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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

area was rinsed thoroughly with water. (2) Mice were anesthetised with ketamine/xylazine, coarsely shaved, and waxed with Zip Wax (Lee Pharmaceuticals, South El Monte, CA) according to the manufacturer's instructions; briefly, a 600 ml beaker was filled with 150 ml of water and was heated on a heat/stirrer plate at medium setting until the water reached 70 °C, at which point a 250 ml beaker filled with 2–3 blocks of wax was placed inside the 600 ml beaker. Wax was heated until it melted. Molten wax was applied with a wooden spatula to the flanks of the mouse and dried for 2–3 min before being removed.

2.7. Microscopy

For GFP visualization, heads from sacrificed animals were removed with scissors and cut open laterally with a MiniMite cordless rotary tool (Dremel, Racine, WI). Sectioned heads were examined microscopically using a Leica MZ10F dissection microscope (Wetzlar, Germany). Skin lesions and whole mice were photographed with a Samsung S1065 digital camera (Samsung, South Korea).

2.8. PCR assays

For qPCR, blood was removed to EDTA coated microfuge tubes (BD Biosciences), homogenized and run directly using Omniklentaq buffer and enzyme (DNA Polymerase Technologies, St. Louis, MO) according to a modified manufacturer's instructions (Kermekchiev et al., 2009). Briefly, a master mix containing, per reaction, 0.2 mM dNTP mix PCR grade (Invitrogen, Carlsbad, CA), 1× ROX reference dye (Invitrogen, Carlsbad, CA), 60× SYBR Green 1 nucleic acid gel stain (Invitrogen, Carlsbad, CA), 1× OmniKlenTaq Buffer (DNA Polymerase Technologies, St. Louis, MO), 1× PEC-2 (DNA Polymerase Technologies, St. Louis, MO), 0.6 μl of Omni Klentaq DNA Polymerase (DNA Polymerase Technologies, St. Louis, MO), 10 pM each of primers SP028 and SP029 (Parker et al., 2008b), 1.25 μl of blood and water to a total volume of 25 μl/reaction. To sample oropharyngeal secretions, we used 6 inch polyester-tipped swabs to swab the bucal cavity (Fisher Scientific, Pittsburgh, PA) and prepared samples for PCR as described by others (Meldgaard et al., 2004). We used identical PCR conditions as for blood except we used 1×SYBR Green one nucleic acid gel stain and 1 μl of Omni Klentaq DNA Polymerase. The PCR reaction was run on an AB 7500 RT-PCR machine (Applied Biosystems, Foster City, CA) as described previously (Parker et al., 2008b).

2.9. Statistics

An unpaired two-tailed *t*-test was used to compare the means of two groups of mice. *P* values below 0.05 were considered statistically significant. Mortality rates were compared using Fisher's exact test.

3. Results

3.1. Rash appearance in C57BL/6 mice

Rash of smallpox and human monkeypox has a particular character that makes it a key component in the clinical differential diagnosis, and could provide an ideal trigger for the initiation of ST-246 and/or CMX001 antiviral therapy providing they are efficacious post rash onset. Accordingly, we investigated the time of rash onset in the C57BL/6 intranasal (IN) challenge model. To this end, we removed the hair from the flanks with Nair and IN infected the mice with ECTV. We found that at a 4300 PFU dose ($\sim$43 × LD_{50}) focal skin changes were observed on all mice at day 8.4 ± 0.7; however, these changes were consistent with folliculitis, although this hypothesis was not confirmed by histopathology (data not shown). At a lower dose of 430 (4.3 × LD_{50}) PFU we could detect papules in 40% (2/5) of mice at day 9.5 ± 0.9. All mice receiving low and high lethal doses of virus survived infection suggesting that the hair removal treatment was somehow protective – possibly as a result of the Nair-induced inflammation of the skin. For this reason, we changed the hair removal process. Hair was removed by waxing mice on the day of infection. Once again we found waxed mice more resistant to lethal infection and that the LD_{50} had increased by at least 1 log (data not shown). Finally, we IN infected the C57BL/6 mice with three different doses of ECTV: 4500 (45 × LD_{50}), 450 (4.5 × LD_{50}) and 45 (0.45 × LD_{50}) PFU, and sacrificed and waxed groups of animals on days 6–16 p.i. We found that mice infected with the highest dose of 4500 (45 × LD_{50}) PFU died by day 8 p.i. and did not present with any lesions. 50% of animals infected with the 450 (4.5 × LD_{50}) PFU dose died by day 13 p.i. – none presented with lesions. As expected, mice infected with the 45 (0.45 × LD_{50}) PFU dose all survived infection; however, some mice (2/6) did present with some rash-like lesions (3–5/mouse) from day 14 p.i. Hematoxylin and eosin (H&E) stained sections of these lesions were examined microscopically which revealed that that the lesions did not contain A-type inclusion bodies typically associated with ECTV replication and were most consistent with bacterial folliculitis. A repeat of this experiment with C57BL/6 mice from a different source gave similar mortality rates at each of the ECTV doses but no rash could be detected in any groups (data not shown).

Thus, clinical observations of "rash" were most likely due to bacterial folliculitis and rash was not reliably detected in C57BL/6 mice following IN infection. Both depilation treatments resulted in a decreased mortality as compared to infected, non-treated controls, which limited their usefulness for evaluation of antiviral efficacy.

3.2. Rash appearance in SKH1 mice

Because rash development in mousepox is dependent on route of infection and mouse genotype, the outbred, SKH1 hairless mouse strain was also evaluated for time of onset of rash. SKH1 mice are known to be susceptible to systemic disease following infection with ECTV, although sensitivity of the strain to mortality by the IN and footpad routes had not been published (Buller and Fenner, 2007). To address this, we tested the SKH1 strain for its sensitivity to ECTV by these routes. We found that the SKH1 strain was, like the C57BL/6 strain, also resistant to lethal footpad infections with doses $\leqslant$1800 PFU (data not shown). Furthermore, when infected IN with ECTV we found that the LD_{50} value was 101 PFU (Reed and Muench, 1938), which was similar to the $\sim$100 PFU LD_{50} of the C57BL/6 strain (Fig. 1A and B) (Parker et al., 2009). When infected via the aerosol route we found the SKH1 LD_{50} to be 85 PFU (Figure S1).

To facilitate the early detection of virus replication in the skin following IN infection, a prerequisite for rash development, SKH1 mice were IN infected with 1000 PFU (1 × LD_{50}) of an ECTV-GFP recombinant. At 2 days p.i. we could detect a few foci with low-fluorescence intensity on the nasal cavity epithelium (data not shown). By day 3 p.i. the posterior vestibule became brightly fluorescent and the foci in the nasal epithelium became larger in size and number. By day 5 p.i. the fluorescence had spread to the meatus and concha. The superior palate consisting of the NALT (nasal associated lymphoid tissue) was also fluorescent. By day 6 p.i. and onward, the whole of the nasal cavity from the vestibule to the nasopharynx was highly fluorescent, although fluorescence was not detected past the nasopharynx (Fig. 2) or in the lungs (data not shown). We also observed small foci of fluorescence beneath

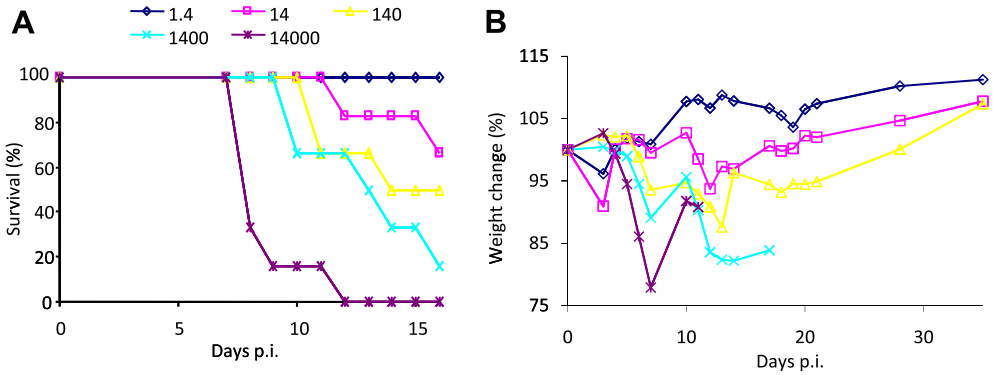

Fig. 1. Groups of SKH1 mice were infected IN with ECTV inoculums of 14000, 1400, 140, 14, and 1.4 PFU. Survival curves (A) and weight change (B) are shown. LD_{50} = 101 PFU. Weight change error bars have been removed for clarity. Data points after day 18 did not change and are therefore not shown in (A). *N* = 5 animals per group.

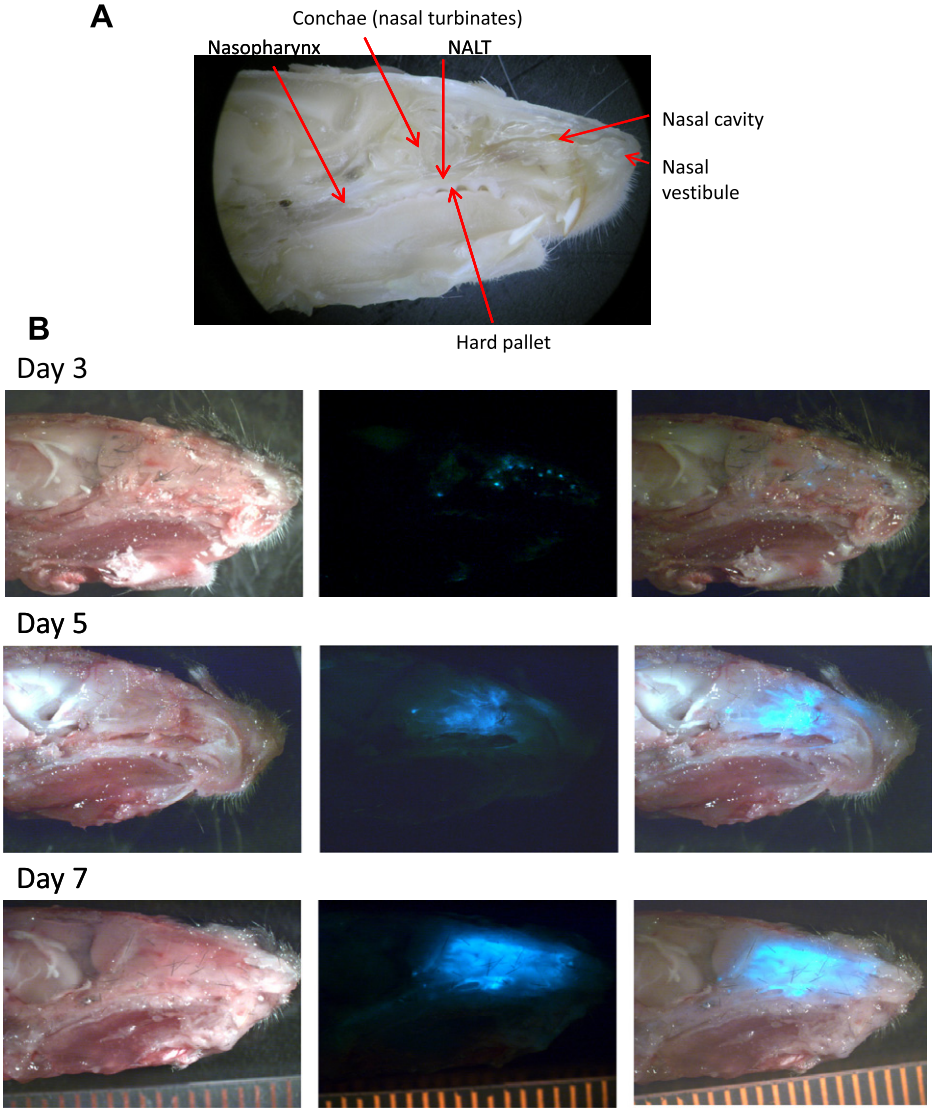

Fig. 2. Mice were infected IN with 1000 (1 × LD_{50}) PFU of ECTV-GFP. (A) Mouse head cut laterally indicating anatomical positions. (B) Infected mouse heads were cut laterally and examined for GFP. First column shows mouse head section under white light; second column shows GFP + areas; third column is overlay.

the skin of infected animals by day 5–6 p.i. (Fig. 3); however, no visible papules or other lesions were observed. These GFP + foci increased in size for the next 2 days before we could detect visible papules (approximately five per mouse). By approximately day 9 we could detect GFP + lesions (approximately 15 per mouse). By day 10 we could detect multiple GFP + areas on the skin (>50 per mouse; data not shown). We observed that approximately 80% of all GFP + foci eventually developed into clinically detectable

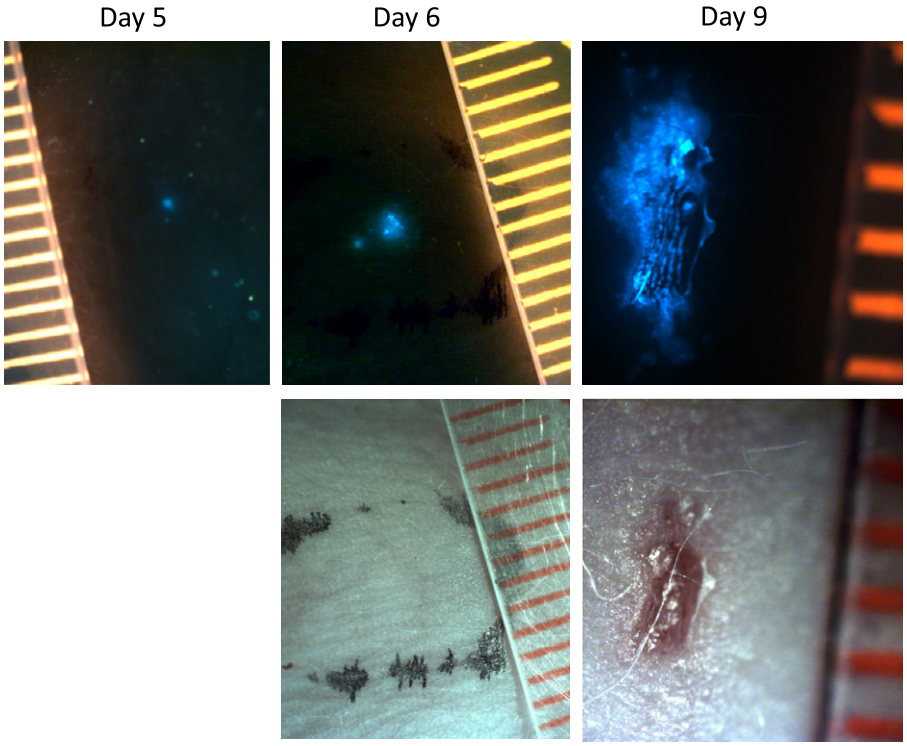

Fig. 3. Groups of SKH1 mice were infected IN with 1000 ($1 \times LD_{50}$) PFU of ECTV-GFP. GFP + foci could be detected in the skin from day 5 and increased in size until day 9. Upper row shows GFP + skin; lower row shows the same sample of skin under white light. No white light lesion was seen at day 5 (not shown). Scale bar = 1 mm. Typical lesions (from the flanks) for the specified day are shown.

lesions. By day 14 p.i. we saw a reduction in the number of GFP + foci and no new lesions. By day 18 we could detect no GFP + foci and all lesions appeared to be healing. Interestingly, we did not observe any lesions that developed from non GFP + areas of the skin, nor could we detect any GFP- lesions.

In ECTV infected mice, papules were detected at 7–9 days p.i. and typically appeared and developed at the same rates. Histologic examination of papules isolated at day 8 p.i. revealed the presence of characteristic A-type inclusion bodies in the epidermis of the superficial and follicular epithelium as well as within sebaceous epithelium (Figure S2). Taken together, these experiments demonstrated that not all foci of virus replication in the skin of SKH1 mice evolved into pocks, and the detection of papules at 7–9 days p.i. was the first reliable, visual sign of virus replication in the skin and the onset of rash.

3.3. The antiviral window for therapeutic intervention in SKH1 and C57BL/6 mice

For rash to be used as a trigger for antiviral treatment, the antiviral window for therapeutic intervention (therapeutic window) must extend well beyond the time of rash onset. Our studies on rash onset following ECTV IN infection of SKH1 mice suggested that papules could be detected as early as 7–9 days p.i. To determine if the therapeutic window of CMX001 extended to at least 7 days p.i. in SKH1 mice, we IN infected mice with three different ECTV challenge doses and initiated CMX001 treatment at days 3, 6, or 9 p.i. Data from three replicate experiments summarized in Table 1 reveal that at the lowest dose of 650 ($6.5 \times LD_{50}$) PFU 60% (9/15) of mice died with an average day of death of 11.4 ± 0.5. At the higher doses of 2050 and 6500 ($65 \times LD_{50}$) PFU mortality rates were 67% (10/15) and 87% (13/15) with an average day of death of 12 ± 0.8 and 10.5 ± 0.4, respectively. CMX001 intervention at day 3 p.i. afforded $\geqslant$93% protection at all doses ($P = 0.005$, 0.0002 and 0.0001 for 650, 2050 and 6500 PFU, respectively); however, no significant protection was achieved when treatment was delayed to day 6, with 60% mortality (9/15) at the 650 ($6.5 \times LD_{50}$) PFU dose and 100% (15/15) and 87% (13/15) mortality at the higher doses of 2050 ($20 \times LD_{50}$) and 6500 ($65 \times LD_{50}$) PFU, respectively. Delaying treatment even further to day 9 p.i. also provided no significant protection, with mortality of 33% (5/15), 87% (13/15) and 87% (13/15) at the 650, 2050 and 6500 doses, respectively. None of the regimens statistically increased the mean day of death. The time to onset of rash was 9.1 ± 0.4 days in the 650 ($6.5 \times LD_{50}$) PFU infected, non-treated controls of the three replicate experiments and at 8.8 ± 0.6 and 8.6 ± 0.4 for the 2050 ($20 \times LD_{50}$) and 6500 ($65 \times LD_{50}$) PFU doses, respectively. This was well-outside of the therapeutic window of CMX001 in SKH1 mice.

Since mouse strains can vary in their capacity to mount an effective immune response against ECTV, and antiviral efficacy is due to synergy between the antiviral therapeutic and the immune system, we also evaluated the CMX001 therapeutic window in the C57BL/6 mouse strain, known for its ability to generate a potent cell-mediated response against ECTV (Esteban and Buller, 2005). Table 2 presents the combined data from the three replicate experiments. Following an 800 ($8 \times LD_{50}$) PFU IN inoculation, 87% (13/15) of infected animals receiving vehicle treatment died with an average day of death of 11.3 ± 0.8. Animals receiving CMX001 on day 0, 4, 5, or 6 p.i. experienced a significant increase in survival ($P = 0.0001$, 0.0007, 0.0007, and 0.05, respectively); however, animals in which treatment was initiated on days 7, 8 or 9 did not have a significant increase in survival. None of the groups had a significant increase in the mean time to death. Weight-change from one representative experiment is shown in Figure S3, and reveals that non-infected and infected mice receiving CMX001 on day 0 lost the least weight. Generally, increased weight-loss accompanied an increase in time to treatment.

Table 1
SKH-1 cumulative protection data from three independent experiments with CMX001 following an IN challenge with ECTV.

Cage #	# mice	Virus (PFU)	Treatment	Initiation of treatment[a]			Day of death (mean ± SEM)	Mortality
				3	6	9		
1	15	Mock	Vehicle	+	–	–		0/15 (0%)
2	15	650	Vehicle	+	–	–	12,10,10,11,14,13, 10,12,11 (11.4 ± 0.5)	9/15 (60%)
3	15	650	CMX001	+	–	–	7	1/15 (7%)[b]
4	15	650	CMX001	–	+	–	12,10,12,11,13,13, 11,11,12 (11.6 ± 0.3)[c]	9/15 (60%)[c]
5	15	650	CMX001		–	+	11,17,9,11,9 (11.4 ± 1.5)[c]	5/15 (33%)[c]
6	12	2050	Vehicle	+	–	–	8,14,14,14,14,9,10 10,14,13 (12 ± 0.8)	10/15 (67%)
7	15	2050	CMX001	+	–	–		0/15 (0%)[b]
8	15	2050	CMX001	–	+	–	11,14,11,11,13,10,12,10,11,10,11,12, 11,14,12 (11.5 ± 0.3)[c]	15/15 (100%)[c]
9	15	2050	CMX001	–	–	+	11,13,7,9,12,9,13,9,11,11,10,10,13 (10.6 ± 0.5)[c]	13/15 (87%)[c]
10	15	6500	Vehicle	+	–	–	11,8,11,12,11,10,8,9,11,12,12,9,12 (10.5 ± 0.4)	13/15 (87%)
11	15	6500	CMX001	+	–	–	11,	1/15 (7%)[b]
12	15	6500	CMX001	–	+	–	9,11,11,9,7,10,10,8, 10,14,14,10,11 (10.3 ± 0.6)[c]	13/15 (87%)[c]
13	15	6500	CMX001	–	–	+	13,11,10,10,9,14,9,9,13,12,10,10,11 (10.8 ± 0.5)[c]	13/15 (87%)[c]

[a] Mice receive a 25 mg/kg loading dose on day 3, 6 or 9 p.i. followed by 2.5 mg/kg maintenance doses every other day for 14 days following initiation of treatment. Doses were administered in 100 μl volumes by gastric gavage.
[b] $P < 0.05$ compared to infected and vehicle treated control.
[c] $P > 0.05$ compared to infected and vehicle treated control.

Table 2
C57BL/6 cumulative protection data from three independent experiments with CMX001 following an IN challenge with ECTV.

Cage #	Virus	# mice	Treatment	Initiation of treatment[a]							Day of death (mean ± SEM)	Mortality
				0	4	5	6	7	8	9		
1	–	15	Vehicle	+								0/15 (0%)
2	–	15	CMX001	+								0/15 (0%)
3	+	15	Vehicle	+							9,10,12,13,8,10,10,10,10,11,12,13,19 (11.3 ± 0.8)	13/15 (87%)
4	+	15	CMX001	+								0/15 (0%)[b]
5	+	15	CMX001		+						20,8,12 (13.3 ± 3.5)[c]	3/15 (20%)[b]
6	+	15	CMX001			+					9,10,20 (13 ± 3.5)[c]	3/15 (20%)[b]
7	+	15	CMX001				+				10,9,10,10,10,10,15 (10.6 ± 0.8)[c]	7/15 (47%)[b]
8	+	15	CMX001					+			10,19,19,9,10,10,13,11,11,13,16,19 (13.3 ± 1.1)[c]	12/15 (80%)[c]
9	+	15	CMX001						+		12,12,15,19,8,9,9,12,11,13,13,13,16 (12.5 ± 0.8)[c]	13/15 (87%)[c]
10	+	15	CMX001							+	10,11,21,10,12,10,11,13,13 (12.3 ± 1.2)[c]	9/15 (60%)[c]

[a] Mice received a 20 mg/kg loading dose followed by 20 mg/kg maintenance doses on days 3, 6, 9, and 12 following the initiation of treatment. Doses were administered in 100 μl volumes by gastric gavage.
[b] $P < 0.05$ compared to infected and vehicle treated control.
[c] $P > 0.05$ compared to infected and vehicle treated control.

Table 3
C57BL/6 cumulative protection data from three independent experiments with ST-246 following an IN challenge with ECTV.

Cage #	Virus	# mice	Treatment	Initiation of treatment[a]							Day of death (mean ± SEM)	Mortality
				0	4	5	6	7	8	9		
1	–	15	Vehicle	+								0/15 (0%)
2	–	15	ST-246	+								0/15 (0%)
3	+	10	Vehicle	+							8,10,10,10,12,11,11,11,12,15 (11 ± 0.6)	10/10 (100%)[d]
4	+	15	ST-246	+								0/15 (100%)[b]
5	+	15	ST-246		+							0/15 (100%)[b]
6	+	15	ST-246			+						0/15 (100%)[b]
7	+	15	ST-246				+				18,10,12,12 (13 ± 1.7)[c]	4/15 (27%)[b]
8	+	15	ST-246					+			8,11,13,15,21,8,8,9 (11.6 ± 1.6)[c]	8/15 (53%)[c]
9	+	15	ST-246						+		11,11,11,12,13,9,9, 11,11,13,8,9,12 (10.7 ± 0.4)[c]	13/15 (87%)[c]
10	+	15	ST-246							+	8,10,10,10,11,12,13,15,8,8,9,10,10 (10.3 ± 0.6)[c]	13/15 (87%)[c]

[a] Mice received daily treatments with 100 mg/kg of ST-246 for 14 days. Doses were administered in 100 μl volumes by gastric gavage.
[b] $P < 0.05$ compared to infected and vehicle treated control.
[c] $P > 0.05$ compared to infected and vehicle treated control.
[d] Five mice were removed due to a flooded cage.

A second antiviral, ST-246, was similarly evaluated in three replicative experiments (Table 3). As shown, 100% (10/10) of infected mice treated with vehicle died with an average day of death of 11 ± 0.6. Mice receiving the ST-246 dosing regimen initiated on day 0, 4, 5, or 6 experienced a significant increase in survival ($P = 0.0001$, 0.0001, 0.0001, and 0.0005, respectively); however, animals receiving the ST-246 regimen commencing on day 7, 8 or 9 did not have a statistically significant increase in survival. None of the treated groups had a significant increase in the mean time to death. Weight-change from one representative experiment is shown in Figure S4 and reveals that non-infected mice and infected mice receiving ST-246 on day 0 lost the least weight.

Generally, increased weight-loss accompanied an increase in time to treatment.

In summary, these data showed that CMX001 did not provide significant protection from lethal ECTV infection when administered to SKH1 mice at 6 days p.i., ~1 day prior to the earliest onset of rash. In the ECTV-infected C57BL/6 mouse strain, ST-246 and CMX001 were efficacious at 6, but not 7 days p.i. suggesting that clinical evidence of rash onset could not be used as a trigger for treatment with ST-246 or CMX001 in ECTV IN infections.

3.4. Disease biomarkers

Since rash onset occurred too late in mousepox to be used as an antiviral treatment trigger in tested IN infection models, we evaluated the utility of other clinical signs and biomarkers. Following an IN infection of C57BL/6 and A/Ncr mice, significant weight loss is observed by day 6 p.i. (Parker et al., 2008b), with signs such as ruffled fur, conjunctivitis, or lethargy by day 7 (data not shown). We also assessed a series of other biomarkers. Body temperature, as measured by telemetry, did not provide reliable data until late in disease when mice became moribund (data not shown). In the blood of infected A/Ncr mice, we observed neutrophilia starting at day 5 following an IN infection. Serum levels of IFN-γ, an indicator of an on-going immune response, and ALT, a sign of liver damage, were significantly elevated in these mice from day 2 and day 6–7 p.i., respectively. Additionally, blood vDNA could be detected from day 5 p.i. Levels of IFN-γ, ALT and vDNA in the blood, as well as neutrophilia, continued to increase until death (Parker et al., 2008b). In the C57BL/6 model we also found significant levels of IFN-γ from day 2 p.i., ALT from days 6 to 7 and vDNA from day 4 (Parker et al., 2009). In the SKH1 system we could detect IFN-γ and vDNA from at least day 4 and day 6, respectively and levels continued to increase until death (data not shown). Of these several candidate biomarkers for indication of infection early in the course of disease, vDNA from blood is the most robust biomarker because it can be used to specifically confirm orthopoxvirus infection down to the species level as early as day 4, which is several days prior to rash development (Li et al., 2005, 2007; Olson et al., 2004; Parker et al., 2008b).

In addition to blood, saliva would be a convenient fluid to detect vDNA early in infection as it could be collected in a less invasive manner than blood. Previously, PCR has been used in the detection of several different virus genomes in saliva; including HIV, dengue, CMV, influenza H1N1, and HHV-7; and in some cases PCR from saliva can be used as an early diagnostic test of infection (Balamane et al., 2010; Bilder et al., 2011; Boppana et al., 2011; Magalhaes Ide et al., 2011; Poloni et al., 2010). Therefore, we compared the time of detectable vDNA in blood with that of oropharyngeal secretions. As shown in Fig. 4, significant rises of vDNA were detected in the oropharyngeal secretions from day 2 p.i. and in the blood from day 4 p.i. compared to the NI controls.

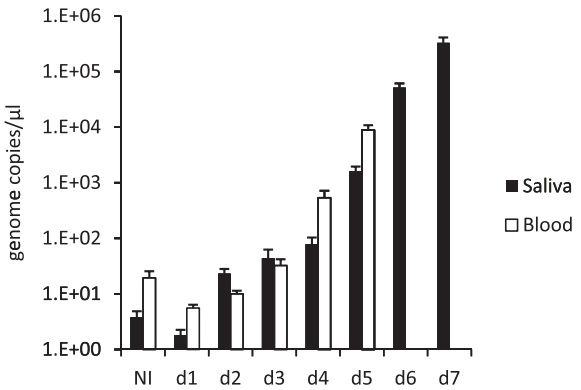

Fig. 4. C57BL/6 mice were infected by the IN route with 450 ($4.5 \times LD_{50}$) PFU of ECTV and oropharyngeal secretions and blood were sampled for viral DNA using qPCR. Saliva was sampled on days 1–7 p.i. Blood samples were taken on days 0–5 p.i. NI indicates non-infected controls. This is a representative experiment out of a total of 2. $N = 5$ animals per group.

4. Discussion

CMX001 and ST-246 are being developed as therapeutics for the treatment of orthopoxvirus infections including VARV, the causative agent of smallpox. Since VARV no longer circulates in human populations, the path to licensure for these antiviral agents is governed under the FDA Animal Rule (CFR, 2011; FDA, 2009). There are at least two critical issues with the use of animal efficacy data for licensure. First, it is challenging to determine an efficacious dose for a drug in humans based on pharmacokinetic/pharmacodynamic data determined experimentally in other species. Second, and the focus of this study, the pathophysiology in the experimental animal model may not completely recapitulate human disease making it difficult to directly compare the stages of disease. Mousepox is arguably the best respiratory challenge rodent model for smallpox. Like smallpox, mousepox requires only a low virus inoculum in the upper respiratory tract to initiate severe, systemic disease; there is no obvious pulmonary involvement during the course of early disease; virus can be detected in respiratory gases during the pre-exanthem period; and disease presents with a characteristic rash, although mousepox rash development is dependent on a number of parameters including mouse strain, virus strain, route of inoculation, and virus dose (Buller and Fenner, 2007; Parker et al., 2010). Unlike typical disease in smallpox, mousepox presents with major pathology in the liver and spleen; additionally, the disease course is shorter with death occurring 7–12 days p.i., depending on virus dose and mouse strain, whereas deaths in smallpox occur approximately 23–28 days p.i. (Fenner et al., 1988). These disease differences add uncertainty to selection of the appropriate trigger for therapeutic intervention in mousepox, and the application of the measured therapeutic window for therapeutic intervention between mousepox and smallpox.

Draft FDA guidance on the selection of the trigger for therapeutic intervention in animal models under the Animal Rule supports the consideration of a "disease-defining manifestation" such as rash in the case of smallpox and/or a "biological parameter/biomarker" (FDA, 2009). Except for rash and virus infectivity, there were very few disease-defining manifestations or biomarkers that were historically measured in smallpox patients and subsequently correlated with disease to allow their potential use as triggers for intervention in animal models. In ordinary smallpox, fever onset was 12–14 days after infection with rash occurring at ~16 days p.i. (Fenner et al., 1988). Virus was routinely detectable in oropharyngeal secretions a day or so prior to onset of rash (Sarkar et al., 1973). Furthermore, VARV was detected in oropharyngeal secretions from 34 of 328 asymptomatic household contacts of cases of smallpox even earlier in the eclipse or incubation period of the disease, and four of the subjects with positive throat swabs developed fever and rash 5–6 days and 7–8 days, respectively, after virus detection (Fenner et al., 1988; Sarkar et al., 1974, 1973). These data suggest that rash and infectivity or vDNA in oropharyngeal secretions could be used to stage disease in animal models, and act as a likely trigger for therapy.

Rash was evaluated in C57BL/6 and SKH1 mice following IN infections. Although C57BL/6 mice purchased from two different sources failed to present consistently with a classic rash under tested conditions, rash onset was observed in similar infections of the SKH1 mice at 7–9 days p.i. with the detection of papules

containing active virus replication as indicated by the presence of A-type inclusion bodies. Other experiments, using only clinical observations, noted rash onset at ~9 days p.i. Though we were able to detect rash in SKH1 mice, it was outside the therapeutic window for CMX001 (ST-246 not tested), which did not provide significant protection from lethal ECTV infection when administered at 6 days p.i. CMX001 and ST-246 had similar therapeutic windows in the C57BL/6 model; however, the use of CMX001 or ST-246 in combination in the SKH1 mousepox model might allow for successful therapeutic intervention after rash onset, since CMX001 (a DNA replication/transcription inhibitor) and ST-246 (a viral egress inhibitor) have completely different mechanisms of action (Parker et al., 2008a). Support for synergy of combination treatment to extend the therapeutic window comes from two studies. Following cowpox virus IN challenge of BALB/c mice, synergistic efficacy of ST-246 and CMX001 was observed at 6 days p.i., whereas monotherapy with ST-246 or CMX001 only provided protection at day 3 p.i. (Quenelle et al., 2007b). Therefore, the window for therapeutic intervention was doubled. If similar outcomes occurred in the ECTV models with dual-therapy, one could predict that the therapeutic window would overlap with the initiation of rash. Secondly, combined treatment of ST-246 and CMX001 was also able to protect A/NCR mice from lethal infection with a highly virulent ECTV recombinant expressing murine IL-4, when single treatments were ineffective (Chen et al., 2011).

Rash development can also be variable in rabbitpox virus (RPXV) and MPXV respiratory animal models. MPXV intranasal infections of dormice result in death from day 8 to 12, a LD_{50} of 12 PFU, and lack of detectable rash over a range of virus doses (Schultz et al., 2009). Rash onset in the RPXV respiratory challenge models is dose-dependent with low dose infections having a prolonged disease course with the development of skin lesions, whereas higher dose infections have a shortened disease course and present with fewer or no lesions (Lancaster et al., 1966; Westwood et al., 1966). In the intravenous and intratracheal monkey (*Macaca fascicularis*) model the opposite is seen; namely, lesions occur earlier at higher doses and later at lower doses; however, in the intrabronchial and aerosol models, lesions appear at approximately the same time regardless of challenge dose (Johnson et al., 2011; Nalca et al., 2010; Stittelaar et al., 2005). Rabbits infected with an aerosol containing a low-dose (<200 PFU) inoculum of RPXV develop highly variable numbers of skin and mucosal lesions around days 9–10 p.i. concomitant with mortality (8–14 p.i.). At higher doses (>200 PFU), aerosolized RPXV produces an overwhelmingly lethal infection with rapid disease progression and death by day 6–7 p.i. If skin lesions occur, they are typically present on the lips, eyelids and/or areas that were shaved before infection at around days 5–7 (Chapman et al., 2010; Garza et al., 2009; Roy and Voss, 2010).

In addition to our study, to our knowledge there are only two published studies where onset of rash can be related to the efficacy of an antiviral treatment in a respiratory challenge animal model. Using the RPXV model, daily administration of 40 mg/kg of ST-246 to rabbits infected with an aerosol dose of 2000 PFU of virus had survival rates of 80–100% when treated at days 0, 1 or 2, but groups treated from day 3 and 4 p.i. had survival rates of 67% and 33%, respectively (Nalca et al., 2008). In control groups, facial and cervical edema was observed at day 3, vDNA could be detected in the blood from day 4 p.i., rash onset occurred at day 5, and 100% mortality or euthanasia took place on day 6 p.i. Using a MPXV model, daily administration of 30 mg/kg of ST-246 to prairie dogs infected with an IN dose of 3×10^5 PFU ($65 \times LD_{50}$) had survival rates of 100% when treated at days 0, 3 or the time of rash onset (day 10) (Smith et al., 2011). In control groups, vDNA was detected in the blood and oral swabs from days 4 to 6, clinical signs were observed beginning on day 8, and 75% mortality occurred at days 10–12 p.i. Of the mousepox, rabbitpox and monkeypox respiratory challenge models used to evaluate ST-246, treatment at the time of rash onset was protective from death only in the monkeypox/prairie dog model. Additional studies will need to be carried out to determine if this result is reproducible as the study group sizes were small at four animals per group, and the animals were wild-caught which may increase experimental variability.

During the smallpox eradication program, onset of rash did not directly lead to a diagnosis of smallpox as even the most experienced clinicians had difficulty identifying cases of smallpox early in the course of disease. As stated in Fenner: "Even countries with an adequate surveillance system, cases were rarely detected and diagnosed until a week or more after (rash) onset" (Fenner et al., 1988). The last recorded case of smallpox, which occurred in Somalia in 1977, is an example of the difficulty in clinical diagnosis. The patient, Mr. Maalin, was admitted to the hospital with a presumptive diagnosis of malaria; on rash onset the diagnosis was changed to chickenpox; and finally 2 days later he self-diagnosed himself with smallpox. Taken together, laboratory and epidemiology findings suggest onset of rash in smallpox may be too late to be a trigger for efficacious antiviral treatment.

Although the pathophysiology of rash development following orthopoxvirus respiratory infection is not well understood, contributing factors are likely route of infection, the level of virus replication in the skin, the responsiveness of the skin-associated lymphoid tissue, and the structure and physiology of the skin itself. This complexity likely plays a part in the variation of rash onset and character in the various orthopoxvirus respiratory models, and confounds its use as a trigger for antiviral treatment. Although ECTV IN infected SKH1 and C57BL/6 strains have a similar 4 day onset of detectable vDNA in blood (data not shown), mean time to death of 10–12 days and an LD_{50} of ~100 PFU, SKH1mice develop a rash and the C57BL/6 mice do not. In this and other orthopoxvirus respiratory challenge models, rash does not appear to be a good indicator of the stage of the disease process, and therefore is not the optimal choice as a trigger for antiviral treatment. Furthermore, we found that the LD_{50} in the SKH1 strain was moderately variable and this is reflected in the mortality rates at the 650, 2050 and 6500 PFU inoculum experiments. These findings, as well as the delayed rash, suggest that the SKH1 strain may not be a suitable model. In ECTV-infected C57BL/6 mice, vDNA was detectable in oropharyngeal secretions from day 2 p.i., and in blood from day 4 p.i., both times are well within the therapeutic window for ST-246 or CMX001.

Detection of vDNA in oropharyngeal secretions during the eclipse period likely represents virus replication from the primary site of infection prior to virus replication in internal organs such as spleen liver and kidney, and as such is an early marker of virus infection. Detection of vDNA in blood represents secondary viremia, a later stage in the replication cycle and results in seeding of the cornified and mucosal epithelium – a prerequisite for rash. Similar to the examined mousepox models, vDNA is detected in both oropharyngeal secretions and blood in MPXV-IN-infected prairie dog from days 4 to 6 (Smith et al., 2011). In MPXV aerosol-infected monkeys (*M. fascicularis*), vDNA can be detected in both oropharyngeal secretions and blood from days 4 (Nalca et al., 2010); in MPXV-intrabronchiol-infected *M. fascicularis* from days 7 and 3, respectively (Johnson et al., 2011); and, in RPXV-IN-infected rabbits from days 4 in the blood (Nalca et al., 2008). vDNA in oropharyngeal secretions is a consistent early marker of virus replication in all examined orthopoxvirus respiratory challenge models.

The use of vDNA in oropharyngeal secretions as a trigger for antiviral treatment would facilitate better comparisons of therapeutic efficacy studies among orthopoxvirus animal models, and permit the prediction of treatment responses in smallpox as VARV

is detected in oropharyngeal secretions of smallpox patients early during the eclipse period (Sarkar et al., 1974). In addition, the detection of vDNA in oropharyngeal secretions would be an excellent biomarker to initiate antiviral treatment in smallpox. Treatment initiated with the detection of vDNA rather than rash would likely result in earlier therapeutic intervention, translating into greater treatment efficacy. Also, the DNA detection assay would provide a high degree of certainty that the disease was indeed poxvirual in its etiopathogenesis (i.e. smallpox or monkeypox), and not some other clinical rash causing disease, such as chickenpox. For example, in 1976 in India shortly after smallpox eradication, 63% of suspected "smallpox cases" were actually cases of chickenpox (Fenner et al., 1988). Similarly, in some cases in the Congo basin, upward of 50% of suspected monkeypox cases have been attributable to chickenpox infections (MacNeil et al., 2009; Meyer et al., 2002; Rimoin et al., 2007). Mitigating against the use of a DNA signature as a treatment trigger for smallpox is the current lack of assay availability at the point-of-care, a deficiency that will be remediated in time (Niemz et al., 2011).

5. Conclusions

Onset of rash is not an effective trigger for antiviral therapeutic intervention in the C57BL/6 and SKH1 mousepox respiratory (IN) challenge models. vDNA in blood samples and/or in saliva is the biomarker of choice to trigger initiation of antiviral therapy in orthopoxvirus models such as the mousepox model that lacks rash or has rash onset outside of the therapeutic window of the test antiviral. Also PCR can be used to provide definitive diagnosis of infection with a particular virus (orthopoxvirus or otherwise). The administration of ST-246 and CMX001 therapeutic treatments to IN-infected C57BL/6 mice was equally efficacious until 6 days p.i.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2012.02.005.

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