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Inactivated parapoxvirus ovis (*Orf virus*) has antiviral activity against hepatitis B virus and herpes simplex virus

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It is known that some viruses are able to induce vigorous immune reactions. This study shows that inactivated parapoxvirus ovis (*Orf virus*), strain D1701 (PPVO), induces an autoregulatory cytokine response that involves the upregulation of IL-12, IL-18, IFN- γ and other T helper 1-type cytokines and their subsequent downregulation, which is accompanied by induction of IL-4. An increase in IL-10 expression was also found in the livers of PPVO-treated mice. PPVO protects mice from lethal herpes simplex virus type 1 infection and guinea pigs from recurrent genital herpes disease. With dosages as low as 500 000 virus particles, PPVO is more potent than the current standard 3TC therapy in hepatitis B virus transgenic mice. No signs of inflammation or any other side effects were observed. PPVO induces IL-12, TNF- α and, together with a suboptimal concentration of Concanavalin A, IFN- γ in human peripheral blood leukocytes as well. The principle of an autoregulatory cytokine induction by an inactivated virus might have advantages over existing immune therapies and it is concluded that inactivated PPVO should be investigated further for its potential use in antiviral therapy.

INTRODUCTION

Viruses can manipulate the immune system by bypassing or suppressing an immune reaction or by activation of the immune system (Lane *et al.*, 1985; Mocarski, 2002; del Val *et al.*, 1992; Mossman, 2002; Grandvaux *et al.*, 2002). Parapoxvirus ovis (PPVO or *Orf virus*), a member of the family *Poxviridae*, causes an acute skin disease of sheep and goats worldwide and may infect humans (Haig & Mercer, 1998). The virus can infect its host repeatedly, in spite of a vigorous inflammatory host immune response, and neutralizing antibodies have not been described (Haig & Mercer, 1998; Haig & McInnes, 2002). A number of efficient immune escape mechanisms have been proposed or described for PPVO (Haig & Mercer, 1998; Haig & McInnes, 2002; McKeever *et al.*, 1988; Haig *et al.*, 1996, 1997; Kruse & Weber, 2001). Like other viruses, PPVO is able to stimulate the innate immune system. PPVO induces phagocytosis, NK cell activity and release of IFN- α , TNF- α , IL-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Buettner *et al.*, 1995; Foerster *et al.*, 1994; Marsig & Stickl, 1988; Mayr *et al.*, 1989). Gene products with immune-modulating functions that have been identified in PPVO include virus orthologues of IL-10 (Fleming *et al.*, 1997) and the vaccinia E3L gene encoding an interferon-resistance factor (Haig *et al.*, 1998). Recently, proteins that bind and inhibit GM-CSF and IL-2 have been described (Dean *et al.*, 2000). The combination of immune escape mechanisms and immune stimulatory activity is a very effective survival strategy for PPVO.

Virus infections may modulate the clinical course of concomitant infections by other pathogens. It was demonstrated previously that virus infections of the liver abolish hepatocellular replication of human hepatitis B virus (HBV) in a non-cytolytic fashion mediated by inflammatory cytokines (Guidotti *et al.*, 1996; Cavanaugh *et al.*, 1998). Based on this observation, it was postulated that virus clearance during human HBV infection is due primarily to this process rather than the destruction of infected cells (Guidotti *et al.*, 1996, 1999). This paradigm shift in virus immunology may impact on future antiviral strategies. We wanted to utilize the paradigm for an immune-therapeutic approach using PPVO to combine immune evasion and immune stimulatory mechanisms. In this report, we demonstrate that inactivated PPVO induces a self-regulatory cytokine response that involves the upregulation of IL-12, IL-18, IFN- γ and other T helper (Th) 1-type cytokines and their subsequent downregulation. Furthermore, we show that PPVO is effective in mice infected with herpes simplex virus type 1 (HSV-1), in a guinea pig model of recurrent genital herpes disease and in a transgenic mouse model of human HBV replication, without any signs of inflammation or other side effects. We conclude that induction of a self-regulatory cytokine response by an inactivated virus might have some advantages over existing immune therapies and that inactivated PPVO should be investigated in detail as a potential antiviral drug.

METHODS

Virus. PPVO strain D1701 was propagated in MDBK cells as described previously (Kruse & Weber, 2001). Briefly, cells were cultured in EMEM with Earle's salt, supplemented with 1 % penicillin/streptomycin (10 000 U penicillin and 10 mg streptomycin ml⁻¹ in physiological saline), 1 % L-glutamine (200 mM), 1 % non-essential amino acids and 10 % (v/v) heat-inactivated FCS. All reagents were from Life Technologies. When the cells were 80–90 % confluent, they were infected with D1701 and incubated at 37 °C in 5 % CO₂ for 7 days. Material was harvested when approximately 80–90 % of the cells showed a cytopathic effect. After the removal of cell debris, the virus was purified from the supernatant through a sucrose gradient. The supernatant was used as a mock control. In some experiments, we have treated mock-infected MDBK cells, essentially as described above, and used the pellet fraction that co-purified in the fraction of the sucrose gradient where the virus was recovered. PPVO was quantified in a plaque assay on MDBK cells and inactivated using binary ethylenimine (Bahnemann, 1990). The success of inactivation was confirmed by plaque titration on MDBK cells.

Cytokine mRNA measurement. Female BALB/cJ mice (4 weeks old, approximately 20 g body weight) were purchased from a commercial supplier (Bomholdtgard). Mice were divided into three treatment groups ($n=20$ animals per group): (i) PPVO strain D1701 (5×10^5 TCID₅₀), diluted in 200 µl non-pyrogenic PBS (Seromed); (ii) complete Freund's adjuvant (CFA) (Sigma); and (iii) non-pyrogenic PBS (placebo). At 6, 12, 24 and 48 h after treatment, five mice were sacrificed and peritoneal cells, liver, axillar, gastric/epigastric/mesenteric lymph nodes and spleen were collected. Total RNA was prepared and cytokine gene expression was quantified using a competitive RT-PCR, as described previously (Siegling *et al.*, 1994). Primer sequences used for competitive RT-PCR are provided in Table 1. PCR products were subjected to agarose (1 %) gel electrophoresis and quantified using a video imaging system (Herolab) with the appropriate software.

Table 1. Primer sequences used for competitive RT-PCR

Primer	Sequence (5'→3')	
	Sense	Antisense
β-actin	TGGAATCCTGTGGCATCCATGAAAC	TAAAACGCAGCTCAGTAACAGTCCG
IFN-γ	AGCGGCTGACTGAACTCAGATTGTAG	GTCACAGTTTTCAGCTGTATAGGG
TNF-α	GGCAGGTCTACTTTGGAGTCATTGC	ACATTCGAGGCTCCAGTGAATTCGG
IL-4	CGAAGAACACCACAGAGAGTGAGCT	GACTCATTTCATGGTGCAGCTTATCG
IL-10	TGCCTTCAGCCAGGTGAAGACT	AAATTCATTTCATGGCCTTGTA
IL-12 p35	AACAAGAGGGAGCTGCCTGCC	CGGGTGCTGAAGGCGTGAAGC
IL-12 p40	TGGACTCCTGATGCCCCTGGA	TGCTTCACACTTCAGGAAAGT
IL-18	CCATGGCTGCCATGTCAGAAGACTCTT	CCTAACTTTGATGTAAGTTAGTGAGAGTGA

Treatment of HSV-1-infected BALB/c mice. BALB/cJ mice were used ($n=10$ animals per group) and PPVO (5×10^5 TCID₅₀) was administered intraperitoneally (i.p.), subcutaneously (s.c.), intramuscularly (i.m.), intravenously or intranasally (i.n.) at 16 h prior to infection. The study included the following controls: PBS (Seromed); supernatant from the virus purification process as another negative control (mock); and CFA (Sigma). For infection, 50 μ l virus suspension (HSV-1 strain walki at 5×10^4 p.f.u.) was applied i.n. PPVO and placebo were administered 7 h prior to infection. Antibodies were administered 6 h after PPVO ($n=8$ animals per group) as follows: (i) PPVO (5×10^5 TCID₅₀); (ii) 0.2 ml PBS; (iii) PPVO+rat anti-mIFN- γ IgG1; (iv) PPVO+rat anti-mouse IgG1; (v) rat anti-mIFN- γ IgG1; and (vi) rat anti-mouse IgG1 (all antibodies from Biosource).

Guinea pig model of genital herpes. Female Hartley guinea pigs (Charles River Laboratories) were infected intravaginally with 2.5×10^5 p.f.u. HSV-2 strain MS. Clinical symptoms were scored as follows: 0, no lesion; 1, erythema; 2, vesicles; 3, confluent lesions; and 4, necrotizing vulvovaginitis. Animals with acute infection (score 3) were randomized and divided into three groups ($n=10$ animals per group). Treatment was started 10 days after healing of the disease (score 0). PPVO (1×10^6 TCID₅₀) was administered i.p. every third day, five times in total. Acyclovir (Glaxo Wellcome) was administered twice daily for 10 consecutive days i.p. at a dosage of 25 mg kg⁻¹ (per dose). Animals were examined daily for 40 consecutive days for herpes lesions; severity was scored on a scale of 0–4.

HBV transgenic mice. HBV transgenic mice [Tg (HBV1.3 fsX^{-3'5'})] that carry a frameshift mutation (GC) at position 2916/2917 (C. Kuhn & H. Schaller, unpublished; Weber *et al.*, 2002) were used ($n=16$; 8 male and 8 female animals per group). PPVO was administered i.p. every third day, three times in total, unless indicated otherwise. Frozen tissue (50 mg) was minced and digested with proteinase K (Roche) over night at 56 °C. Nucleic acids were extracted using the phenol/chloroform procedure. Southern blot analysis was performed using 20 μ g *Pst*I-restricted genomic DNA. Before electrophoresis, DNA was digested with RNase A (Qiagen). Quantitative analysis of hepadnaviral nucleic acid was performed essentially as described recently (Weber *et al.*, 2002).

Histological and immunohistological analyses. Analyses were performed as described recently (Weber *et al.*, 2002). Briefly, liver specimens from one or two lobes were fixed in 4 % formaldehyde solution overnight at room temperature and embedded in paraffin. For immunohistochemical analysis, a polyclonal rabbit antibody against the HBV capsid antigen (HBcAg) (Dako) was used. Staining was carried out using the Vectastain ABC kit, as described by the manufacturer (Vector Laboratories).

Studies with human peripheral blood leukocytes. Heperanized whole blood samples (100 μ l) were diluted 1:10 with endotoxin-free culture medium and cultivated at 37 °C for 4 (TNF, IL-12) or 48 (IFN- γ) h in the presence or absence of low dose Concanavalin A (ConA, 12 μ g ml⁻¹) (Sigma), phytohaemagglutinin (PHA, 1:5000) (Sigma), mAb OKT-3 (1 μ g ml⁻¹) and different concentrations of PPVO. Supernatants were collected and measured by commercially available ELISA kits (IFN- γ and IL-12 p70, Biosource; TNF- α , Immulite).

Statistical analysis. Cytokine expression profiles were analysed using the parameter-free Wilcoxon test. HBV DNA reduction was analysed using variance analysis with subsequent post-hoc comparison of means. Survival analyses were performed using log rank analysis (STATISTICA, StatSoft).

RESULTS

PPVO induces a complex and autoregulatory cytokine response

Only marginal levels of cytokine mRNA were detectable in peritoneal lavage cells of mice that were treated i.p. with placebo. Data for mice that were treated with PPVO or CFA as a control were normalized for these values, which were set as 1 (not shown in the graphs). Shortly after PPVO administration, but not after CFA treatment, we found a strong (50-fold) upregulation of cytokines, particularly of IFN- γ expression (Fig. 1A). The PPVO-induced expression profile for TNF- α and IL-12 p40 (and IL-12 p35, data not shown) was not different from that observed in CFA-treated mice for the first 24 h after administration (Fig. 1B, C). Instead, we found a significant increase in IL-18 expression in PPVO-treated mice (Fig. 1D). We also found a significant upregulation of IL-15 expression, which remained stable over the whole time of observation (data not shown). Both IL-4 and IL-2 were rarely detectable and IL-1 and IL-10 levels were not influenced significantly (data not shown). After PPVO treatment, IFN- γ gene expression was also induced significantly in lymph nodes (Fig. 2A) and spleen (data not shown). The early peak of IFN- γ gene expression in lymph nodes was followed by upregulation of IL-4 (Fig. 2B), resulting in a drop in the IFN- γ :IL-4 ratio from 11 to 1.3 at 6 and 48 h, respectively. In the livers of PPVO-treated mice, we found IFN- γ induction at 6 h (Fig. 2C) and induction of TNF- α at 6 and 12 h after administration (data not shown). In contrast to the other organs investigated, we found induction of IL-10 expression in the livers of PPVO-treated mice (Fig. 2D). This was not observed in CFA-treated animals.

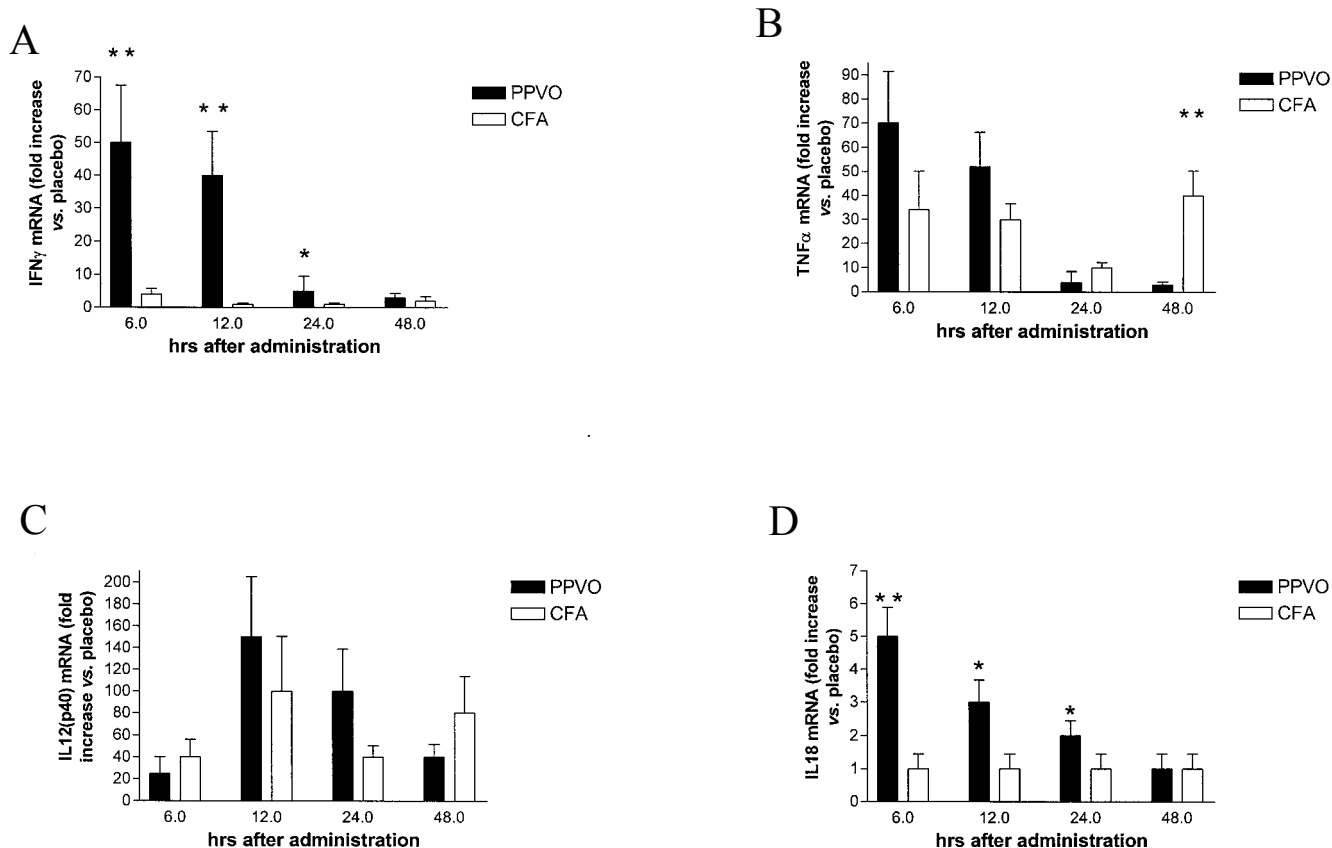


Fig. 1. BALB/c mice ($n=5$ animals per group) were treated i.p. with PPVO or CFA. Peritoneal lavage cells were harvested and analysed for cytokine expression using quantitative RT-PCR. Treatment with placebo (PBS) resulted in signals around or below the limits of detection. Data were normalized for the placebo control. PPVO induces a strong IFN- γ expression (A). PPVO-induced expression profile for TNF- α (B) and IL-12p40 (C) differed only gradually from that of CFA-treated mice. IL-18 mRNA (D) expression was induced only by PPVO but not by CFA. Indicated differences are significant in comparison to CFA controls (**, $P<0.01$; *, $P<0.05$; Wilcoxon test).

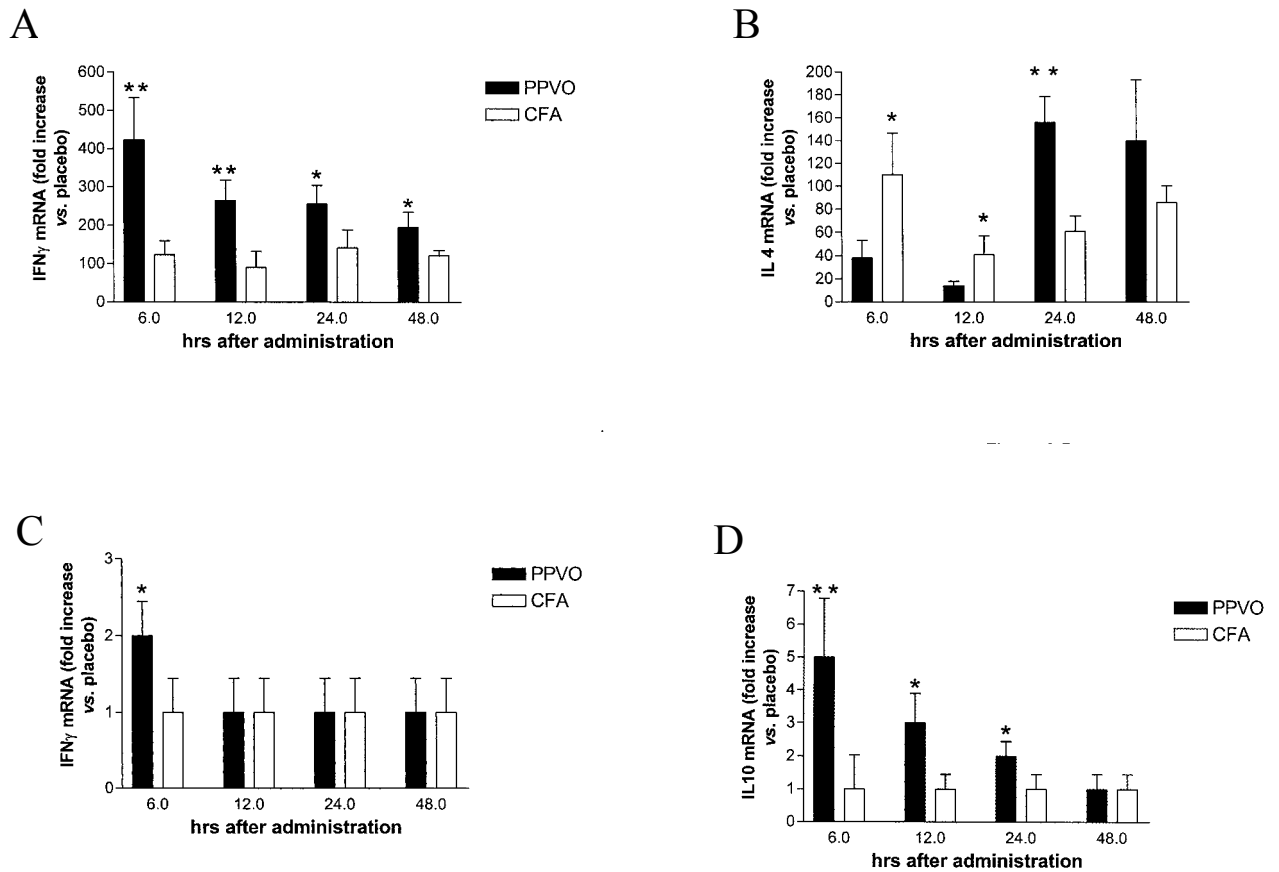


Fig. 2. BALB/c mice ($n=5$ animals per group) were treated i.p. with PPVO or CFA. (A, B) Axillar, mesenteric, gastric and portal lymph nodes were harvested and one of each analysed (pool) for cytokine expression. Treatment with placebo (PBS) resulted in signals around or below the detection limits. Data were normalized for the placebo control. (A) PPVO but not CFA induces a strong IFN- γ expression in the lymph nodes of these mice. (B) After 24 h, a significant increase in IL-4 expression was observed in lymph nodes from PPVO- but not CFA-treated mice. (C) IFN- γ expression was also induced in the livers of PPVO- but not CFA-treated mice. (D) IL-10 expression was upregulated for 24 h in the livers of PPVO-treated mice. Indicated differences are significant in comparison to CFA controls (**, $P<0.01$; *, $P<0.05$; Wilcoxon test).

To exclude cell debris as a cause of cytokine induction, we included material from mock-infected cells as a control in a subsequent experiment. The pellet fraction from mock-infected cells did not induce cytokine expression comparable to PPVO. Induction of IFN- γ and TNF- α mRNA expression in peritoneal lavage cells is depicted in Fig. 3. Similar results were obtained from explanted lymph nodes (data not shown).

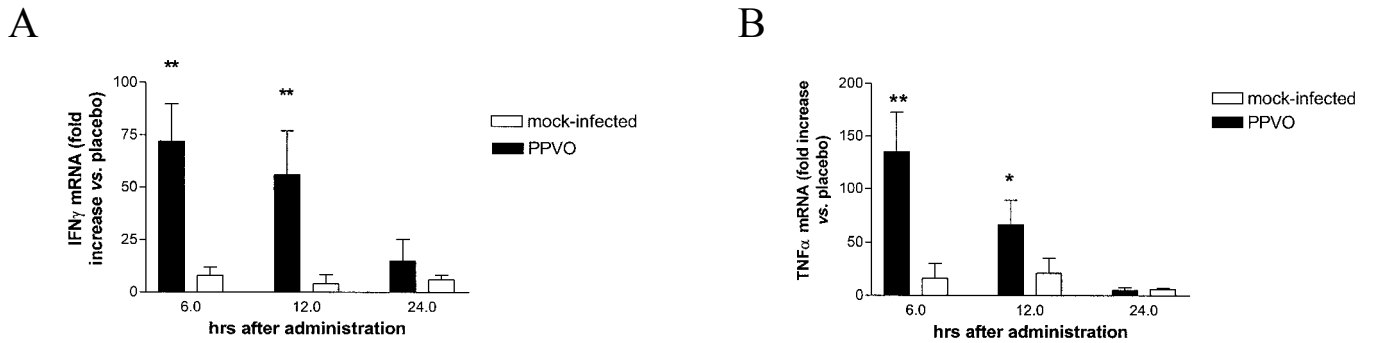


Fig. 3. BALB/c mice ($n=5$ animals per group) were treated i.p. with PPVO or material from mock-infected cells. Peritoneal lavage cells were harvested and analysed for cytokine expression using quantitative RT-PCR. PPVO induced IFN- γ (A) and TNF- α (B) mRNA expression, whereas mock-infected cells did not. Indicated differences are significant (**, $P<0.01$; *, $P<0.05$; Wilcoxon test).

PPVO protects mice from infection with human HSV-1 and is active in a guinea pig model of recurrent genital herpes

BALB/c mice were pre-treated with PPVO or placebo using various routes of administration and were infected after 16 h with a lethal dose of HSV-1 i.n. As depicted in Fig. 4(A), mice were protected independently of the route of administration ($P=0.01-0.04$, log rank analysis). Control mice were treated with CFA or supernatant from PPVO-infected culture that was cleared of virus content. Both controls were not active (data not shown). The PPVO-mediated antiviral effect could be blocked using a mAb against IFN- γ (Fig. 4B) but not with antibodies against IL-12 or IL-18 (data not shown). The antiviral activity of PPVO varies with the titre of HSV used for lethal challenge; another variable is the strain of mice. We also observed a time-dependency of PPVO activity. Optimal protective effects were observed when PPVO was administered 6–12 h prior to challenge. In contrast, minimal or no antiviral effects were observed in this mouse model when PPVO was administered later than 24 h after the challenge (data not shown).

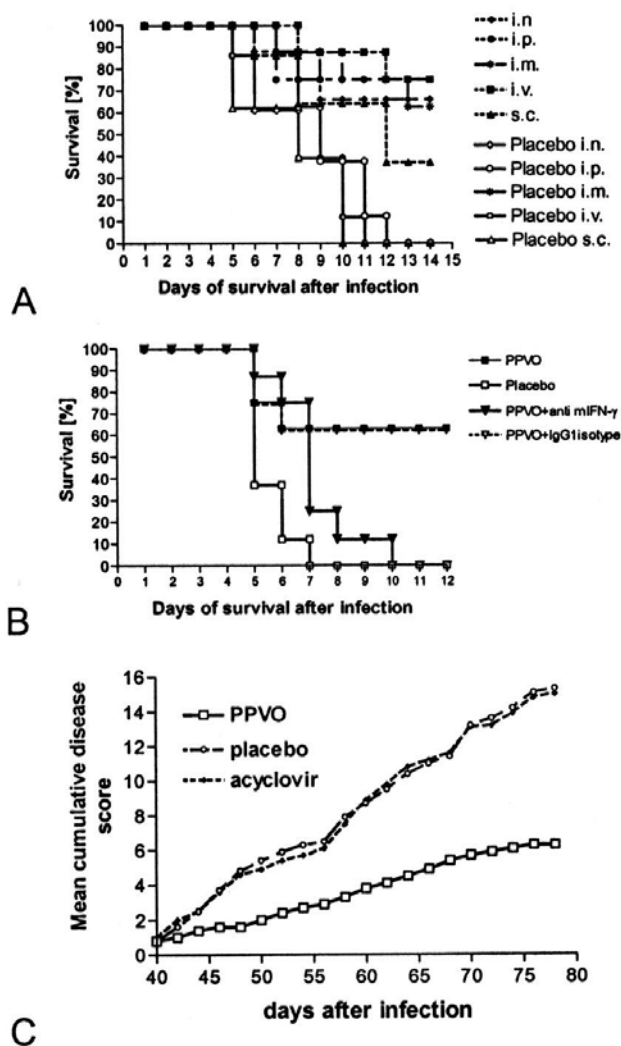


Fig. 4. Mice were treated with PPVO or placebo and infected 16 h later i.n. with a lethal dose of human HSV-1. (A) Treatment with PPVO (5×10^5 TCID₅₀) protected BALB/c mice ($n=10$ animals per group) from the lethal virus challenge. Survival curves of the animals that were treated with either PPVO or placebo (PBS) are shown ($P=0.01-0.04$; log rank analysis). The effect was not dependent on the route of administration. (B) Administration of an IFN- γ -neutralizing antibody abolished antiviral activity of PPVO. The IFN- γ -neutralizing antibody or an isotype control antibody was administered i.p. immediately before infection. The survival of PPVO+anti-IFN- γ antibody-treated mice was significantly reduced in comparison to PPVO-treated animals ($P=0.03$; log rank analysis) and to animals that got PPVO plus the control antibody ($P=0.03$) but not to placebo-treated mice. (C) Guinea pigs were infected intravaginally with HSV-2 and animals with acute infection (score 3) were randomized. Treatment with PPVO, acyclovir or placebo started 10 days after healing of the primary disease (score 0) ($n=10$ animals per group). PPVO was administered every third day i.p., five times in total. Placebo and acyclovir were administered daily for 10 consecutive days i.p. Animals were examined daily for 40 consecutive days for herpes lesions and the lesion severity was scored.

To test the activity of PPVO in a second species, we used a guinea pig model of genital herpes. Disease symptoms during periods of recurrence were absent to mild in PPVO-treated guinea pigs (score 0–2) but tended to be severe in placebo- or acyclovir-treated animals (score 3–4). This is reflected in the cumulative score of herpes disease symptoms, which was reduced significantly ($P < 0.01$, Student's *t*-test) in PPVO but not in acyclovir-treated guinea pigs (Fig. 4C). Starting 10 days after treatment, HSV could be detected in vaginal secretions from acyclovir- or placebo-treated animals, whereas no virus was detected in vaginal secretions derived from PPVO-treated guinea pigs (data not shown).

PPVO inhibits HBV replication in a transgenic mouse model

We have used HBV transgenic mice [strain Tg (HBV1.3 fsX^{-3'5'})] that carry a frameshift mutation (GC) at position 2916/2917 (C. Kuhn & H. Schaller, unpublished; Weber *et al.*, 2002). PPVO-treated mice showed approximately 70–80 % less HBV DNA in the liver and 100 times less HBV DNA in the plasma in comparison to placebo-treated mice ($P = 0.04$ and 0.002 , respectively; variance analysis with post-hoc comparison of means) (Fig. 5A, B). Significant effects on HBV replication were observed after a 3 week treatment using $30 \text{ mg } 3\text{TC kg}^{-1}$ three times a day *per os*. PPVO activity was dose-dependent in plasma (Fig. 5C) and in the liver (data not shown). The antiviral effect was maintained after repeated administrations (Fig. 5D). Administration of a mAb against IFN- γ reduced the antiviral effect significantly in the livers (Fig. 5E) and plasma (data not shown) of HBV transgenic mice. Southern blot analysis was performed to compare the levels of replicative intermediates of HBV DNA in the livers. HBV DNA replication was unaffected in placebo-treated mice (Fig. 6A) but was almost undetectable in the livers of mice treated with PPVO (Fig. 6B). PPVO treatment has also led to almost undetectable levels of HBV-specific RNAs in the liver of these mice (data not shown). In addition, HBcAg, which is indicative of ongoing HBV replication, was markedly reduced in PPVO-treated mice (Fig. 6D) but not affected in placebo-treated mice (Fig. 6C). The intranuclear capsid antigen was also affected by PPVO treatment. No infiltration of lymphocytes or other cells has been detected histologically and liver enzymes were normal during and after treatment.

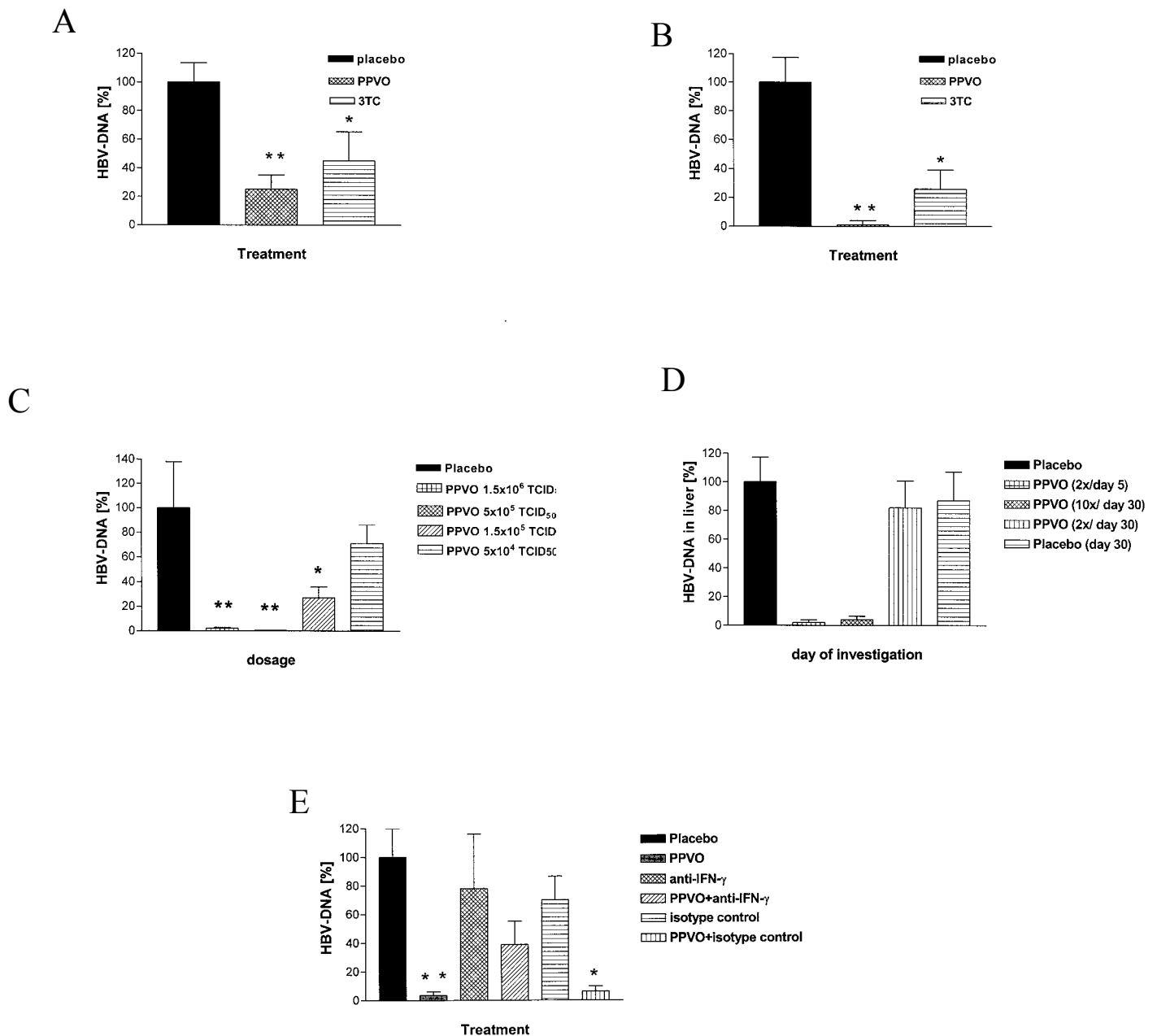


Fig. 5. PPVO is active against human HBV. PPVO or placebo was administered i.p. every third day, three times in total, to HBV transgenic mice ($n=10$; 5 male and 5 female mice per group). 3TC was administered orally three times a day at 30 mg kg^{-1} for 21 consecutive days. Treatment with PPVO reduced viral DNA when compared to placebo in the livers (A) and in the plasmas (B) to a greater extent than 3TC (**, $P<0.01$ and $P<0.05$; variance analysis and post-hoc comparison of means). The differences in the plasmas are significant ($P<0.05$). The effect was dose dependent in plasma (C) and liver (data not shown). (D) HBV transgenic mice were treated every third day with placebo ($n=10$ animals per group) or PPVO ($5 \times 10^5 \text{ TCID}_{50}$) ($n=15$ animals per group). Treatment started on day 1. Five mice from each group were analysed 1 day after the second treatment and livers were analysed for HBV DNA. Five more mice from the PPVO-treatment group were left untreated until day 30 (PPVO, 2x/day 30). The remaining mice were treated 10 times in total and livers were analysed on day 30 for HBV DNA. (E) Administration of an IFN- γ -neutralizing mAb significantly reduced the antiviral effect of PPVO in the livers of HBV transgenic mice. **, $P<0.01$; *, $P<0.05$ (variance analysis and post-hoc comparison of means).

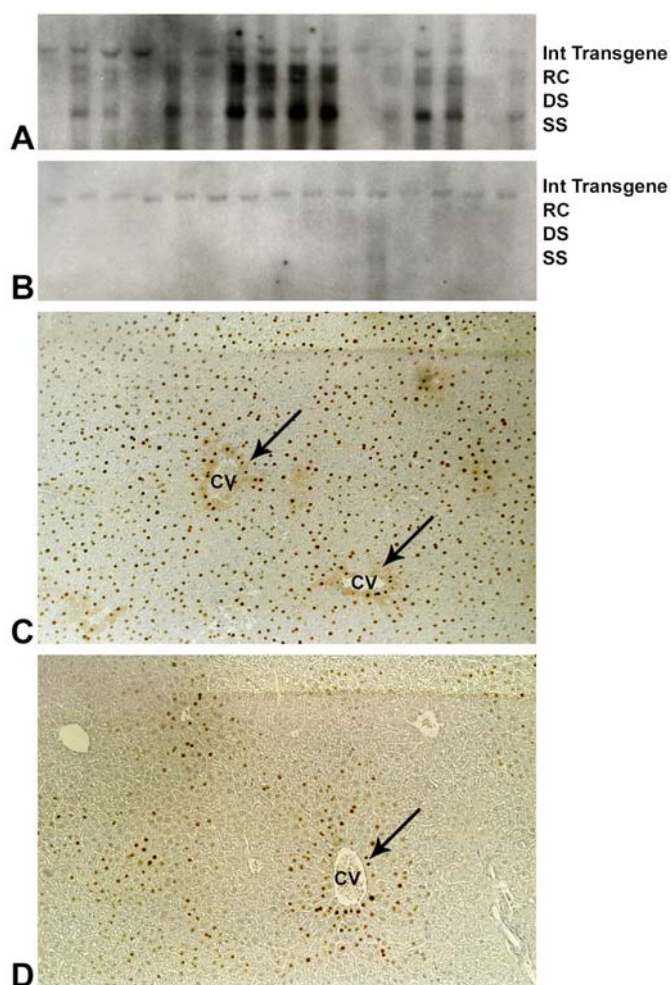


Fig. 6. PPVO-treatment abolishes hepadnavirus replication. In placebo-treated HBV transgenic mice, replicative intermediates of viral DNA can be detected by Southern blot analysis (A). Int Transgene, integrated transgene; RC, relaxed circular HBV DNA; DS, double-stranded linear HBV DNA; SS, single-stranded HBV DNA. These replicative intermediates were absent in PPVO-treated mice (B). (C, D) Immunohistological analysis of HBcAg expression in the livers. Diffuse cytoplasmic staining in periportal areas [arrows, central veins (CV)] indicates viral capsids and ongoing HBV replication in placebo-treated mice (C). Cytoplasmic HBcAg was strongly reduced in PPVO-treated mice (D). The nuclear HBcAg-specific stain (for empty capsids) was also markedly reduced after PPVO treatment (D).

PPVO induces IL-12 and IFN- γ in human peripheral blood leukocytes

To assess whether PPVO may be useful for humans, we tested the cytokine-inducing capacity in human whole blood assays. PPVO alone induced TNF- α (>500 pg ml $^{-1}$) and IL-12 (>25 pg ml $^{-1}$) in a dose-dependent manner but, without co-stimulation, did not induce significant levels of IFN- γ (data not shown). Together with low dose ConA (1 μ g ml $^{-1}$), however, it superinduced IFN- γ (>5 - to 12-fold, $P < 0.01$) as well. Co-administration of low-dose PHA or anti-CD3 mAb also amplifies IFN- γ secretion. Co-stimulation seems to be necessary for PPVO-mediated IFN- γ stimulation to induce IL-12/IL-18 receptor expression on resting lymphocytes and so making the cells responsive to IL-12/IL-18. In the *in vivo* mouse models, T cells are pre-activated by the viral antigens. The cytokine-inducing effect could not be blocked by addition of bactericidal/permeability-increasing protein (BPI), suggesting that this effect is not due to endotoxin contamination.

DISCUSSION

In this report, we demonstrate that inactivated PPVO induces an autoregulatory cytokine response that involves the upregulation of IL-12, IL-18, IFN- γ and other Th1-type cytokines and their subsequent downregulation, which is accompanied by induction of IL-4. This cytokine response mediates antiviral activity against HSV and HBV *in vivo*.

Mechanisms of IFN- γ induction

Expression of the IFN- γ -inducing cytokine IL-12 was elevated in mice treated with PPVO to levels that were also observed after CFA administration, although CFA induced much less IFN- γ . Moreover, CFA was not able to protect the mice against virus infections. IL-18, a cytokine shown recently to be a powerful inducer of IFN- γ (Micallef *et al.*, 1996), was induced only in PPVO-treated mice, an observation suggesting a role of IL-18 in PPVO-mediated biological effects *in vivo*. Upregulation of mRNA for both IFN- γ -inducing cytokines could be found at the site of PPVO application only, whereas IFN- γ upregulation was found both at the site of PPVO injection and in lymphoid organs. These data suggest that the IFN- γ -inducing cytokines are induced locally by PPVO, circulate systemically (for cytokines they have an untypical long half-life) and upregulate IFN- γ secretion by antigen-triggered IL-12/IL-18-responsive T cells at the site of injection of PPVO as well as in peripheral immune organs such as lymph nodes and spleen. IFN- γ mediates directly or indirectly its systemic antiviral efficacy (upregulation of MHC molecules, activation of CTL and NK cells, activation of macrophages and induction of opsonizing antibodies, etc.). Since administration of neutralizing antibodies against IFN- γ , but not against IL-12 or IL-18, abolished antiviral activity against HSV and reduced activity against HBV, other scenarios of IFN- γ induction are possible. Schijns *et al.* (1998) demonstrated that, following an infection with mouse hepatitis virus, mice with a targeted disruption of the IL-12 p40 and/or p35 gene were still capable of producing a polarized Th1-type cytokine response, as evidenced by high IFN- γ and non-detectable IL-4 production. Therefore, IL-12 and IL-18 may complement each other in PPVO-mediated IFN- γ induction; it has been shown recently that IL-18 has antiviral activity against HBV (Kimura *et al.*, 2002). Since we have used an inactivated virus preparation, it is unlikely that *de novo*-synthesized viral proteins mediate cytokine induction. The effects we have observed were induced by treatment with preparations of whole PPVO but, using a vaccinia virus-based library of PPVO DNA, we have been able to identify PPVO candidate proteins that are responsible for the IFN- γ -inducing activity (A. Friebe *et al.*, unpublished data). Consistent with previous studies that were performed *in vitro* (Buettner *et al.*, 1995), vaccinia virus in these experiments did not possess antiviral activity *in vivo*.

In human cells, PPVO could also induce IFN- γ (together with a suboptimal T cell receptor stimulus) and this effect was blocked partially by anti-IL-12 and anti-IL-18 mAbs. When both mAbs were used together, IFN- γ production was blocked almost completely (data not shown).

Possible advantages of inactivated PPVO over antiviral cytokine monotherapies

In addition to its IFN- γ -stimulating activity, IL-18 has also pro-Th2 effects. It has been reported recently that IL-18 enhances IL-4 production by ligand-activated NK T lymphocytes (Leite-de-Moraes *et al.*, 2000). Therefore, IL-18 could also mediate the increase in IL-4. On the other hand, IL-4 has been demonstrated to downregulate the IL-18 receptor α chain, thereby negatively regulating IL-18 and IL-18-mediated effects (Smeltz *et al.*, 2001). We conclude that the PPVO-mediated IL-4 response might be part of cytokine networking and responsible for the downmodulation of the initial Th1 immune response. Further studies to address this question are in progress.

Our results are consistent with the finding that inflammatory cytokines are capable of abolishing HBV replication and HBV gene expression non-cytopathically (Guidotti & Chisari, 1996; Cavanaugh *et al.*, 1998; Guidotti *et al.*, 1996, 1999). The therapeutic use of viruses that are not inactivated would have certain risks and could lead to uncontrollable effects. However, the application of therapeutic cytokines is limited. The half-life of recombinant IFN- γ is low and the protein would have to have been administered at high dosages, which, in turn, would lead to serious side effects. In contrast to a single systemic application of recombinant IFN- γ , PPVO appears to upregulate other effector cytokines also (TNF, etc.) and, in parallel, it induces regulatory cytokines, such as IL-4, detectable after 24–48 h, in lymph nodes, and IL-10 in the liver. This may explain the high efficiency in virus clearing without significant evidence for harmful tissue destruction, particularly in transgenic mice. It has been shown that IL-12 administration is therapeutically useful in HBV transgenic mice (Cavanaugh *et al.*, 1997). Most of the antiviral activity of IL-12 is mediated via IFN- γ induction, with the longer *in vivo* half-life of IL-12 explaining its higher efficacy as compared to IFN- γ . Importantly, although we have observed a more pronounced Kupffer cell reaction in the livers of PPVO-treated HBV transgenic mice, no signs of toxicity or inflammation have been observed histologically and liver enzymes were found in a normal range upon and after treatment with PPVO (data not shown). IL-10, which was induced in the liver after PPVO administration, is known to downregulate T cell activation by antigen-presenting liver sinusoidal cells (Knolle *et al.*, 1998). We speculate that the lack of any inflammation in the livers of PPVO-treated mice might be related to the prolonged induction of IL-10 expression and the constant efficacy after repeated dosing by some of the unique immune escape mechanisms mediated by PPVO-encoded proteins (Haig & Mercer, 1998; Haig & McInnes, 2002; McKeever *et al.*, 1988; Haig *et al.*, 1996, 1997; Kruse & Weber, 2001).

Also, we did not find inflammation in pathological examinations of HSV-infected guinea pigs. It has been described recently that IFN- γ is responsible for the clearance of virus infection from the CNS (Binder & Griffin, 2001). Using the guinea pig model of recurrent genital herpes, we could answer three questions: (i) the effects of PPVO are not mouse specific; (ii) we are able to target infections even at immune-privileged sites such as the CNS; and (iii) this is possible without side effects.

Putative therapeutic options

Interestingly, PPVO induced the cytokine network in human blood cells. It stimulates TNF- α and IL-12 secretion directly and, in pre-activated T cells, IFN- γ . Blocking experiments with BPI demonstrated that this effect was not due to endotoxin contamination. Thus, PPVO might express similar effects in humans as in mice.

In summary, our data show that inactivated PPVO (strain D1701) has antiviral activity and that the induction of a cytokine cascade by inactivated PPVO might have advantages over existing immune therapies. More studies are needed to investigate the interaction of inactivated PPVO with the immune system of chronically infected animals. We conclude from our data that inactivated PPVO should be investigated further as a potential antiviral drug.

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