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Original Article

Inactivated Orf-virus shows disease modifying antiviral activity in a guinea pig model of genital herpesvirus infection



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Abstract *Background:* Inactivated Orf virus (iORFV) has been used as a preventative as well as a therapeutic immunomodulator in veterinary medicine in different species. iORFV elicits strong effects on cytokine secretion in mice and human immune cells leading to an auto-regulated loop of initial up-regulation of inflammatory and Th1-related cytokines followed by Th2-related cytokines that attenuate immunopathology. The therapeutic potential of iORFV has been recognized in several models for difficult-to-treat disease areas such as chronic viral diseases, liver fibrosis or various forms of cancer.

Methods: Guinea pigs were infected with Human Herpesvirus (HSV)-2 strain MS and treated with iORFV, Acyclovir (ACV) or placebo, respectively. Clinical score of herpes lesions and viral shedding was assessed over a period of 40 days. In addition, viral DNA in dorsal root ganglia was quantified at the end of the study.

Results: Disease symptoms were minimal or absent in iORFV-treated guinea pigs but tended to be severe in animals treated with either ACV or placebo. The cumulated disease score was significantly reduced in iORFV-treated but not in ACV- or placebo-treated guinea pigs. In addition, treatment with iORFV, but not ACV or placebo, led to significant reduction of viral DNA load in dorsal root ganglia.

Conclusion: iORFV effectively suppressed recurrences in guinea pigs experimentally infected with HSV. iORFV did not only reduce recurrent disease episodes but was, compared with ACV, more effective in reducing latency as measured by viral DNA detected in dorsal root ganglia of infected animals.

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Introduction

Orf virus (ORFV) is an epitheliotropic DNA virus that belongs to the genus *Parapoxvirus* of the *Poxviridae* family.¹ ORFV causes *orf*, an acute skin disease of sheep and goats worldwide.²

Inactivated Orf virus (iORFV) has been introduced as a preventative as well as therapeutic immunomodulator in veterinary medicine in different species.^{3–7}

The therapeutic potential of iORFV has been recognized in various models of difficult-to-treat diseases such as hepatitis B virus infection, liver fibrosis or various forms of cancer.^{10,11} An iORFV-based agent is currently in clinical phase testing in patients infected with human hepatitis B virus (HBV).¹²

iORFV has strong effects on cytokine secretion in mice and human immune cells leading to an auto-regulated loop of initial up-regulation of inflammatory and Th1-related cytokines followed by Th2-related cytokines that attenuate immunopathology.^{8,9}

Moreover, iORFV elicited significant antiviral activity in mice challenged with human herpesvirus 1 (HSV-1). Neutralization of iORFV-induced IFN γ using monoclonal anti-IFN- γ antibodies abolished iORFV-mediated activity.

Here, we aimed to study iORFV-mediated antiviral activity in an animal model with a higher relevance in terms of clinical aspects compared to an acute virus challenge model in mice. We choose the guinea pig model of genital herpesvirus infection. Genital herpes is a very common sexually transmitted diseases problem globally with estimated 417 million people between the ages of 15 and 49 being infected in 2012.¹³

While current practice to use nucleoside analogs as an anti-Herpes therapy impacts recurrent disease, these compounds do not prevent viral shedding.^{14,15} Therefore, there remains a significant need to further improve antiviral therapy for genital herpes disease.

Immunotherapeutic vaccines have been described as emerging potential novel treatment options for genital herpes. The approaches are currently at clinical and pre-clinical stages.¹⁶

With this study, we provide evidence that iORFV effectively suppresses recurrences in guinea pigs experimentally infected with HSV-2. In addition, iORFV was, compared with acyclovir (ACV), more effective in reducing latency as measured by viral DNA detected in dorsal root ganglia of infected animals.

Further studies are required to unravel the details involved in this potential therapeutic principle.

Material and methods

Virus

Orf virus, strain D1701, was propagated in bovine kidney cells (MDBK) essentially as described previously.⁸ The virus was purified through a sucrose gradient and inactivated using binary ethylenimine (BEI).

Guinea pig model of genital herpes

Female Dunkin Hartley guinea pigs (Charles River Wiga, Sulzfeld, Germany) were infected intravaginally with

2.5×10^5 PFU HSV-2 strain MS. The animals were observed for clinical disease and the clinical symptoms were scored: 0: no lesion; 1: erythema; 2: vesicles; 3: confluent lesions; 4: necrotizing vulvovaginitis. All animals with acute infection (score 3) were randomized, divided into three groups ($n = 10$ animals/group) and treated with iORFV, Acyclovir (ACV) or placebo, respectively, starting approximately 10 days after healing of the primary disease (score 0). iORFV (1×10^6 TCID₅₀) was administered intraperitoneally (i.p.) every third day, 5 times in total. Acyclovir (ACV, 9-(2-Hydroxyethoxymethyl)guanine, Glaxo Wellcome, RTI, NC) was administered daily for 14 consecutive days i.p. After completion of the treatment, animals were examined daily until the end of the experiment (day 80) for herpes lesions and the lesion severity was scored on a 0–4 scale. Wet swabs were used to harvest vaginal secretions every fourth day starting after therapy. Swabs were placed in tubes with 1 ml Dulbecco's MEM and processed essentially as described.¹⁷ Virus titers were determined using standard plaque test procedures.¹⁷

Preparation of dorsal root ganglia, DNA isolation and real time quantitative PCR

At the end of the experiment on day 80, animals were sacrificed using T61 (Hoechst, Frankfurt, Germany) injection under deep anesthesia with pentobarbital-sodium (Narcoren, Rhone-Merieux [Merial], Hallbergmoos, Germany). Six sacral dorsal root ganglia (sDRG) of each animal were extracted and pooled. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen No. 51304; Qiagen, Hilden, Germany) following the instructions of the manufacturer. DNA was stored at -20°C until use.

Real time quantitative PCR was performed using the TaqMan[®] PCR Core Reagent kit (PE Applied Biosystems No. 402930) with standard cycling conditions (45 cycles consisting of 15 s at 95°C and 1.5 min cycle at 60°C) as single reporter assays essentially as described previously.¹⁷ Purified HSV-DNA as well as DNA isolated from non-infected guinea pigs served as standards. Results were analyzed with the standard curve method and given as copies of HSVDNA/ng of guinea pig DNA.

The primers gp-tq-1 (gtgttctggaagccaactgct) and gp-tq-2 (caccagtcaagaggtcccacc), and the probe (6-carboxyfluorescein-cacggagccatgatgcgtggct-N,N,N',N'-tetramethyl-6-carboxyrhodamine) were specific for guinea pig DNA binding to sequences encoding the neutrophil cationic peptide 2 (Genbank Acc. No. D379774).

Primers HSVgHtq-1 (ccgcttcggctcaatctc) and HSVgHtq-2 (tttatcacctatctcaccgcca), and the probe (6-carboxyfluorescein-cgcgctgccccttcgca-N,N,N',N'-tetramethyl-6-carboxyrhodamine) recognize HSV-2 glycoprotein H sequences. Primers were used at 300 nM and probes at 200 nM in a 50 μl reaction volume.

Statistics

Data were evaluated for statistical significance using one-way analysis of variance (ANOVA) with Bonferroni's post-test. Student's *t*-test was used for confirmation. Tests were

performed using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA, USA).

Results

iORFV-treatment has preventative effects on recurrent genital herpes

We used a guinea pig model of recurrent genital herpes disease. The disease symptoms during periods of recurrence were minimal or absent in iORFV-treated guinea pigs (score 1, Fig. 1a) but tended to be severe in placebo or animals

treated with ACV (score 3, Fig. 1c). When cumulated over the observation period, the score of disease symptoms was significantly reduced ($p < 0.01$, t -test) in iORFV-treated but not in ACV-treated guinea pigs (Fig. 1d). During the 80-day follow-up period, no animal developed clinical disease score 4 (necrotizing vulvovaginitis). Clinical episodes of severity grade 3 (confluent lesions) were observed in the placebo- or ACV-treatment groups but not in the group of guinea pigs that received iORFV (Table 1). In addition, in the iORFV-treated group 2/10 animals did not develop any signs of recurrent disease whereas all animals in either the placebo or ACV treatment groups developed at least mild recurrent disease characterized by erythema and vesicles.

Table 1 HSV-2-infected guinea pigs with acute infection were randomized, divided into three groups ($n = 10$ animals/group) and treated with iORFV, Acyclovir (ACV) or placebo, respectively, starting approximately 10 days after healing of the primary disease. After completion of the treatment, animals were examined daily for 80 days for herpes lesions and the lesion severity was scored: 0: no lesion; 1: erythema; 2: vesicles; 3: confluent lesions; 4: necrotizing vulvovaginitis.

Treatment group	Number of animals with no clinical signs	Number of animals with episodes \geq score 3	Number of clinical episodes \geq score 3
iORFV	2/10	0/10	—
Placebo	0/10	4/10	6
ACV	0/10	3/10	10

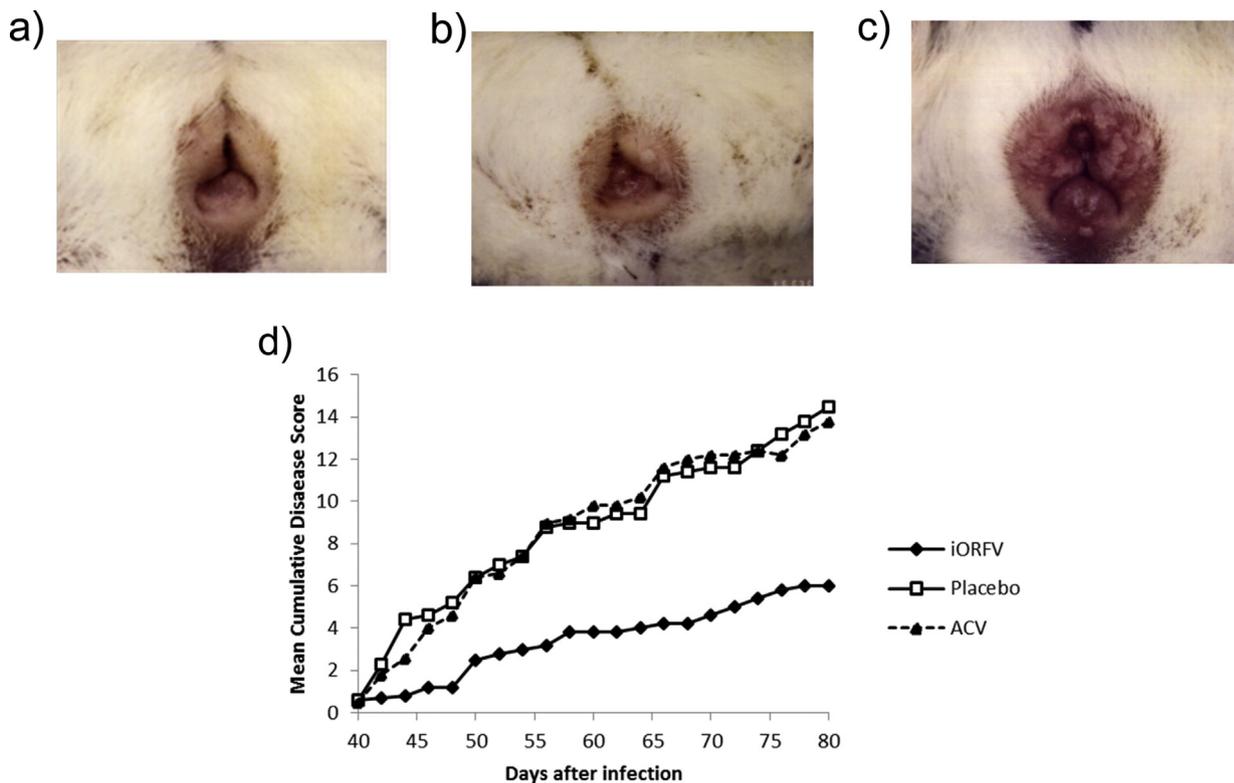


Figure 1. Guinea pigs were infected intravaginally with HSV-2. Pictures show various scores of clinical herpes disease: a: score 1; b: score 2; c: score 3. Animals with acute infection (score 3) were randomized. Treatment with iORFV, acyclovir (ACV), or placebo started 10 days after healing of the primary disease (score 0) ($n = 10$ animals/group). iORFV was administered every third day intraperitoneally (i.p.), five times in total. Placebo and ACV were administered daily for 14 consecutive days i.p. After completion of the treatment cycle, animals were examined daily for 40 consecutive days for herpes lesions and the lesion severity was scored (d).

In addition, the number of days with lesions was reduced by more than 50% in animals treated with iORFV when compared to animals treated with placebo or ACV, respectively. This observed effect was statistically significant vs. placebo-treated as well as ACV-treated animals ($p < 0.05$, t -test) (Fig. 2).

iORFV-treatment reduces viral shedding

Importantly, recurrent viral shedding as measured by percent of days where virus was detected in vaginal swabs was significantly lower in animals treated with iORFV compared to animals treated with ACV or placebo, respectively ($p < 0.01$, t -test). Virus was detected in 1.7% of vaginal swabs obtained from iORFV-treated animals whereas 26.8% of swabs from guinea pigs treated with ACV and 35.8% from placebo-treated animals were positive for HSV-2. The difference observed between ACV-treated and placebo-treated groups was not statistically significant in this experiment. Results are shown in Fig. 3.

iORFV reduces viral load in dorsal root ganglia

Similar to the findings for recurrent lesion days and clinical scores, iORFV-treatment reduced latent viral load of dorsal root ganglia (DRG). Animals were sacrificed at the end of the experiment on day 80 and DRG were analyzed for HSV-specific DNA. Viral DNA in DRG of guinea pigs treated with iORFV ($1.5 \times 10^3 \pm 1.4 \times 10^3$) was significantly reduced vs. placebo-treated animals ($6.3 \times 10^3 \pm 6.5 \times 10^3$) ($p < 0.05$, t -test) as well as animals treated with ACV ($8.3 \times 10^4 \pm 1.1 \times 10^5$) ($p < 0.05$, t -test) (Fig. 4).

Discussion

Herpes simplex virus 2 (HSV-2) is transmitted through sexual contact and causes persistent infection that may result in frequent recurrent symptomatic genital ulcers. After primary infection, persistent infection is being established in the sacral ganglia. When reactivated, the virus follows the axon to the skin or mucosa and viral shedding may either result in recurrent genital disease or is asymptomatic. Specifically, latency of the virus in the ganglia of infected individuals is an unsolved issue. Despite the progress that was achieved with new therapies for viral diseases caused by hepatitis C virus (HCV) or human immunodeficiency virus (HIV) the situation for herpes therapies did not change. Hence, there remains a significant need to improve the standards of antiviral treatment for recurrent HSV disease, or – ideally – prevent recurrent disease. Available medicines like nucleoside analogs have been demonstrated some potential in reducing recurrent disease but did not promise control over viral shedding.^{13–15,18} Recently, N-methanocarbathymidine (N-MCT) was effective as therapy for acute and recurrent genital HSV-2 disease in the guinea pig model of HSV-2 infection and decreased acute vaginal virus shedding more effectively than acyclovir.¹⁹ However, N-MCT was not as effective as acyclovir in reducing the number of days with recurrent virus shedding. Augmentation of local herpes-specific immune responses is seen as a critical aspect to immunotherapy.¹⁶ The concept of IFN- γ -mediated clearance of viral infections from the central nervous system was established previously²⁰ although some questions about the role of the immune system in controlling recurrent disease remain unclear. iORFV has been described as a powerful inducer of IFN- γ in various

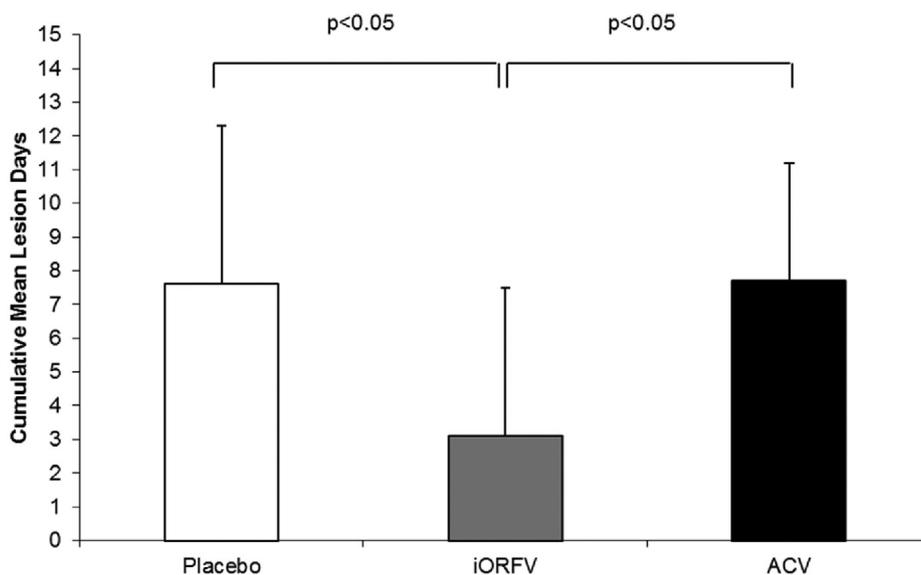


Figure 2. iORFV treated animals experienced 3.3 ± 4.4 days with lesions during the observation time whereas animals receiving a placebo had 7.6 ± 4.7 days with clinical lesions. Guinea pigs treated previously with ACV experienced mean 7.7 ± 3.5 days with lesions. The reduction observed in the iORFV group was statistically significant vs. the groups of guinea pigs treated with either placebo or ACV, respectively ($p < 0.05$, t -test).

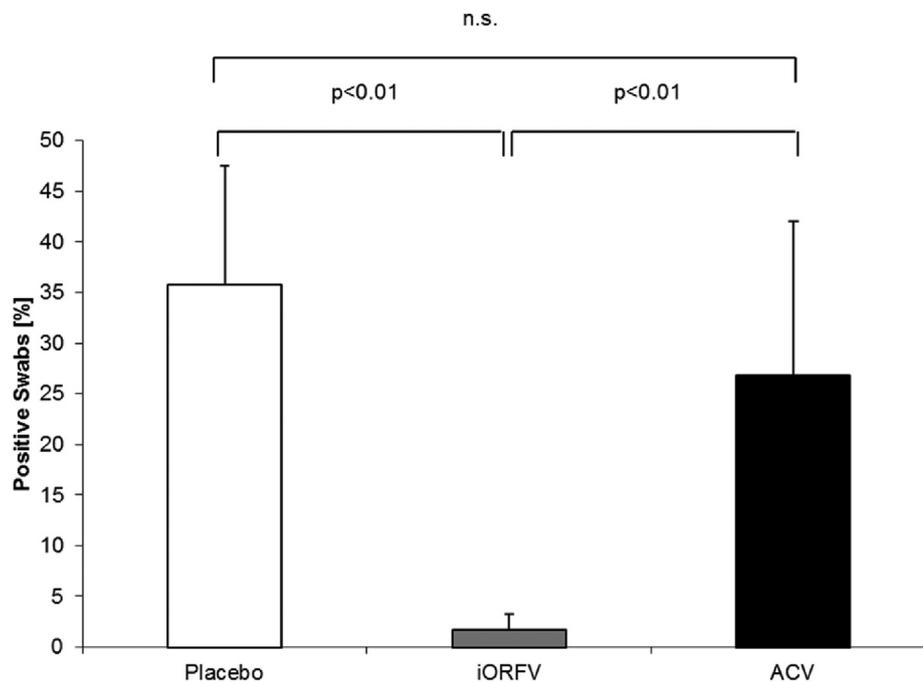


Figure 3. Recurrent viral shedding as measured by percent of days where virus was detected in vaginal swabs was significantly lower in animals treated with iORFV when compared to those obtained from animals treated with ACV or placebo, respectively ($p < 0.01$, t -test).

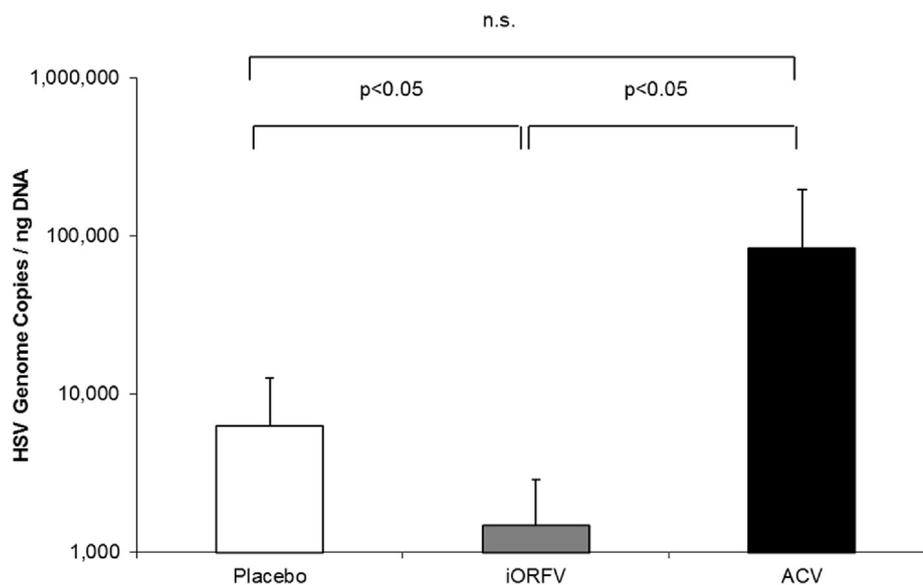


Figure 4. On day 80 after infection, HSV-2 infected guinea pigs treated either with iORFV, ACV or placebo were sacrificed and dorsal root ganglia were analyzed for HSV-2 DNA by real time quantitative PCR. Viral DNA in DRG of guinea pigs treated with iORFV ($1.5 \times 10^3 \pm 1.4 \times 10^3$) was significantly reduced vs. placebo-treated animals ($6.3 \times 10^3 \pm 6.5 \times 10^3$) ($p < 0.05$, t -test) as well as animals treated with ACV ($8.3 \times 10^4 \pm 1.1 \times 10^5$) ($p < 0.05$, t -test). Figure shows mean data from $n = 8$ animals/group. The difference between placebo and ACV-treatment groups was not significant.

species.¹⁰ In addition, activity of iORFV was found in various animal models of viral infections or viral disease including models of human herpesvirus disease.⁸ In this study, we analyzed the effects of iORFV-treatment on herpesvirus-mediated disease in greater detail in the guinea pig model of genital recurrent herpes disease. Specifically, we aimed to explore whether the immunomodulatory effects

of iORFV would prevent or modulate recurrent disease. Therefore, we initiated treatment after primary disease symptoms had. ACV treatment was not effective in modifying recurrent herpes disease. This is consistent with other studies that did not demonstrate therapeutic effects on herpes disease in the guinea pig even when ACV was given 72 h or 96 h post infection.^{21,22} Again in other studies, a

minor or late reduction of mean lesion scores was seen after acyclovir treatment was started 72 h p.i.^{23,24}

However, in our study, treatment with iORFV showed significant antiviral activity that led to a reduction of recurrent herpes disease. Our results suggest a correlation of reduction of ganglionic HSV-specific DNA and the clinical symptoms over time. The inhibitory effect on viral shedding in iORFV-treated animals is consistent with the other findings. We hypothesize that reduction of HSV in vaginal swabs is due to IFN- γ -mediated direct antiviral activity as demonstrated in mice.⁸

The HSV-2 specific IgG titer was slightly and non-significantly decreased in blood of iORFV-treated vs. placebo- or ACV-treated animals (not shown). Further studies are required to unravel the details involved in this potential therapeutic principle.

Conflict of interest

OW is an employee of Bayer AG, AS has been an employee of Bayer HealthCare AG, AF declares no conflicts of interest.

Acknowledgments

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