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Evaluation of disease and viral biomarkers as triggers for therapeutic intervention in respiratory mousepox – An animal model of smallpox

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ABSTRACT

The human population is currently faced with the potential use of natural or recombinant variola and monkeypox viruses as biological weapons. Furthermore, the emergence of human monkeypox in Africa and its expanding environs poses a significant natural threat. Such occurrences would require therapeutic and prophylactic intervention with antivirals to minimize morbidity and mortality of exposed populations. Two orally-bioavailable antivirals are currently in clinical trials; namely CMX001, an ether-lipid analog of cidofovir with activity at the DNA replication stage and ST-246, a novel viral egress inhibitor. Both of these drugs have previously been evaluated in the ectromelia/mousepox system; however, the trigger for intervention was not linked to a disease biomarker or a specific marker of virus replication. In this study we used lethal, intranasal, ectromelia virus infections of C57BL/6 and hairless SKH1 mice to model human disease and evaluate exanthematous rash (rash) as an indicator to initiate antiviral treatment. We show that significant protection can be provided to C57BL/6 mice by CMX001 or ST-246 when therapy is initiated on day 6 post infection or earlier. We also show that significant protection can be provided to SKH1 mice treated with CMX001 at day 3 post infection or earlier, but this is four or more days before detection of rash (ST-246 not tested). Although in this model rash could not be used as a treatment trigger, viral DNA was detected in blood by day 4 post infection and in the oropharyngeal secretions (saliva) by day 2–3 post infection – thus providing robust and specific markers of virus replication for therapy initiation. These findings are discussed in the context of current respiratory challenge animal models in use for the evaluation of poxvirus antivirals.

1. Introduction

Variola virus (VARV), the etiological agent of smallpox, and monkeypox virus (MPXV) are considered possible biological weapons for bioterrorists and rogue nations (Parker et al., 2008c). The mortality rates are approximately 40% and 10%, respectively, and both viruses are transmitted by respiratory tract secretion; however, in the case of MPXV, other routes of inoculation are likely in various species. Furthermore, MPXV, which causes a milder 'smallpox-like' disease, appears to be increasing its environs and infecting increasing numbers of humans in Africa (Parker et al., 2007; Rimoin et al., 2010). Of equal concern is the possibility that VARV/MPXV will be genetically modified to encode human IL-4 which could significantly increase virulence, as has been demonstrated with ectromelia virus (ECTV) and myxoma virus (Chen et al., 2011; Jackson et al., 2001; Kerr et al., 2004). Moreover, a growing section of the community cannot be safely vaccinated with traditional vaccines against VARV/MPXV (due to contraindications; such as various skin conditions or an immunocompromised or immunosupressed status) and the only available antiviral treatment is cidofovir (CDV). The utility of CDV in a public health emergency is limited by its inherent nephrotoxicity and intravenous (IV) delivery method (Parker et al., 2008a). To address

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the pressing need for effective antivirals, an orally bioavailable ester of CDV (CMX001) and a virus egress inhibitor (ST-246) are being developed (Hostetler, 2007; Painter and Hostetler, 2004; Parker et al., 2008d; Quenelle et al., 2007a; Yang et al., 2005) and have been evaluated in the mousepox model (Fenner, 1981; Parker et al., 2008a). As described in a 2009 FDA guidance document, it would be important to use a 'disease defining manifestation' relevant to human disease to initiate therapy in an animal model for the generation of efficacy data (FDA, 2009). In an animal model of smallpox/human monkeypox, the appearance of rash would be an ideal trigger as it appears 10–12 days following infection, and contributes to clinical differential diagnosis (Fenner et al., 1988). Here we show that rash cannot be used as a trigger for intervention in intranasally (IN) ECTV-infected C57BL/6 and the hairless SKH1 mice as it appears at a time in the course of disease when the initiation of antiviral therapy is no longer effective; however, the detection of viral DNA (vDNA) in blood or oropharyngeal secretions (saliva) can be used to initiate efficacious antiviral treatment and simultaneously provide a conclusive diagnosis of infection by the specific orthopoxvirus in question.

2. Materials and methods

2.1. Cells and virus

BSC-1 cells (ATCC CCL 26) were grown in Eagle's minimum essential medium (MEM) containing 10% fetal calf serum (FCS) (Hyclone III, Logan, UT), 2 mM L-glutamine (GIBCO, Grand Island, NY), 100 U/ml penicillin (GIBCO, Grand Island, NY), and 100 μg/ml streptomycin (GIBCO, Grand Island, NY). A plaque-purified isolate of the Moscow (Mos) strain of ECTV (ATCC VR-1374) designated MOS-3-P2, was propagated in an African green monkey kidney cell line, BSC-1 (Chen et al., 1992). Virus was purified through a sucrose cushion as described elsewhere (Moss, 1998). Virus infectivity was estimated as described previously (Wallace & Buller, 1985). Briefly, virus suspensions were serially diluted in PBS + 1% FCS (Fetal Clone II, HyClone), adsorbed to monolayers for 1 h at 37 °C, and overlaid with a suspension of 1% carboxyl methyl cellulose in Dulbecco's Modified Eagle Media (DMEM) + 5% FCS. After 4 days at 37 °C, virus plaques were visualized and virus inactivated by the addition to each well of 0.5 ml of a 0.3% crystal violet/10% formalin solution. We also used a virus encoding eGFP (ECTV-GFP) to detect viral replication in the mouse. eGFP, driven by the VACV p7.5 early/late promoter, was inserted in an ECTV interrupted TNFR homolog sequence (ECTV insertion site between 167,940 and 168,192 bp), and was selected for using MPA transient dominant selection (Falkner and Moss, 1990).

2.2. Animals

Four to six week old female, immunocompetent, SKH1 and C57BL/6 mice were obtained from Charles River (SKH1) (Wilmington, MA), the National Cancer Institute (C57BL/6) (Frederick MD) and Harlan laboratories (C57BL/6 repeat from different source experiment) (Indianapolis, IN), respectively. SKH1 mice carry the *hairless* (Hr^{hr}) gene containing a modified polytropic retrovirus stably integrated into exon 6 of the gene, resulting in aberrant splicing of over 95% of *Hr* transcripts (Benavides et al., 2009; Smith et al., 1982). The *Hr* gene encodes a transcriptional co-repressor, highly expressed in the mammalian skin especially the hair follicle. This strain was used previously to evaluate antivirals following IV injection of vaccinia virus (Quenelle et al., 2004).

Mice were housed in filter-top microisolator cages and fed commercial mouse chow and water, ad libitum. The mice were housed in an animal biosafety level 3 containment areas. Animal husbandry and experimental procedures were in accordance with PHS policy, and approved by the Institutional Animal Care and Use Committee.

2.3. Antiviral compounds

CMX001, a lipid (hexadecyloxypropyl) conjugate of CDV, was synthesized and supplied by Chimerix Inc., (Durham, NC). Dilutions of CMX001, 2.5, 20 and 25 mg/kg were prepared fresh prior to each experiment by dissolving the appropriate amount of compound in sterile, distilled water, and storing them at 4 °C over the course of the experiment. The 20 and 25 mg/kg doses were used as loading doses in the SKH1 and C57BL/6 experiments, respectively. In SKH1 experiments, maintenance doses were used at 2.5 mg/kg every other day for 14 days following the loading dose. In C57BL/6 experiments, a 20 mg/kg maintenance dose was used on days 3, 6, 9, and 12 following the loading dose. ST-246 was synthesized and supplied by SIGA technologies Inc., (Corvallis, OR). 100 mg/kg dilutions of ST-246 were prepared fresh prior to each experiment by dissolving the compound in aqueous 0.75% methylcellulose (Sigma, St. Louis, MO) containing 1% tween (CMC) and stored at 4 °C for the course of the experiment. For both compounds, mice were dosed via gastric gavage with a total volume of 100 μl.

2.4. Viral challenges

Mice were anesthetised with 0.1 ml/10 g body weight of ketamine HCl (9 mg/ml) and xylazine (1 mg/ml) by intraperitoneal injections. ECTV and ECTV-GFP were diluted in PBS without Ca^{2+} and Mg^{2+} to the required concentration. For IN challenges, anesthetised mice were laid on their dorsal side with their bodies angled so that the anterior end was raised 45° from the surface; a plastic mouse holder was used to ensure conformity and virus or saline was slowly loaded into each nare (5 μl/nare). Mice were subsequently left in situ for 2–3 min before being returned to their cages. Groups of five animals were treated at various times post infection (p.i.) with vehicle or test article. For aerosol challenges, mice were exposed to aerosolized ECTV suspended in MEM using a nose-only inhalation exposure system (CH Technologies, Westwood, NJ) as previously described (Parker et al., 2008d). Mice were monitored for disease signs daily, and weighed every day until 21 days p.i. After 21 days p.i., mice were weighed on days 28, 35 and 42. Each experiment was repeated thrice in various combinations.

To confirm infection, surviving mice were bled for ELISAs (as described previously Buller et al., 1983; Stabenow et al., 2010) at day >21 p.i. to confirm the presence or absence of ECTV antibodies, as appropriate.

2.5. Histopathology

Skin lesions were processed as described previously (Stabenow et al., 2010). Briefly, lesions were removed from mice using scissors and scalpels, placed in 10% neutral buffered formalin for 24 h, and then transferred to 70% ethanol prior to trimming, processing and embedding in paraffin. Paraffin sections were stained with hematoxylin and eosin (H&E) and examined microscopically.

2.6. Hair removal

Hair was removed in one of two ways: (1) Mice were anesthetised with ketamine/xylazine and treated with Nair hair removal cream (Church & Dwight, Princeton, NJ) according to the manufacturer's instructions; briefly, a thick, even layer of cream was applied to the flanks of the mouse and left for 3–6 min before being wiped off with a damp cloth. Following cream removal the treated