox and variola virus, the causative agent of smallpox, infections in cynomolgus macaque models. These similarities suggest that CPXV infection in non-human primates should be pursued further as an alternative model of smallpox. Further development of the CPXV primate model, unimpeded by select agent and biocontainment restrictions, should facilitate the development of MCMs for smallpox.

Four factors drive the need for improved non-human primate (NHP) models of orthopoxvirus infection: (i) the increased threat of intentional release of variola virus (VARV), the causative agent of smallpox, as a bioterrorism weapon, (ii) the increased frequency of sporadic cases and spread of zoonotic orthopoxviruses including cowpox (CPXV) (Vorou et al., 2008) and particularly monkeypox virus (MPXV) (Parker et al., 2007; Rimoin et al., 2010) in humans, (iii) the uncertain future of VARV research due to increased interest in eradication of the known VARV stocks (Lane & Poland, 2011; McFadden, 2010; Tucker, 2011), and (iv) the lack of an NHP model that fully mimics the natural route or course of infection as mandated by the FDA ‘Animal Rule’. To fulfil the requirements of the ‘Animal Rule’, a medical countermeasure (MCM) must demonstrate efficacy in at least two animal models in which the route and dose of virus administration, time to onset of disease and time-course/progression of infection optimally reflects human disease (FDA, 2009). To this end, animal model development in multiple species and using natural routes of transmission must be explored to effectively evaluate MCMs. CPXV-induced disease may serve as an ideal model for orthopoxviruses because (i) CPXV is a biosafety level-2 (BSL-2) pathogen, (ii) CPXV shares homology with MPXV and VARV (Lefkowitz et al., 2005; Seet et al., 2003; Xing et al., 2006), and (iii) CPXV produces disease in mice and NHPs (Bray et al., 2000; Martinez et al., 2000).

Recent studies suggest that CPXV inoculation of NHPs may serve as an additional model of VARV infection that would increase our knowledge of orthopoxvirus disease progression in NHPs. We have recently described the disease progression of CPXV Brighton Red strain after intravenous (i.v.) inoculation of cynomolgus macaques (Macaca fascicularis). i.v. inoculation resulted in a rapid, fulminant haemorrhagic disease that shared several characteristics with human haemorrhagic smallpox (Johnson et al., 2011b). Other groups have explored common marmosets as an alternative NHP model of orthopoxvirus disease after an outbreak of CPXV in a German zoo (Martina et al., 2006; Mätz-Rensing et al., 2006). A follow-up study exploring CPXV infection of marmosets revealed that the LD50 via intranasal inoculation in marmosets was <10³ p.f.u. and the Calpox variant produced some typical signs of poxvirus disease (Kramski et al., 2010). Establishment of the marmoset cowpox model is significant, but critical reagents are not readily available for marmosets, and i.v. MPXV infection of macaques is the most widely established NHP orthopoxvirus model for testing MCMs (Chen et al., 2005; Earl et al., 2008; Edghill-Smith et al., 2006).
Four groups of two cynomolgus macaques were inoculated with serial 10-fold dilutions of CPXV from $5 \times 10^5$ to $5 \times 10^4$ p.f.u. by IB inoculation using a paediatric bronchoscope as described previously (Johnson et al., 2011a). The inoculum was delivered in a volume of 5 ml and deposited in the left tertiary bronchus. NHPs were monitored daily. Blood was drawn for analysis and physical exams were performed periodically throughout the study (days 0, 2, 4, 6, 8, 10, 12, 16, 21 and 28). All animal handling and clinical procedures were performed while NHPs were anaesthetized with Ketamine or Telazol. Complete necropsies were performed when NHPs met established clinical end-point criteria or at study end (day 28). NHPs were deeply anaesthetized with Telazol or Ketamine and humanely euthanized by i.v. pentobarbital overdose. All animal procedures adhered to National Institutes of Health (NIH) policies, including those set forth in the Guide for the Care and Use of Laboratory Animals, and were approved by the National Institute of Allergy and Infectious Diseases Animal Care and Use Committee.

Clinical signs of disease following CPXV IB inoculation included dyspnoea, inappetance and minor cutaneous erythema. Lesion severity was mild compared with MPXV- and VARV-induced disease in humans with seven of eight NHPs developing six or fewer lesions. One NHP receiving $5 \times 10^5$ p.f.u. of CPXV developed 445 lesions (Table 1). All NHPs in the $5 \times 10^7$ p.f.u. and $5 \times 10^6$ p.f.u. groups succumbed on days 7 and 11 post-inoculation (p.i.) and days 11 and 15 p.i., respectively. IB inoculation with $5 \times 10^5$ p.f.u. of CPXV resulted in one of two NHPs succumbing on day 14 p.i., and administration of $5 \times 10^4$ p.f.u. of CPXV did not result in lethal disease. Although the group size is small, data obtained in this study suggest that $5 \times 10^5$ p.f.u. of CPXV may be the LD$_{50}$ following CPXV IB inoculation. By comparison, inoculation of $5 \times 10^5$ p.f.u. of CPXV i.v. was 100 % lethal. The LD$_{50}$ of CPXV i.v. has yet to be determined (Johnson et al., 2011b), but is likely to be several log$_{10}$ p.f.u. lower than IB CPXV inoculation.

Histopathological changes in lymph nodes or liver following CPXV IB inoculation were similar to those observed following lethal i.v., IB, intratracheal or aerosol challenge of MPXV in NHPs (Goff et al., 2011; Johnson et al., 2011a; Zaucha et al., 2001), i.v. inoculation of CPXV in NHPs (Johnson et al., 2011b), naturally occurring MPXV in a prairie dog (Langohr et al., 2004) and patients with smallpox (Bras, 1952; Lillie, 1930; Martin, 2002). Analysis of haematoxylin and eosin (H&E) stained tissue sections revealed the following morphological changes: lymph...

### Table 1. IB CPXV infection of cynomolgus macaques results in severe disease

<table>
<thead>
<tr>
<th>Dose (p.f.u.)</th>
<th>NHP Day of clinical end-point</th>
<th>Peak no. of skin lesions</th>
<th>Peak viraemia (log$_{10}$ gene copies ml$^{-1}$)</th>
<th>Peak viral load in nasal eluates (log$_{10}$ gene copies ml$^{-1}$)</th>
<th>Viral load in indicated tissue (log$_{10}$ p.f.u. g$^{-1}$)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lung</td>
</tr>
<tr>
<td>$5 \times 10^4$</td>
<td>8034</td>
<td>NA§</td>
<td>6</td>
<td>5.1</td>
<td>6.3</td>
</tr>
<tr>
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<td>5014</td>
<td>NA</td>
<td>4</td>
<td>6.4</td>
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<tr>
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<td>5094</td>
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<td>2</td>
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<td>3.1</td>
</tr>
<tr>
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<td>1</td>
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</tr>
<tr>
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<td>8028</td>
<td>11</td>
<td>4</td>
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<td>7.2</td>
</tr>
<tr>
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<td>4083</td>
<td>15</td>
<td>445</td>
<td>8.0</td>
<td>7.8</td>
</tr>
<tr>
<td>$5 \times 10^7$</td>
<td>2023</td>
<td>11</td>
<td>6</td>
<td>8.0</td>
<td>7.8</td>
</tr>
<tr>
<td>9051</td>
<td>7</td>
<td>2</td>
<td>5.9</td>
<td>6.0</td>
<td>7.3</td>
</tr>
</tbody>
</table>

* Tissues were assayed for viral load at time of necropsy on day of clinical end-point (3606, 8028, 4083, 2023 and 9051) or at study end (day 28).
† Lymph node was average of inguinal bronchial and submandibular lymph node tissues.
‡ Central nervous system was average of thoracic and lumbar spinal cord.
§ Indicated NHPs survived to study end (day 28).
‖ No virus detected.
nodes indicated oedema and histiocytosis in non-survivors independent of dose and lymphoid hyperplasia in surviving NHPs. There were multifocal neutrophilic infiltrates within the liver (Fig. 1a) at all doses, and some liver sections displayed hepatocellular degeneration. Intrahepatocyte, intracytoplasmic inclusion bodies were also detected in one NHP in the $5 \times 10^6$ p.f.u. dose group (Fig. 1b).

IB CPXV infection shares many similarities with other orthopoxvirus airway inoculation models; however, comparison of the IB CPXV infection with human VARV disease suggests that lung disease is exaggerated in CPXV IB-inoculated NHPs. IB CPXV inoculation resulted in consolidated lungs with oedema and atelectasis in non-surviving NHPs (Fig. 1c). H&E staining of lung tissue from non-surviving NHPs from the $5 \times 10^7$, $5 \times 10^6$ and $5 \times 10^5$ p.f.u. dose groups revealed severe necrotizing pleuritis, alveolar oedema, necrotic bronchiolar epithelium and diffuse vascular congestion (Fig. 1d). CPXV IB inoculated NHPs shared similarities with aerosolized MPXV-infected animals; both displayed fibrinous pleuritis with pleural adhesions and multifocal, white, plaque-like thickenings of the visceral pleura (Zaucha et al., 2001). Similar to MPXV IB inoculation, CPXV IB inoculation resulted in pneumonia, congestion and pleural adhesions (Johnson et al., 2011a). Bronchopneumonia, bronchitis, bronchiolitis, oedema and, rarely, atelectasis was observed in 25 of 177 fatal VARV human cases reviewed by Bras (1952) and 17 of 54 cases reviewed by Councilman (Bras, 1952; Councilman et al., 1904). During the USA 2003 MPXV outbreak, coughing was a common symptom and dyspnoea was observed less frequently (Chapman et al., 2010; Reynolds et al., 2006).

Viral load in the blood and nasal swabs was measured by qPCR and viral load in 72 tissues collected at necropsy were determined by plaque assay of tissue homogenates on Vero cells as described previously (Johnson et al., 2011a). Viral load data are summarized in Table 1. Viral load in whole blood and nasal swabs was initially detected between days 2 and 6 for all groups, and peak viral load in blood as measured by qPCR ranged from 4.9 to 8.0 log$_{10}$ gene copies ml$^{-1}$ with surviving NHPs generally developing lower peak viral load than non-survivors (Table 1). The four NHPs that succumbed at higher doses ($5 \times 10^6$ or $5 \times 10^7$ p.f.u.) of CPXV had high viral titres in several tissues, indicating a systemic infection. Each of the NHPs in the $5 \times 10^6$ and $5 \times 10^5$ p.f.u. dose groups had virus concentrations ranging from 4.5 to 9.2 log$_{10}$ p.f.u. g$^{-1}$ in tissue samples from the lymph nodes, lungs, liver and nares at moribund endpoint. Three of four NHPs in these groups also had detectable virus in the skin (data not shown), central nervous system and spleen. Similar to human patients with MPXV (Müller et al., 1988), CPXV was isolated from livers of four of five moribund animals.

The immune response to CPXV infection was evaluated by measuring the neutralizing antibody activity and cytokine concentrations in serum. Neutralizing antibody activity was detected using a flow cytometry-based assay modified from Earl et al. (2003) and serum cytokine concentrations were measured using the Biosource human cytokine multiplex-25 bead array assay kit (Invitrogen). Seven of eight infected NHPs developed detectable neutralizing antibody against CPXV, and the neutralizing antibody activity rapidly increased between days 8 and 10 p.i. and plateaued in surviving NHPs by day 16 (Fig. 2a). The NHP that received $5 \times 10^6$ p.f.u and succumbed on day 7 did not develop any detectable neutralizing antibody. However, the remaining NHPs that succumbed did develop neutralizing antibody by day 10 p.i. The peak neutralizing antibody titre was not maintained and declined by moribund endpoint. The data suggest that the neutralizing antibody response alone was probably not sufficient to resolve CPXV infection. The serum concentrations of 25 cytokines and chemokines were analysed over the course of the study and increases in interleukin (IL)-6 and monocyte chemo-attractant protein-1 (MCP-1) serum concentrations were

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**Fig. 1.** Gross necropsy and histopathology of H&E stained tissue sections of intrabronchially cowpox inoculated NHPs. (a) Hepatic centrilobular necrosis with monocytic infiltrates ($\times 10$). (b) Intracytoplasmic inclusion bodies within hepatocytes (arrows) and neutrophilic infiltrates ($\times 40$). (c) Lung consolidation and oedema (arrow) observed at gross necropsy of a $5 \times 10^6$ p.f.u. NHP (NHP 8028). (d) H&E lung tissue section demonstrating severe pleuritis and alveolar infiltrates (arrow) ($\times 5$).
observed in NHPs infected with $5 \times 10^6$ and $5 \times 10^7$ p.f.u. of CPXV, although the peak concentrations varied widely (Fig. 2b). In addition, concentrations of macrophage inflammatory protein-1β (MIP-1β) and alpha interferon (IFN-α) were elevated in NHPs inoculated with $5 \times 10^6$ p.f.u. of CPXV. The increases in MIP-1β, IL-6 and MCP-1 demonstrated similar trends when compared to VARV infection in NHPs (Jahrling et al., 2004).

Similarities between VARV-, MPXV- and CPXV-induced disease include target organs, viraemia, upregulation of MCP-1, IL-6 and MIP-1β, similar histopathology findings and development of neutralizing antibody. The small sample size evaluated limited the utility of statistical analysis of this dataset, but the observed disease and similarity in disease presentation when compared to VARV infection of humans suggests that CPXV infection of NHPs infected with $5 \times 10^6$ and $5 \times 10^7$ p.f.u. of CPXV, although the peak concentrations varied widely (Fig. 2b). In addition, concentrations of macrophage inflammatory protein-1β (MIP-1β) and alpha interferon (IFN-α) were elevated in NHPs inoculated with $5 \times 10^6$ p.f.u. of CPXV. The increases in MIP-1β, IL-6 and MCP-1 demonstrated similar trends when compared to VARV infection in NHPs (Jahrling et al., 2004).

Fig. 2. (a) The level of neutralizing antibodies in CPXV-infected NHPs was measured by a flow cytometric assay using a VACV expressing GFP (VACV–GFP) as the target virus. CV-1 cells ($5 \times 10^4$) were incubated with VACV–GFP at an m.o.i. of 2.5 in the presence of 44 μg cytosine arabinoside and sera (diluted 1:1000) from infected NHPs for 18–24 h. Prior to addition to the cells, the sera and virus were allowed to complex for 1 h at 37 °C. Following incubation, cells were trypsinized (Trypsin + 2.5 % EDTA), washed twice in PBS + 2 % FBS, and analysed by flow cytometry for GFP expression. (b) The concentration of selected cytokines and chemokines in serum samples from CPXV-infected NHPs indicate a strong, but variable pro-inflammatory response. Serum cytokine concentrations were measured using the Biosource human cytokine multiplex-25 bead array assay kit (Invitrogen) from serum samples drawn throughout the study.
cynomolgus macaques by airway exposure may provide an effective model of human VARV infection. By further developing CPXV airway models in parallel with recognized poxvirus models, we hope to establish CPXV infection as a suitable BSL-2 NHP model that would provide greater access of orthopoxvirus models to the research community, thus facilitating the development of improved vaccines and anti-orthopoxvirus therapeutics.

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References


