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A study of the vaccinia virus interferon-γ receptor and its contribution to virus virulence

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Vaccinia virus (VV) strain Western Reserve gene B8R encodes a 43 kDa glycoprotein that is secreted from infected cells early in infection as a homodimer. This protein has amino acid similarity with the extracellular domain of cellular IFN-γ receptor (IFN-γR) and binds and inhibits IFN-γ from a wide range of species. Here we demonstrate that the B8R protein also inhibits equine IFN-γ. The 5′ end of the B8R mRNA has been mapped by primer extension analysis and the contribution of IFN-γRs to VV virulence was studied by the construction of a deletion mutant lacking the B8R gene (vΔB8R) and a revertant virus (vB8R-R) in which the B8R gene was re-inserted into the deletion mutant. A recombinant virus that expressed a soluble form of the mouse IFN-γR was also constructed and studied. The virulence of these viruses was tested in rodent models of infection. In mice, the loss of the VV IFN-γR did not affect virulence compared with WT and revertant viruses, consistent with the low affinity of the VV IFN-γR for mouse IFN-γ. However, expression of the mouse soluble IFN-γR increased virus virulence slightly. In rabbit skin, loss of the VV IFN-γR produced lesions with histological differences compared with WT and revertant viruses. Lastly, the affinity constants of the VV IFN-γR for human and mouse IFN-γ were determined by surface plasmon resonance.

Introduction

Vaccinia virus (VV) is a poxvirus and has a large double-stranded DNA genome, complex virus particle and cytoplasmic site of replication (Moss, 2001). The VV genome has been sequenced for strains Copenhagen (Goebel *et al.*, 1990), Tian Tan (accession no. AF095689), modified virus Ankara (MVA) (Antoine *et al.*, 1998) and most of Western Reserve (WR) [Smith *et al.* (1991) and references therein]. These data show that VV encodes approximately 200 genes and those located in the central region are highly conserved, encoding enzymes, transcription factors and structural proteins necessary for virus replication. In contrast, the regions of the genome adjacent to each terminus are more variable and encode genes that are mostly non-essential for virus replication in cell culture (Perkus *et al.*, 1991) but which affect features such as host range, virulence and immunomodulation. Near the right end of the VV WR genome several genes were predicted to encode soluble glycoproteins (Smith *et al.*, 1991) and some of these proteins showed amino acid similarity to cell proteins that function as receptors for host cytokines or interferons. Gene B15R encodes a soluble receptor for interleukin (IL)-1β (Alcamí & Smith, 1992; Spriggs *et al.*, 1992), gene B18R encodes a soluble and cell surface receptor for type I interferons (IFN) (Colamonici *et al.*, 1995; Symons *et al.*, 1995; Alcamí *et al.*, 2000) and gene B8R encodes a soluble receptor for type II IFN or IFN-γ (Alcamí & Smith, 1995; Mossman *et al.*, 1995). Gene B8R is the subject of this paper.

IFN-γ is a pleiotropic cytokine that promotes the Th1 immune response and also has direct antiviral activity (Farrar & Schreiber, 1993). It is a secreted, 17 kDa glycoprotein that binds to the IFN-γR on the surface of cells. This interaction triggers dimerization of the receptor (Fountoulakis *et al.*, 1991, 1992; Greenlund *et al.*, 1993), leading to signal transduction. The crystal structure of IFN-γ showed that the protein is largely α-helical and exists as a dimer (Ealick *et al.*,

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1991; Samudzi *et al.*, 1991; Walter *et al.*, 1995). The IFN-γR is an 85–90 kDa glycoprotein with a type I membrane topology, it has 2 fibronectin type III repeats and is a member of the class II cytokine receptor superfamily (Farrar & Schreiber, 1993). Only a single IFN-γR exists and transgenic mice lacking this protein show an enhanced sensitivity to infection with VV and other viruses (Huang *et al.*, 1993). Likewise, treating animals with IFN-γ increases their resistance to virus infection (Karupiah *et al.*, 1993; van den Broek *et al.*, 1995).

The VV B8R protein has amino acid similarity to the T7 protein of myxoma virus (Howard *et al.*, 1991) that binds and inhibits rabbit IFN-γ (Upton *et al.*, 1992). Subsequently, the VV B8R gene was shown to encode a 43 kDa secreted glycoprotein that inhibits IFN-γ from human, cow, rabbit, rat and chicken but not mouse (Alcamí & Smith, 1995; Mossman *et al.*, 1995; Puehler *et al.*, 1998). The broad species specificity was unexpected because IFN-γ and IFN-γR from cells are largely species-specific. Nonetheless, deletion of the B8R gene from VV was reported to cause virus attenuation in a mouse model (Verardi *et al.*, 2001). Recently, the VV B8R protein was shown to be a homodimer in the absence of ligand, unlike the cellular IFN-γR that dimerizes upon ligand binding (Alcamí & Smith, 2002).

Here we have studied the role of the VV IFN-γR in virus virulence by the construction of WT and deletion viruses and a recombinant virus expressing a soluble form of the mouse IFN-γR. These viruses replicated normally in cell culture but showed differences *in vivo*.

Methods

■ **Cells and viruses.** BS-C-1, RK_{13}, HeLa D980R, TK^-143 and Cos-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS). Equine dermal cells were grown in minimal essential medium (MEM) containing 10% FBS and were obtained from Dr Jay Patel (Akzo Nobel). The Western Reserve (WR) strain of VV was used throughout and was grown, titrated and purified as described (Mackett *et al.*, 1985). Cocal virus was obtained from Dr W. James (Sir William Dunn School of Pathology, University of Oxford) and Semliki Forest virus (SFV) was obtained from Dr Jay Patel (Akzo Nobel).

■ **Reagents.** Radioiodinated human IFN-γ (^{125}I-hIFN-γ; specific activity 50–125 μCi/μg) was obtained from New England Nuclear Life Science Products. Purified recombinant hIFN-γ (3×10^7 U/mg) was obtained from PeproTech Ltd (London, UK) and recombinant mouse IFN-γ (5×10^6 U/mg) was obtained from Genzyme and R&D Systems. Equine IFN-γ was obtained by transfection of Cos-7 cells with pCIneo/Eq-IFNγ (a gift from Dr Jay Patel). The supernatant was harvested 5 days later, centrifuged at 10000 ***g*** to remove cellular debris and the equine IFN-γ was titrated as described below. One unit of equine IFN-γ was defined as that which reduced SFV plaque formation by 50%. Typically Cos-7 supernatants contained approximately 70 U/ml IFN-γ.

Hamster monoclonal antibody (mAb) against the mIFN-γ receptor (alpha chain) was obtained from Genzyme. Biotinylated goat anti-hamster IgG was obtained from Vector Laboratories and streptavidin–horseradish peroxidase was from Amersham.

■ **Primer extension analysis.** RNA was isolated from RK_{13} cells that had been mock-infected or infected with VV at 10 p.f.u. per cell for 6 h in the presence or absence of 100 μg/ml cycloheximide or for 16 h in the presence or absence of 40 μg/ml cytosine arabinoside (AraC) as described previously (Ausubel *et al.*, 1990). Oligonucleotide 5′ CTTATAACTAGTTATTTTAGCGTGTATAC 3′ was labelled at the 5′ end with ^{32}P by incubation with [γ-^{32}P]ATP (10 μCi/ml, 3000 Ci/mM, Amersham) and T4 polynucleotide kinase (New England Biolabs) and hybridized to 10 μg of denatured RNA samples. The nucleotide primer was extended with reverse transcriptase (RNase H^-) (GibcoBRL) and the sample was electrophoresed on a 6% polyacrylamide gel alongside a ^{35}S-labelled DNA sequencing ladder and detected by autoradiography.

■ **Isolation of RNA and preparation of cDNA from EL-4 cells.** Total cellular RNA was isolated from the murine thymoma cell line EL-4.NOB1 using RNAzol (Biogenesis Ltd, Poole, UK), following the manufacturer's instructions. Complementary DNA was synthesized using 5 μg of RNA, oligo(dT) and avian myeloblastosis virus reverse transcriptase (Promega).

■ **Construction of recombinant viruses.** A virus deletion mutant lacking 96% of the B8R ORF was constructed using transient dominant selection (Falkner & Moss, 1990). A plasmid was assembled that contained the DNA flanking the 5′ and 3′ regions of the B8R ORF. These fragments were amplified by PCR using *Pyrococcus furiosus* DNA polymerase and VV WR DNA as template. The left flanking region was amplified with oligonucleotides 5′ CTAGAATTCAACGCAGAGGTCACACG 3′ (B8R1F) and 5′ ATCCCCGGGTGTTGTTTGTTATTTGAC 3′ (B8R1R) that contain *Eco*RI and *Sma*I sites, respectively (underlined). The right flanking region was amplified with oligonucleotides 5′ CCAGGATCCTTAGCATGCTTAACTTGAC 3′ (B8R2F) and 5′ TCAAAGCTTCACTTGCAGTTGGG 3′ (B8R2R) that contain *Bam*HI and *Hin*dIII sites (underlined), respectively. These fragments were digested with the appropriate restriction enzymes and cloned sequentially into plasmid pSJH7 (Hughes *et al.*, 1991) that had been cut with the same enzymes. The resultant plasmid, termed pΔB8R, contained 319 and 302 nucleotides of the 5′ and 3′ flanking sequence, respectively, including 30 nucleotides of coding sequence at the 3′ end of the gene. The DNA sequence was determined and shown to be correct.

Plasmid pΔB8R was transfected into VV-infected cells and mycophenolic acid (MPA)-resistant recombinant viruses were isolated as described (Falkner & Moss, 1990). These were grown on hypoxanthine guanine phosphoribosyltransferase-negative D980R cells in the presence of 6-thioguanine (TG) (Kerr & Smith, 1991) and plaque isolates corresponding to WT (vB8R) or deletion mutant (vΔB8R) were identified by PCR using oligonucleotides that span the B8R gene locus.

A revertant virus (vB8R-R) was constructed by transfecting a plasmid (pB8R-R) into vΔB8R-infected cells. This plasmid was constructed by digesting pSTH1 (Engelstad & Smith, 1993) with *Pst*I and *Sph*I and the resulting 1003 bp fragment containing the entire B8R ORF and flanking regions was cloned into pΔB8R that had been cut with the same enzymes. The DNA sequence was determined and shown to be correct. MPA-resistant intermediate viruses were isolated as above and resolved into deletion mutant and revertant viruses (vB8R-R) on D980R cells in the presence of 6-TG as described.

A recombinant virus that expressed a soluble version of the mouse IFN-γR (vsmIFN-γR) was constructed as follows. The extracellular domain of the mouse IFN-γR alpha chain (amino acids 1–257) was amplified by PCR with EL-4.NOB1 cDNA as a template using the primers 5′ ACAACACC**ATGGGCCCGCAGGCGGC** 3′ (IFN-γR/B8R5′) and 5′ AAATTAGTTA**TGAATCCTTTCTGTCATC** 3′ (IFN-γR/B8R3′). This was fused to the B8R promoter using splicing by overlap extension (Horton *et al.*, 1989). The 5′ and 3′ flanking regions of the B8R ORF were amplified by PCR with VV WR DNA as a template using the primers

5′ **GCCGCCTGCGGGCC**CATGGTGTTGTTTGTTATTTG 3′ (B8R5′/IFN-γR) and B8R1F (above) and 5′ **GATGACAGAAAGGATTCA**TAACTAATTTTTATTAATGATACAAAAACG 3′ (B8R3′/IFN-γR) and B8R2R (above). VV sequences are shown in plain type, mouse IFN-γR alpha chain sequences in bold type and initiation and stop codons are underlined. These three PCR products were reamplified using the B8R1F and B8R2R primers and the assembled DNA fragment was then digested with *Eco*RI and *Hin*dIII and cloned into pSJH7 that had been cut with the same enzymes, to form plasmid pB8R/smIFNγR. DNA sequence analysis showed the sequence was correct.

Plasmid pB8R/smIFNγR was transfected into vΔB8R-infected cells and a virus expressing the soluble murine IFN-γR alpha chain (vsmIFN-γR) under control of the B8R promoter was isolated by transient dominant selection as above. This virus was used to generate a vΔB8R-revertant virus (vΔB8R-R) in which the mouse vIFN-γR gene was removed and the B8R locus was restored to that of the parental virus vΔB8R. Cells infected with vsmIFN-γR were transfected with pΔB8R and the revertant virus was generated by transient dominant selection as above.

■ **Preparation of supernatants from virus-infected cells.** TK$^-$143B cells were infected at 5 p.f.u. per cell. At the indicated times supernatants were harvested and centrifuged at 3000 r.p.m. for 10 min at 4 °C to remove cellular debris. Virus particles were removed by centrifugation at 16500 r.p.m. in an SW41 Ti rotor for 60 min at 4 °C and the supernatants were stored at −20 °C.

■ **Construction and expression of a B8R–Fc fusion protein.** The VV WR B8R gene fused to the Fc region of human IgG1 was constructed in pCOSFCLINK (a generous gift from Dr Peter R. Young, SmithKline Beecham Pharmaceuticals, King of Prussia, PA, USA). The chimaera was obtained by PCR using VV WR DNA as template and the oligonucleotides 5′ GTCGAATTCCAAACAACACCATGAG 3′ (*Eco*RI site, underlined) and 5′ ATTGGTACCTGAATATTTAGTCAAG 3′ (that inserts a Gly and Thr via a *Kpn*I site, underlined, after amino acid 272 of the B8R protein before the hinge region of human IgG1 that was provided by the pCOSFCLINK plasmid). The resultant plasmid was called pCOS B8R-Fc. The sequence of the B8R gene was confirmed as identical to that reported previously. The plasmid was transfected into DHFR$^-$ CHO cells and stable transfectants were selected in increasing concentrations of methotrexate (MTX; Sigma) up to a final concentration of 10 μM. B8R–Fc fusion protein production was quantified using an ELISA for human IgG1 Fc. CHO cells expressing B8R–Fc fusion protein were adapted to grow in defined medium (CHO-S-SFM II; GibcoBRL) and the B8R–Fc fusion protein was purified from the supernatant on a protein A–Sepharose column (Pharmacia). Expression of the 35K–Fc fusion protein has been described (Alcamí *et al.*, 1998).

■ **Biological assays for IFN.** The biological activity of hIFN-γ was measured by the inhibition of cocal virus plaque formation as described (Alcamí & Smith, 1995). The biological activity of equine IFN-γ was measured by the inhibition of SFV plaque formation on equine dermal cells. Cells were pretreated with 10 U of equine IFN-γ for 24 h. The cells were then infected with 100 p.f.u. of SFV and plaques were stained and counted 48 h p.i.

■ **Binding assays.** Covalent cross-linking of ^{125}I-hIFNγ to supernatants from VV-infected cells was performed as described (Alcamí & Smith, 1995).

■ **BIAcore analysis.** Surface plasmon resonance (SPR) experiments used a BIAcore 2000 (BIAcore, St. Albans, UK) (Nicholson *et al.*, 1998). The purified mAb R10ZD9 (anti-human IgGFc; a gift from Dr P. Anton van der Merwe) was immobilized to CM5 sensor chips (BIAcore) using the Amine Coupling Kit (BIAcore) with a flow rate of 10 μl/min. Immobilized R10ZD9 was regenerated with no loss of IgGFc binding by sequential injections of 10 mM NaOH and 0·1 M glycine, pH 2·5. B8R–Fc and 35K–Fc (control) fusion proteins were coupled by injection at 30 μg/ml for 10 min at a flow rate of 5 μl/min. For kinetic experiments, mouse and human IFN-γ were injected in 50 μl at 50 μl/min at various concentrations between 100 pM and 1·0 μM. Kinetic constants were derived using the curve-fitting facility of the BIAevaluation program (version 3.0, BIAcore) using a simple 1:1 binding model. To correct for refractive index changes, binding responses generated in the control surface (35K–Fc) were subtracted from responses generated in the B8R–Fc surface.

■ **Ligand blotting.** Proteins from supernatants from VV- or baculovirus-infected cells and purified Fc fusion proteins were resolved by SDS–PAGE under non-reducing conditions on a 10% gel and transferred onto 0·45 μM nitrocellulose (Schleicher and Schuell). The filters were blocked in 3 % (w/v) non-fat skimmed milk in 10 mM Tris–HCl (pH 7·4), 140 mM NaCl, 0·02% (w/v) NaN_3 overnight at 4 °C, and then probed with 5 ng/ml ^{125}I-hIFN-γ in blocking buffer with or without 1 mg/ml cold IFN-γ for 4 h at room temperature. Blots were then washed, dried in air and exposed to X-ray film.

■ **Immunoblotting for mouse IFN-γR alpha chain.** Virus-free supernatants from 6×10^4 cells were resolved by SDS–PAGE under non-reducing conditions on a 12·5% polyacrylamide gel and transferred onto nitrocellulose (Hybond-ECL; Amersham). After blocking in 5% (w/v) nonfat skimmed milk in PBS–0·1 % (v/v) Tween 20, the membrane was incubated sequentially with a hamster mAb against mouse IFN-γR alpha chain (Genzyme) at 1 μg/ml, biotinylated anti-hamster IgG conjugate (Vector Laboratories) at 1·5 μg/ml, and finally streptavidin–horseradish peroxidase conjugate (1:1500; Amersham). Immunoreactive bands were visualized by enhanced chemiluminescence.

■ **Assays of viral virulence.** Intranasal infection of female, BALB/c mice (5–6 weeks old) was performed as described (Symons *et al.*, 1995). Each day, mice were weighed individually and monitored for signs of illness. Those having lost 30% of their initial body weight were sacrificed.

For intradermal infection, 10^4 p.f.u. of recombinant VV in 10 μl of PBS was injected into the ear pinnae of female BALB/c mice between 8 and 12 weeks of age. The diameter of lesions was measured daily as described (Tscharke & Smith, 1999).

Female New Zealand White rabbits (1·5 kg) were injected intradermally with 10^4, 10^5 or 10^6 p.f.u. of the indicated viruses/site in 0·1 ml of PBS along the flanks of the animal. Animals were monitored daily for signs of illness and the lesion size was measured with fine calipers. At 3 and 5 days p.i. animals were sacrificed and lesions excised for immunohistochemical analysis.

■ **Immunohistochemistry.** Rabbit dermal samples were prepared and immunostained using anti-rabbit CD43 antibody (IgG1; Serotec) to detect rabbit T cells, monocytes and macrophages, or mouse mAb 5B4/2F2 against the VV A27L protein (Czerny & Mahnel, 1990) as described previously (Ng *et al.*, 2001). Sections were counterstained with 0·1% (w/v) cresyl violet acetate, dehydrated, mounted and examined as described previously (Ng *et al.*, 2001). Several 10 μm sections were examined to ensure that those shown are representative.

Results

The 5′ end of the B8R mRNA was mapped by primer extension analysis (Fig. 1a). An oligonucleotide complementary to the B8R mRNA (Fig. 1b) was labelled with ^{32}P at its 5′ end and hybridized to RNA extracted from VV-infected cells

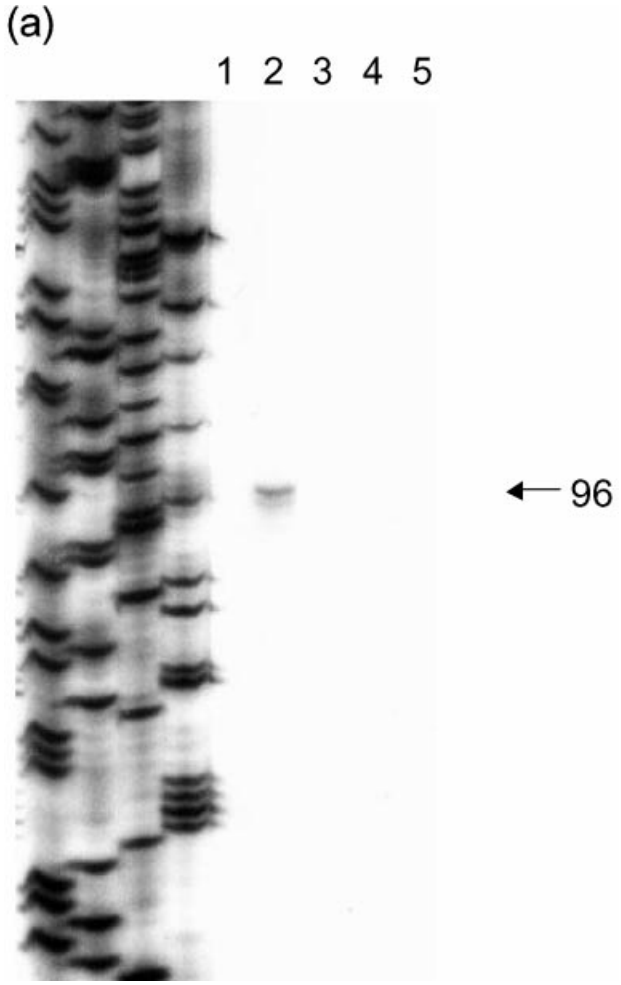

Fig. 1. Primer extension analysis. (a) RNA was extracted from mock-infected cells (lane 5) or WR-infected cells harvested at either 6 h p.i. in the presence (lane 2) or absence (lane 3) of cycloheximide, or at 16 h p.i. in the presence (lane 1) or absence (lane 4) of AraC and was hybridized to a ^{32}P-labelled oligonucleotide primer (b). After reverse transcription, the extension products were electrophoresed on a 6% polyacrylamide gel alongside a sequencing ladder. An autoradiograph is shown. (b) The position of the deduced 5′ end of the B8R mRNA is shown (dot) and the position of the oligonucleotide primer (of complementary sequence) is underlined. The consensus sequence for early promoters is shown.

that had been incubated with or without cycloheximide or AraC (an inhibitor of virus DNA replication and therefore of intermediate and late gene expression). Reverse transcription products were analysed alongside a sequencing ladder (Fig. 1a).

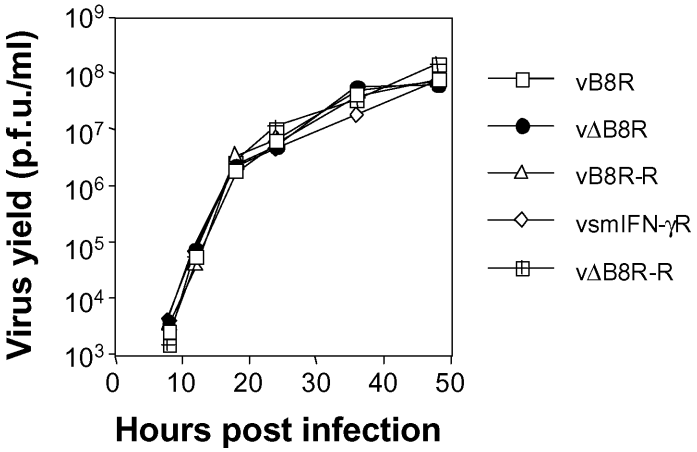

Fig. 2. Replication of recombinant viruses in cell culture. Flasks of BS-C-1 cells were infected with the indicated viruses at 0·01 p.f.u. per cell. At the indicated times the cells were scraped into the culture medium and the virus infectivity was determined in duplicate by plaque assay on BS-C-1 cells.

A major product of 96 nucleotides and a minor product of 95 nucleotides were obtained using RNA extracted from cells infected for 6 h in the presence of cycloheximide (lane 2), but not with RNA from mock-infected cells or from cells infected for 6 h in the absence of cycloheximide or for 16 h in the presence or absence of AraC. The presence of B8R RNA 6 h p.i. in only the presence of cycloheximide is consistent with the build up of early transcripts in the absence of protein synthesis. These data indicate that the B8R gene is transcribed early during infection predominantly from a site 27 nucleotides upstream of the initiating methionine codon of the B8R ORF. This RNA start site is approximately 14 bp downstream of a motif, 5′ AAAAATTTAAAATATA 3′, that is similar to the consensus sequence for this region of early VV promoters (Davison & Moss, 1989).

Construction of deletion and revertant viruses

The role of the B8R gene in VV replication *in vitro* and *in vivo* was investigated using WT (vB8R), deletion mutant (vΔB8R) and revertant (vB8R-R) viruses. The relative contributions of the VV IFN-γR and soluble mouse IFN-γR to VV virulence in mice was compared by constructing a recombinant virus (vsmIFN-γR) that expressed the mouse IFN-γR alpha chain truncated after Ser-257 so that it would be secreted from the cell. The vsmIFN-γR was compared with parental (vΔB8R) and revertant (vΔB8R-R) viruses.

The genomes of these five viruses were analysed by Southern blot analysis (see supplementary data 1 at JGV Online: http://vir.sgmjournals.org). This showed that the genomes of the recombinant viruses were as predicted.

Growth properties of the recombinant viruses *in vitro*

The plaque phenotype of all viruses was normal on BS-C-1 cells (data not shown). However, this analysis might not

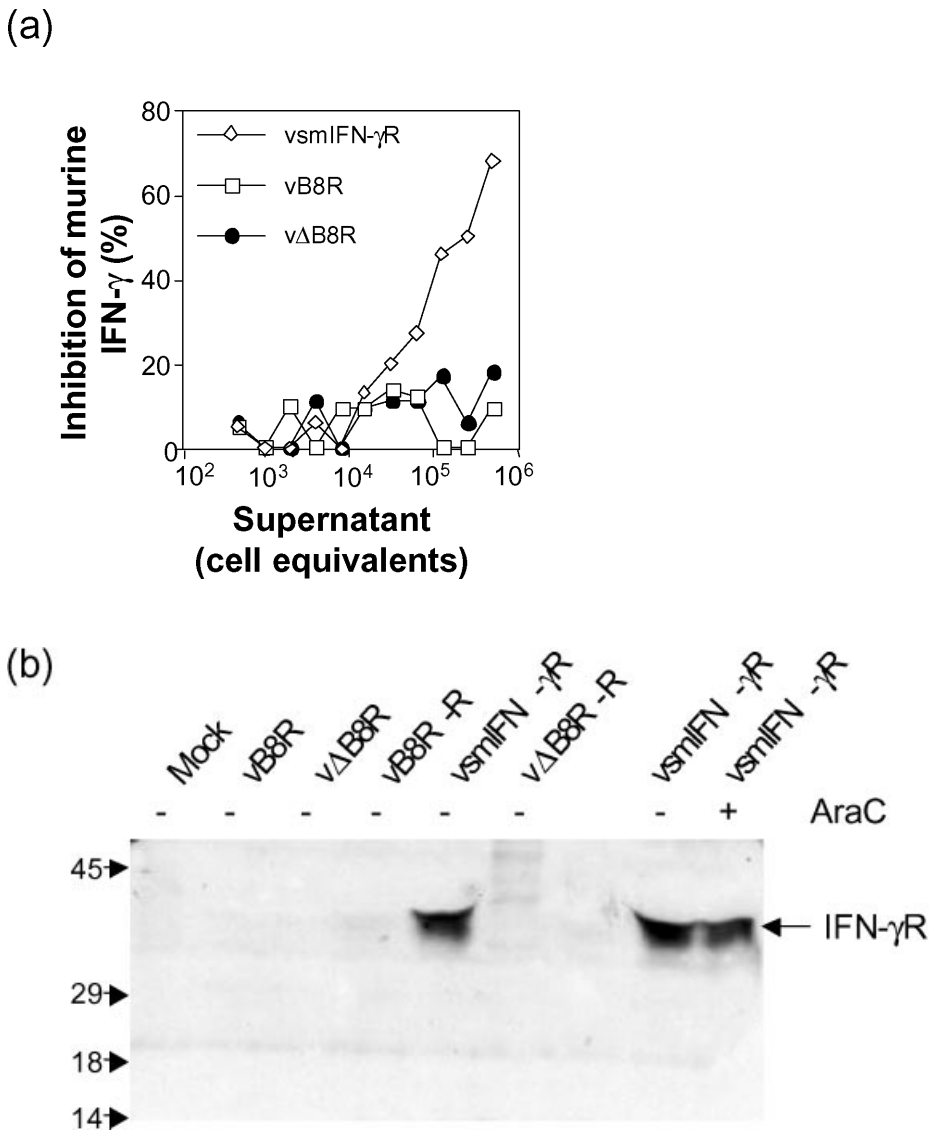

Fig. 3. Expression of mouse soluble IFN-γR by vsmIFN-γR. (a) Inhibition of mouse IFN-γ activity. TK⁻143 cells were infected with the indicated viruses and at 24 h p.i. the culture supernatants were prepared (Methods) and tested for inhibition of mouse IFN-γ by bioassay. Cocal virus was grown on murine L929 cells that had been pretreated with mouse IFN-γ (Alcamí & Smith, 1995) or untreated. Increasing amounts of supernatants from cells infected with vsmIFN-γR, WR or vΔB8R were added to the IFN-γ prior to addition to L929 cells. The inhibition of the antiviral effect of IFN-γ was measured by the restoration of cocal virus plaque formation. Data are expressed as the percentage inhibition of mouse IFN-γ. (b) Immunoblot. TK⁻143 cells were infected with the indicated viruses and at 24 h p.i. the culture supernatants were prepared (Methods) and analysed by immunoblotting using a mAb against the mouse IFN-γR. The position of the mouse soluble IFN-γR is indicated by an arrow and the positions of molecular size markers are indicated in kDa.

detect minor differences in virus replication and therefore the growth properties were investigated after infection of BS-C-1 cells at 0·01 p.f.u. per cell. Over a 48 h period the rate of increase in virus titre and the final titre attained were indistinguishable for all viruses (Fig. 2).

Expression of IFN-γRs by recombinant viruses

The ability of the viruses to express soluble proteins that bound IFN-γ was measured by ligand blotting and bioassay. First, proteins in the supernatants of mock-infected cells or cells infected with vB8R, vΔB8R and vB8R-R were tested for binding to human IFN-γ by cross-linking with EDC followed by SDS–PAGE and autoradiography. vB8R and vB8R-R expressed a protein that cross-linked with human IFN-γ and the formation of this complex was inhibited by a 100-fold excess of cold human IFN-γ but not IFN-α (data not shown). In contrast, vΔB8R did not express this protein, confirming that B8R was the only VV gene encoding such an activity.

The expression of soluble mouse IFN-γR by vsmIFN-γR was demonstrated by bioassay (Fig. 3a). Mouse IFN-γ

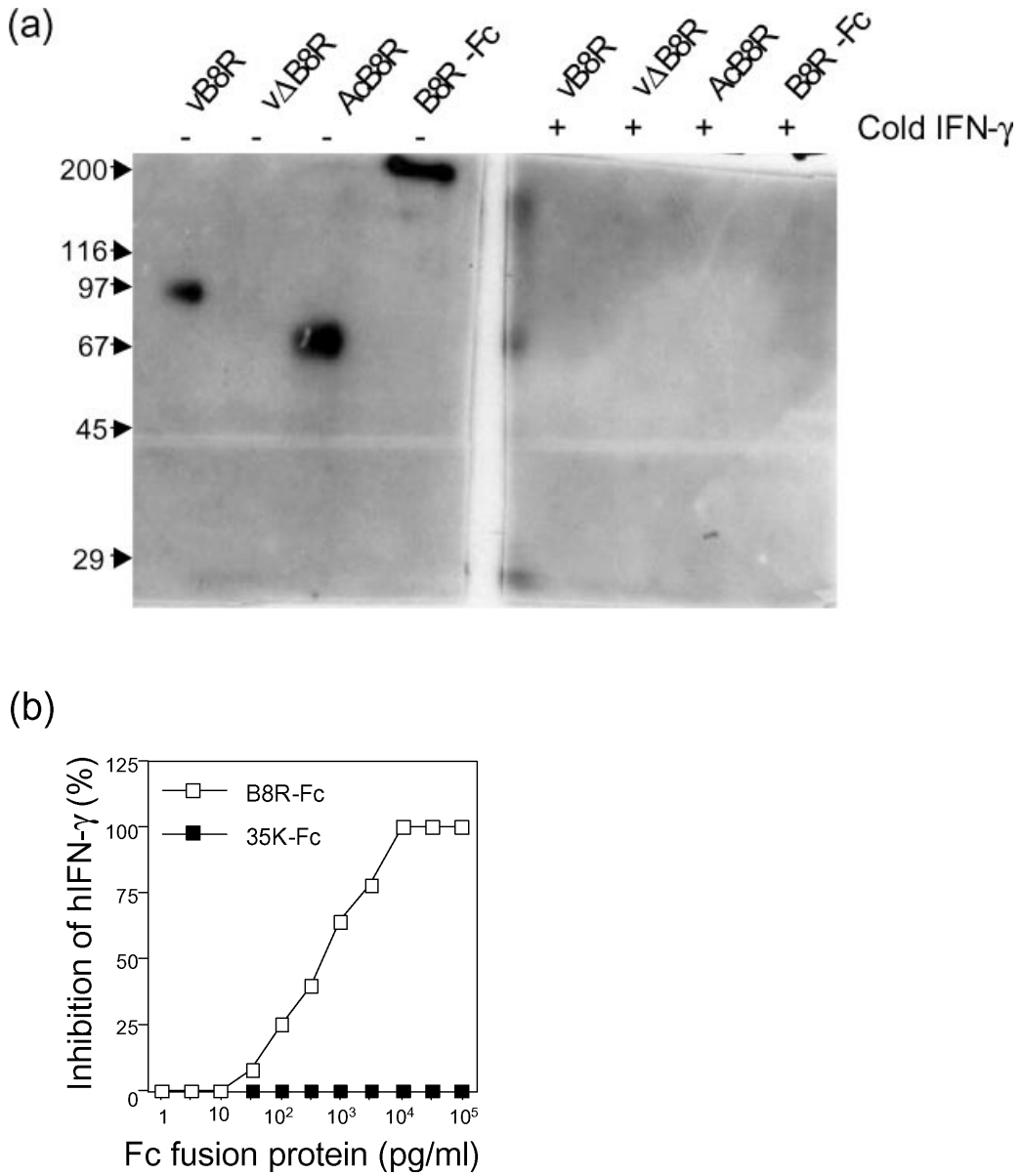

Fig. 4. Expression of B8R–Fc. (a) Detection of B8R–Fc by ligand blotting. COS cells were transfected with plasmid pCOS B8R–Fc (B8R–Fc) and after 3 days the supernatants were collected and separated by SDS–PAGE followed by ligand blotting (Methods). Supernatants from cells infected with vB8R, vΔB8R or AcB8R were analysed in parallel. Nitrocellulose filters were incubated with ^{125}I-hIFN-γ in the presence or absence of a 100-fold excess of cold human IFN-γ. After washing to remove unbound ^{125}I-hIFN-γ an autoradiograph was prepared. The positions of molecular size markers are shown in kDa. (b) Inhibition of human IFN-γ activity by B8R–Fc. HeLa cells were treated with human IFN-γ in the presence of increasing concentrations of purified B8R–Fc or 35K–Fc, or cells were untreated. The cells were then infected with cocal virus and the number of plaques was determined at 48 h p.i. Data are expressed as the percentage inhibition of human IFN-γ.

prevented the formation of plaques by cocal virus on mouse L929 cells and this activity was inhibited in a dose-dependent manner by the supernatants from cells infected with vsmIFN-γR but not by those from WR- or vΔB8R-infected cells. This showed that vsmIFN-γR expressed a soluble protein that inhibited mouse IFN-γ and confirmed that the IFN-γR made by VV WR does not inhibit mouse IFN-γ. Immunoblotting with a mAb specific for the mouse IFN-γR alpha chain confirmed expression of the mouse soluble IFN-γR (38–40 kDa) (Fig. 3b). The mouse IFN-γR gene was expressed from the VV B8R promoter to mimic the temporal regulation of the B8R gene. Early expression was confirmed by infecting cells with vsmIFN-γR with or without AraC and observing similar levels of protein.

Expression of B8R as an Fc-fusion protein

To produce recombinant B8R protein from a mammalian source, the B8R protein was fused to the Fc domain of human

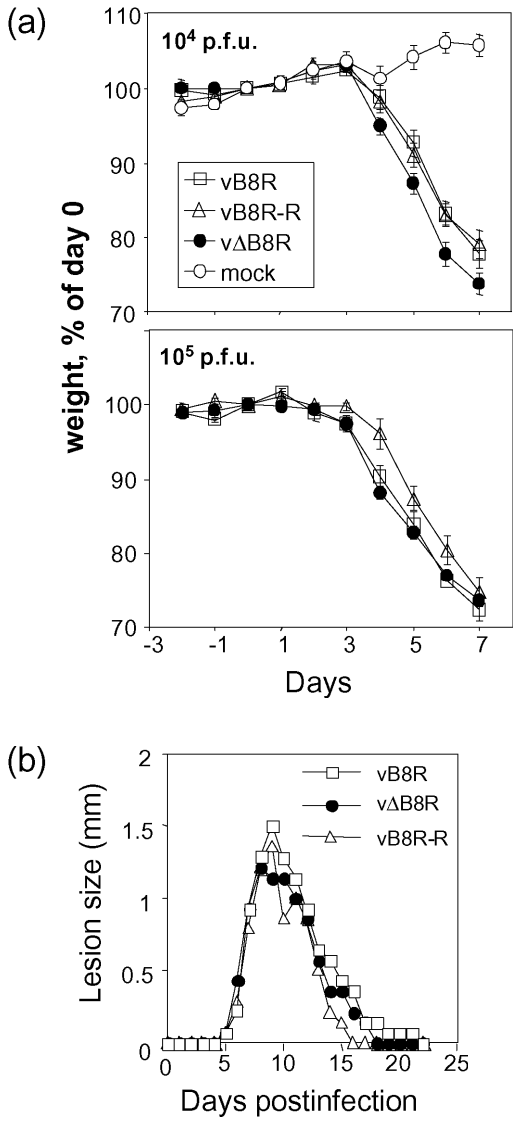

Fig. 5. Virulence of vΔB8R and control viruses. (a) Intranasal model. Groups of five mice were either mock-infected or infected with the 10^4 or 10^5 p.f.u. of the indicated viruses and their body weights were determined each day. Data are presented as the mean (±SEM) body weight of the group of animals compared to the mean weight of the same group on day 0. (b) Intradermal model. Groups of seven mice were infected by intradermal injection of 10^4 p.f.u. of the indicated viruses and the lesion sizes were measured daily. Data are presented as the mean.

IgG1 and expressed from transfected COS cells using methodology described previously for the CC chemokine-binding protein of VV strain Lister (Alcamí *et al.*, 1998). Supernatants from cells transfected with B8R–Fc, or from mammalian cells infected with WR or vΔB8R, or from Sf21 insect cells infected with a recombinant baculovirus that expresses the VV IFN-γR, AcB8R (Alcamí & Smith, 1995), were analysed by ligand blotting after electrophoresis in the absence of reducing agent (Fig. 4a). Cells infected with VV WR, but not vΔB8R, expressed an 85–90 kDa protein, consistent with the B8R protein existing as a homodimer (Alcamí & Smith, 2002). The size of the VV IFN-γR made by insect cells (~ 67 kDa) or as a B8R–Fc fusion (nearly 200 kDa) also indicated B8R was a homodimer. The specificity of each protein for IFN-γ was demonstrated by the inhibition of binding with a 100-fold excess of cold IFN-γ. The biological activity of purified B8R–Fc was demonstrated by its ability to inhibit the activity of human IFN-γ in a dose-dependent manner (Fig. 4b). Equivalent concentrations of 35K–Fc had no such inhibitory activity.

Affinity of B8R for human and mouse IFN-γ

The affinity of B8R for human and mouse IFN-γ was determined by surface plasmon resonance using BIAcore. B8R–Fc was bound to the surface of the sensor chip via the Fc domain that was recognized by an anti-Fc mAb. Different concentrations of human or mouse IFN-γ were then passed across the chip surface and the association, dissociation and K_d values were calculated from the association and dissociation kinetics. Although the association rates for human and mouse IFN-γ (5×10^6 and 1×10^6 mol/s, respectively) differed by only 5-fold, the dissociation rate for human IFN-γ ($4{\cdot}3 \times 10^{-4}$ mol/s) was approximately 100-fold slower than for mouse IFN-γ (4×10^{-2} mol/s). Consequently, B8R had a much higher affinity constant (9×10^{-11} M) for human IFN-γ than mouse IFN-γ (4×10^{-8} M) and this was consistent with the ability of B8R to inhibit human but not mouse IFN-γ in bioassay (Alcamí & Smith, 1995).

Contribution of B8R and mouse soluble IFN-γR *in vivo*

Although the B8R protein was dispensable for virus replication in cell culture it was likely to play a role *in vivo* and this was tested in three models. In a mouse intranasal infection model the mortalities (data not shown), weight loss (Fig. 5a) and signs of illness (not shown) of vB8R, vΔB8R and vB8R-R were not statistically different ($P < 0{\cdot}05$, Student's *t*-test) from each other on any day following infection with 10^4 or 10^5 p.f.u. Similarly, in a murine intradermal model (Tscharke & Smith, 1999) the resultant lesion size was indistinguishable for all three viruses (Fig. 5b). These results were consistent with the low affinity of the VV vIFN-γR for mouse IFN-γ and its inability to inhibit the biological activity of mouse IFN-γ *in vitro*.

Next the role of the VV IFN-γR was examined in a rabbit intradermal model, because the virus protein is known to inhibit the biological activity of the rabbit IFN-γ. At 3 days p.i. with 10^6 p.f.u. the lesions were examined histologically (Fig. 6). Infection with vΔB8R caused a greater infiltration of $CD43^+$ inflammatory cells than infection with WT or revertant viruses, although changes in lesion sizes were not apparent. Conversely, the level of VV antigen as measured using a mAb to the A27L gene product showed that after infection with vΔB8R there was a reduced level of VV antigen in the dermis.

To study the contribution of a soluble IFN-γR to VV virulence in a mouse model we constructed a recombinant

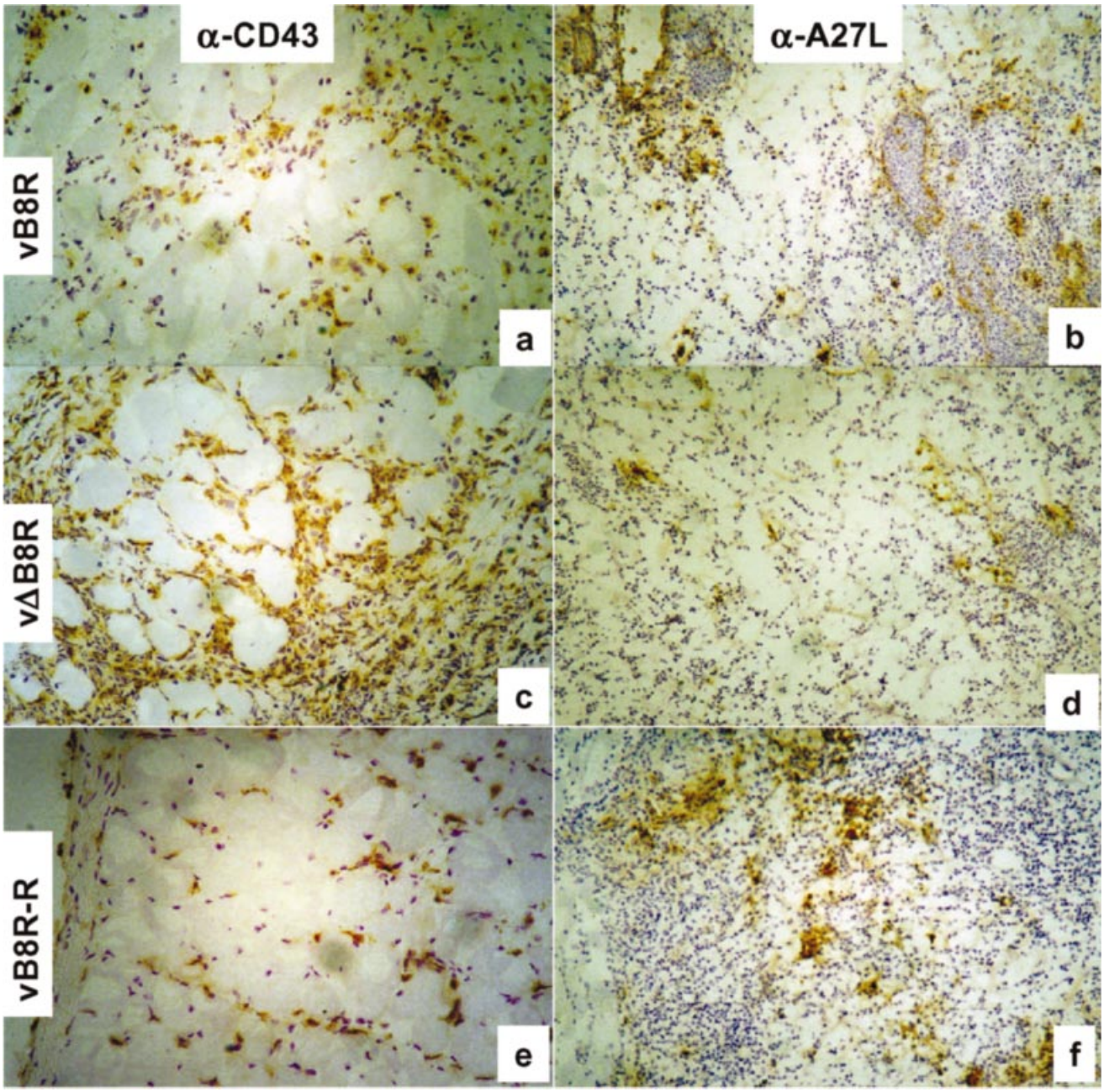

Fig. 6. Histological analysis of infected rabbit skin. New Zealand White rabbits were infected with the indicated viruses and at 3 days p.i. were sacrificed and the lesion was processed for immunohistochemistry using antibodies against rabbit CD43 (α-CD43) and against the VV A27L gene product (α-A27L) as described in Methods.

virus expressing the soluble mouse IFN-γR from the B8R locus under control of the B8R promoter. After intranasal infection, vsmIFN-γR was more virulent than vΔB8R and vΔB8R-R control viruses. On days 4, 5, 6 and 8 p.i. with 10^4 p.f.u. there was a statistically significant ($P < 0{\cdot}05$, Student's *t*-test) greater weight loss following infection with vsmIFN-γR than with each control virus (Fig. 7a). Furthermore, after infection with 10^4 or 10^5 p.f.u. the signs of illness (Alcamí & Smith, 1992) appeared sooner and were more severe with significant differences from controls on days 4, 5, 6 and 7 p.i. with each dose. In the intradermal mouse model, there was a slight reduction in mean lesion size after infection with vsmIFN-γR compared to controls but this difference was not statistically significant (Fig. 7b).

Inhibition of equine IFN-γ

Finally, we tested the ability of the VV IFN-γR to inhibit equine IFN-γ. Data presented show that the supernatants from

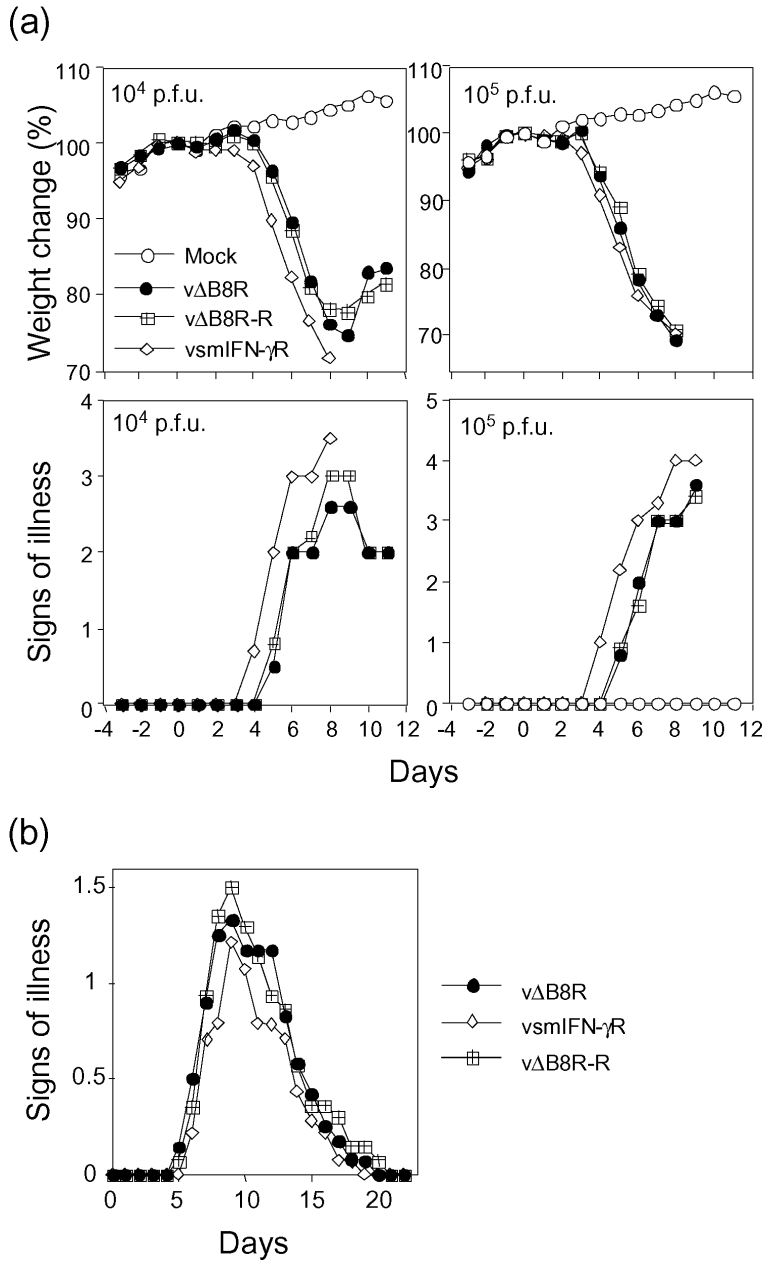

Fig. 7. Virulence of recombinant virus expressing the mouse soluble IFN-γR and control viruses. (a) Groups of five mice were either mock-infected or infected with the 10^4 or 10^5 p.f.u. of the indicated viruses and their body weights and signs of illness were recorded each day. Data are presented as the mean body weight of the group of animals compared to the mean weight of the same group on day 0 (top panels) and the mean signs of illness for each group (bottom panels). (b) Intradermal model. Groups of six or seven mice were infected by intradermal injection of 10^4 p.f.u. of the indicated viruses and the lesion sizes were measured daily. Mean values are shown.

cells infected with VV strain WR, but not vΔB8R, possess an activity that inhibits the biological activity of equine IFN-γ in a dose-dependent manner as measured by the reversal of the inhibition of SFV plaque formation by equine IFN-γ on equine dermal cells (Fig. 8). The VV IFN-γR has now been demonstrated to inhibit IFN-γ from man, cow, rat, rabbit, horse and chicken but not mouse.

Discussion

This report provides a further characterization of VV gene B8R gene and the encoded vIFN-γR including the contribution of this protein to virus virulence. Data presented demonstrate that gene B8R is transcribed early during infection, is non-essential for virus replication in cell culture and is the only VV WR gene encoding an IFN-γR. The range of IFN-γs that the VV IFN-γR inhibits is extended to include equine IFN-γ and the affinity constants of the B8R protein for human and mouse IFN-γ were determined. *In vivo*, the VV B8R protein did not affect virulence in mouse models, but affected the outcome of infection in rabbit skin. In contrast, a recombinant VV expressing mouse soluble IFN-γ had enhanced virulence compared to controls in the murine intranasal model.

Previously, the VV B8R protein was shown to inhibit IFN-γ from a wide range of species, although it distinguished between the closely related mouse and rat IFN-γ. Here the

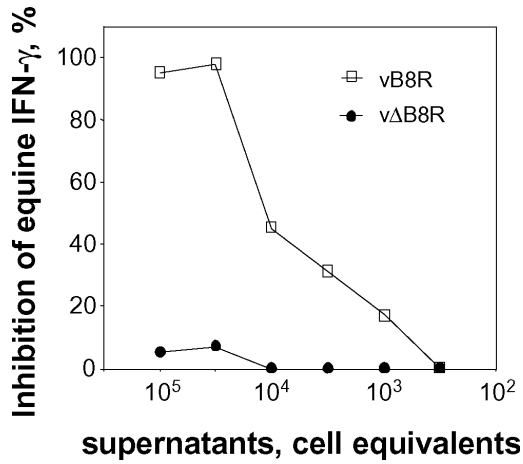

Fig. 8. VV vIFN-γR inhibits the biological activity of equine IFN-γ. Equine dermal cells were pretreated for 24 h with equine IFN-γ with or without increasing amounts of supernatants from vB8R or vΔB8R-infected cells. The cells were then infected with SFV and the number of plaques was counted 2 days later. Data are expressed as the percentage inhibition of the antiviral activity of IFN-γ. Data shown are from one experiment, which was repeated twice with similar results.

range of IFN-γs that B8R inhibits is extended to equine IFN-γ. The rationale for investigating equine IFN-γ was that early vaccinators took material from horses and used this for smallpox vaccination when cowpox virus was scarce. In addition the Ankara strain of VV, from which MVA was derived, was isolated from a horse in Turkey. This prompted Baxby (1981) to suggest that the unknown host of VV might be horses. If this is true, then the terms vaccinia, vaccine and vaccination might have been equinia, equine and equination!

The K_d for human and mouse IFN-γ was determined. As expected from the biological inhibition of human but not mouse IFN-γ, the B8R protein had a much higher affinity for human than mouse IFN-γ. The K_d for human IFN-γ (9×10^{-11} M) compares favourably with the affinity of human and mouse IFN-γR for their ligands (10^{-11}–10^{-10} M) (Aguet *et al.*, 1988; Hemmi *et al.*, 1989) thus explaining the ability of B8R to block the biological activity of human IFN-γ. In contrast, the affinity of B8R for mouse IFN-γ (4×10^{-8} M) is unlikely to be high enough to compete with the natural mouse cell surface receptor. The affinity of the soluble IFN-γR α chain is lower than that of the cell surface receptor ($K_d = 10^{-9}$ M) (Walter *et al.*, 1995). This is consistent with the finding that although the IFN-γR beta chain does not form an independent binding site for IFN-γ, it does contribute to the binding affinity for IFN-γ.

The low affinity for mouse IFN-γ was consistent with the lack of virus attenuation in two mouse models after deletion of the B8R gene. In contrast, in a rabbit model there was an increase in infiltration of $CD43^+$ leukocytes after infection with the deletion mutant compared to controls. This enhanced infiltration was accompanied by a corresponding decrease in the level of VV antigen. Our study has a different conclusion to another paper that reported that disruption of B8R reduced VV strain WR virulence after intranasal infection of $CB6F_1$ mice or intraperitoneal injection of nude mice (Verardi *et al.*, 2001). Possible reasons for this discrepancy are that different mouse strains were utilized, or that the insertion of strong, bidirectional, synthetic VV promoters and the β-galactosidase gene into the B8R locus affected virulence, or that the deletion mutants described by Verardi *et al.* contained an additional mutation that contributed to the attenuated phenotype. The latter possibility was not addressed by the construction of revertant viruses.

To study the contribution of an IFN-γR in a mouse model, we constructed a virus that expresses the mouse IFN-γR. To make the situation as analogous as possible to the VV B8R protein, the mouse IFN-γR gene was expressed from the B8R promoter and was truncated before the region encoding the transmembrane anchor so that the protein was secreted from the cell. However, one difference between the mouse soluble IFN-γR and the VV IFN-γR is the ability of the latter protein to dimerize in the absence of IFN-γ (Alcamí & Smith, 2002). This property may give the VV protein enhanced ability to neutralize IFN-γ. The secreted smIFN-γR inhibited the biological activity of the mouse IFN-γ and the recombinant virus expressing this protein had enhanced virulence in a mouse model. The changes *in vivo* observed by either insertion of mouse soluble IFN-γR (in the mouse model) or deletion of VV B8R (in the rabbit model) were modest compared to changes in virulence resulting from the deletion of genes encoding proteins affecting the egress of virus from infected cells, such as A34R (McIntosh & Smith, 1996), A36R (Parkinson & Smith, 1994) and F12L (Zhang *et al.*, 2000), but were similar to changes resulting from deletion of other immunomodulators (Moore & Smith, 1992; Alcamí & Smith, 1996).

The B8R protein affects the outcome of infection in rabbits but not in mice and deletion of the gene would be predicted to be an attenuating mutation in other species in which the host IFN-γ was inhibited by the VV protein. Consistent with this, VV MVA does not make an IFN-γR (Blanchard *et al.*, 1998) and is attenuated in man compared to other VV strains, although the contribution of B8R to this phenotype has not been determined. It will be interesting to examine the immune response to infection with viruses lacking the B8R protein (in rats or rabbits) or containing the mouse IFN-γR (in mice).

In conclusion, the VV B8R protein is shown to be a non-essential immunomodulator that affects virus virulence in a rabbit but not mouse model of infection. The K_d values of the B8R protein are consistent with the observed inhibition of human but not mouse IFN-γ by this protein.

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