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Inhibition of Interferons by Ectromelia Virus

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Ectromelia virus (EV) is an orthopoxvirus (OPV) that causes mousepox, a severe disease of laboratory mice. Mousepox is a useful model of OPV infection because EV is likely to be a natural mouse pathogen, unlike its close relatives vaccinia virus (VV) and variola virus. Several studies have highlighted the importance of mouse interferons (IFNs) in resistance to and recovery from EV infection, but little is known of the anti-IFN strategies encoded by the virus itself. We have determined that 12 distinct strains and isolates of EV encode soluble, secreted receptors for IFN-γ (vIFN-γR) and IFN-α/β (vIFN-α/βR) that are homologous to those identified in other OPVs. We demonstrate for the first time that the EV vIFN-γR has the unique ability to inhibit the biological activity of mouse IFN-γ. The EV vIFN-α/βR was a potent inhibitor of human and mouse IFN-α and human IFN-β but, surprisingly, was unable to inhibit mouse IFN-β. The replication of all of the EVs included in our study and of cowpox virus was more resistant than VV to the antiviral effects induced in mouse L-929 cells by IFN-α/β and IFN-γ. Sequencing studies showed that this EV resistance is likely to be partly mediated by the double-stranded-RNA-binding protein encoded by an intact EV homolog of the VV E3L gene. The absence of a functional K3L gene, which encodes a viral eIF-2α homolog, in EV suggests that the virus encodes a novel mechanism to counteract the IFN response. These findings will facilitate future studies of the role of viral anti-IFN strategies in mousepox pathogenesis. Their significance in the light of earlier data on the role of IFNs in mousepox is discussed.

The interferons (IFNs) are a large family of multifunctional cytokines that inhibit virus replication and spread via their direct antiviral and indirect immunoregulatory activities (70, 76). Multiple IFN-α subtypes and IFN-β are produced by virus-infected cells and bind to a single cellular IFN-α/β receptor (IFN-α/βR) (12, 56). IFN-γ is produced mainly by NK and T cells upon recognition of virus-infected cells and binds to a distinct cellular IFN-γ receptor (IFN-γR) (1, 13, 31). Disruption of the gene for either IFN-β (26), IFN-γ (24, 42), IFN-γR (39), IFN-α/βR (54), or both IFN receptors (74) renders mice highly susceptible to viral infection. Both types of IFN restrict viral replication via the induction of an antiviral state in cells bearing the appropriate receptor (70). They also have nonoverlapping roles in the activation and regulation of innate and adaptive immune responses to viral infection (12, 13).

The idea that IFNs play a central role in antiviral defense has been reinforced by the discovery of numerous viral mechanisms of IFN inhibition (35). The fundamental importance to viruses of IFN blockade is well illustrated by consideration of the poxvirus family (51). These large, cytoplasmic DNA viruses encode a number of gene products that interfere with the actions of IFNs as well as other cytokines (3). For example, members of the orthopoxvirus (OPV) genus, which includes *Vaccinia virus* (VV), *Cowpox virus* (CPV), *Ectromelia virus* (EV), and *Variola virus* (VaV), encode factors that sequester extracellular IFNs and block intracellular IFN-induced antiviral effects (66).

Outside the cell, poxviruses inactivate IFN-α/β and IFN-γ via their expression of two soluble, abundantly secreted proteins, the viral IFN-α/βR (vIFN-α/βR) (8, 23, 71) and viral IFN-γR (vIFN-γR) (4, 52, 53, 73). These bind to their respective IFNs with high affinity, preventing their interaction with cellular receptors. The vIFN-α/βR, encoded by the VV strain Western Reserve (WR) B18R gene, is an immunoglobulin superfamily glycoprotein with limited homology to the cellular IFN-α/βR. It acts both in solution and when associated with the cell surface (8). The vIFN-γR is a direct, soluble homolog of the cellular IFN-γR that is encoded by the VV WR gene B8R. Uniquely among IFNRs, the vIFN-α/βR and vIFN-γR bind IFNs from a broad range of host species. Deletion of the vIFN-γR or vIFN-α/βR gene from the VV genome attenuates the virus in a mouse model of infection (71, 75).

Inside the cell, IFNs induce expression of gene products that produce an antiviral state, in which viral replication is inhibited (70). Two major enzymes induced by IFN are protein kinase R (PKR) and 2′,5′-oligoadenylate synthetase (2′,5′-A synthetase), which are both activated by binding to double-stranded RNA (dsRNA), which is often produced during viral infection. Activated PKR and 2′,5′-A synthetase inhibit cellular translation via different pathways, preventing the expression of viral genes. There is also evidence that activation of both systems results in the induction of apoptosis (17, 27, 46, 82).

VV replication in cultured cell lines is poorly inhibited by IFN treatment (57, 59, 80), and the virus can protect coinfected viruses from IFN-induced antiviral effects (78, 79). This resistance is mediated by two intracellular VV proteins encoded by the genes E3L (20) and K3L (11). Recombinant VVs lacking either gene are sensitive to IFN treatment in in vitro cell culture (9, 11). Moreover, deletion of the E3L gene from VV renders the virus apathogenic in mice (15). The 190-amino-acid E3L gene product (34) is a dsRNA-binding protein (dsRBP) with a conserved C-terminal dsRNA-binding domain

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(18). Specific, sequence-independent binding of dsRNA by this viral dsRBP prevents the activation of PKR (20) and 2′,5′-A synthetase (60), allowing translation of viral mRNAs to continue. E3L is also required for VV replication in cells from a wide range of hosts (10).

The VV K3L gene encodes an 88-amino-acid homolog of the eukaryotic translation initiation factor eIF-2α (veIF-2α) (34). Phosphorylation of eIF-2α by activated PKR results in translational inhibition. The veIF-2α acts as a nonphosphorylatable PKR pseudosubstrate, competitively inhibiting eIF-2α phosphorylation and therefore, like E3L, viral mRNA translation. Recent work has also shown that IFN-γ signals transduced via Stat1 activation are inhibited by the VH1 phosphatase encoded by the VV H1L gene (55).

Study of the role of OPV virulence factors in pathogenesis requires an appropriate animal model of infection. EV is particularly useful in this regard because it is a natural mouse pathogen that causes mousepox, a generalized infection of laboratory mice that can be mimicked experimentally by footpad inoculation with extremely small doses of the virus (32). In vivo studies have underlined the importance of IFN-α/β and especially IFN-γ in the recovery of mousepox-resistant C57BL/6 mice from infection with EV (41, 43, 44). However, little information is currently available regarding the ability of EV to inhibit mouse IFNs.

Previous studies have demonstrated that multiple EV strains and isolates express soluble, secreted receptors and/or binding proteins that inhibit tumor necrosis factor (TNF), interleukin-1β (IL-1β), CC-chemokines, and IL-18 (14, 47, 68, 69). Binding and sequencing studies have indicated that the Moscow strain of EV expresses homologs of the OPV vIFN-α/βR and vIFN-γR (22, 23, 52), but nothing is known of their ability to block the biological activities of mouse IFNs.

Here, we demonstrate that 12 EV strains and isolates derived from diverse temporal and geographic sources express functional vIFN-γRs and vIFN-α/βRs. Crucially, we found that the EV vIFN-γR inhibited the biological activity of mouse IFN-γ and that the vIFN-α/βR inhibited mouse IFN-α but, surprisingly, not mouse IFN-β. The growth of all of the EVs in mouse L-929 cells was highly resistant to the antiviral effects of mouse IFNs. This resistance is presumably mediated partly by an EV homolog of the VV E3L gene product (EV dsRBP) but not by an EV veIF-2α, as the EV K3L homolog does not encode a functional gene product.

MATERIALS AND METHODS

Cells and viruses. BSC-1, L-929, and HeLa cells were cultured in Glasgow minimum essential medium supplemented with 10% fetal calf serum (FCS). *Spodoptera frugiperda* (Sf-21) insect cells and *Autographa californica* nuclear polyhedrosis virus were cultured in TC-100 medium (Sigma) containing 10% FCS.

VV strain WR, recombinant VV WR vΔB18R (71), CPV strain Brighton Red (BR), and all EV strains were grown in BSC-1 cells. The sources of the viruses VV WR, CPV BR, and the EV strains and isolates Hampstead, Moscow, Ishibashi I-111, MP-1, MP-2, MP-3, MP-4, MP-5, Hampstead egg, Naval, Mill Hill, and Cornell have been described elsewhere (68). The recombinant baculoviruses AcB8R and AcB15R have been described before (5, 6). *Cocal virus* was obtained from W. James (Sir William Dunn School of Pathology, University of Oxford).

Reagents. Human recombinant [^{125}I]IFN-γ (125 μCi/μg) was obtained from DuPont-New England Nuclear. Recombinant human, mouse, and rat IFN-γ with specific activities of 3×10^7 U/mg (human) and 10^7 U/mg (mouse and rat) were all obtained from Peprotech EC. Recombinant mouse IFN-α and IFN-β (specific activities, 5×10^6 and 9.4×10^6 U/mg, respectively) were obtained from Calbiochem. Human natural IFN-α (specific activity, 1.5×10^8 U/mg) derived from Namalwa cells was originally obtained from Wellcome (Beckenham, United Kingdom; now Glaxo SmithKline). Human IFN-β (specific activity, 2×10^8 U/mg) was a gift from G. L. Smith (Wright-Fleming Institute, Imperial College School of Medicine, London, United Kingdom).

Preparation of medium for binding and biological assays. FCS-free medium from OPV-infected BSC-1 cell cultures (5.8×10^5 adherent cells per ml of medium, or cell equivalents per milliliter) was collected at 48 h postinfection. After the removal of cellular material by centrifugation, BSC-1 supernatants were adjusted to 20 mM HEPES (pH 7.4), and any infectious VV, EV, or CPV present in them was inactivated with 4,5′,8-trimethypsoralen and exposure to UV light (72). Binding medium was RPMI 1640 containing 20 mM HEPES (pH 7.4) and 0.1% (wt/vol) bovine serum albumin. In some instances supernatants were concentrated and dialyzed against phosphate-buffered saline as described previously (6). Supernatants from Sf-21 cells infected with the recombinant baculoviruses AcB8R and AcB15R were prepared as described previously (5, 6).

Binding assays with [^{125}I]IFN-γ. Medium from 10^4 OPV-infected BSC-1 or baculovirus-infected Sf-21 cells was incubated with 1.51 nM human [^{125}I]IFN-γ in a volume of 25 μl for 2 h at room temperature and cross-linked with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC; 40 mM) as described (6). Samples were analyzed by denaturing sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. The binding specificities of OPV vIFN-γRs were further examined by the addition of excess unlabeled recombinant human, mouse, or rat IFN-γ in binding assays prior to the 2-h incubation.

Activity assay for IFN-γ and IFN-α/β. The biological activity of IFNs was measured by their inhibition of cocal virus plaque formation, as described previously (6, 71). Human IFNs were assayed in cultures of human HeLa cells, and mouse and rat IFNs were assayed in cultures of mouse L-929 cells. Cell monolayers in 24-well tissue culture plates were treated for 24 h at 37°C in 0.5 ml of medium containing 10% FCS in the presence or absence of IFN and viral supernatant. Monolayers were then rinsed and infected with approximately 100 PFU of cocal virus. Plaques were counted 18 to 24 h postinfection. OPV supernatants alone did not produce plaques in cell monolayers (data not shown).

Assay of OPV susceptibility to IFN-induced antiviral effects. Monolayers of L-929 cells in 10-cm^2 six-well tissue culture dishes were treated in 3 ml of medium containing 10% FCS and either no IFN, mouse IFN-γ, or mouse IFN-α/β. Mouse IFN-α/β consisted of equal doses (units per well) of recombinant mouse IFN-α and IFN-β. After 24 h at 37°C, monolayers were rinsed and infected with 4,000 PFU of OPV. At 4 days postinfection, cells and medium were harvested together, and the number of PFU in each well was measured by duplicate titration on BSC-1 cell monolayers.

Extraction of viral DNA and sequencing of viral genes. Viral DNA was prepared from BSC-1 cells infected with EV by extraction from viral cores (30). The vIFN-α/βR genes from EV strains Naval and Hampstead were PCR amplified with *Taq* DNA polymerase and the upstream oligonucleotide B18R-1 (5′-CCC GGATCCTATCGTATCCACCTACC-3′) and the downstream oligonucleotide B18R-2 (5′-CCCGTCGACTATATGTTCTCTATCGG-3′), based on the sequence of the B18R gene of VV WR.

The EV Hampstead and CPV BR homologs of the VV veIF-2α gene K3L were PCR amplified and sequenced with the upstream oligonucleotide EVK3L-1 (5′-AGATATAAAAACATAAGTTTATCC-3′) and the downstream oligonucleotide EVK3L-2 (5′-CATTGGTAAATCCTTGTAGGC-3′), which were based on the available veIF-2α gene sequence from EV Moscow (accession no. U67964).

The EV Hampstead and CPV BR homologs of the VV dsRBP gene E3L were PCR amplified and sequenced using the upstream oligonucleotide EVE3L-3 (5′-AATCTCCAGAACCAGCATCACC-3′) and the downstream oligonucleotide EVE3L-4 (5′-TGTGACAGATATCATCTTTAGAG-3′), which were based on the sequence of the E3L gene from VV Copenhagen (accession no. M35027).

DNA sequencing was carried out by the DNA sequencing service of the Department of Biochemistry (University of Cambridge), and the sequence data were analyzed using the Genetics Computer Group software package.

Nucleotide sequence accession numbers. The accession numbers of the sequences reported in this paper are AJ319804, AJ319805, AJ312291, AJ312292, AJ312293, and AJ312294.

RESULTS

EV isolates. In order to assess natural or passage-induced variations in the expression or function of viral IFN inhibitors

between different EVs, we included a collection of 12 EV isolates and strains from 10 temporally and geographically diverse laboratory outbreaks in our experiments. These were EV Hampstead, EV Moscow, EV MP-1, EV MP-2, EV MP-3, EV MP-4, EV MP-5, EV Cornell, EV Naval, and the plaque-purified virus EV Ishibashi I-111. Two egg-passaged strains derived from EV Hampstead, EVs Hampstead Egg and Mill Hill, were also included in the study. All of these viruses have been described elsewhere (68).

Binding of mouse IFN-γ by the EV vIFN-γR. A previous study (52) showed that EV Moscow expresses a secreted vIFN-γR which could be chemically cross-linked to ^{32}P-labeled human, mouse, and rabbit IFN-γ, enabling its detection by SDS-PAGE and autoradiography. Using a similar assay in which medium from uninfected or OPV-infected BSC-1 cells was incubated with human [^{125}I]IFN-γ prior to cross-linking, we confirmed that EV Moscow encodes a secreted vIFN-γR which forms a 54-kDa complex with the radiolabeled IFN. This activity was also identified in seven other EV isolates (EVs Hampstead, Ishibashi, and MP-1, -2, -3, -4, and -5) (Fig. 1a). Similar activities were detected in supernatants from BSC-1 cells infected with the WR strain of VV and from insect cells infected with a recombinant baculovirus expressing the VV WR B8R gene.

The earlier study (52) suggested that the EV vIFN-γR binds mouse IFN-γ with higher affinity than the homologous VV vIFN-γR, which does not inhibit mouse IFN-γ in assays of biological activity (6). We found that a 50-fold molar excess of unlabeled mouse IFN-γ almost completely displaced human [^{125}I]IFN-γ from its complex with the EV Hampstead vIFN-γR (Fig. 1b), while a 300-fold excess had no effect on the binding of human [^{125}I]IFN-γ by the VV WR vIFN-γR (Fig. 1c). Results similar to those for EV Hampstead were obtained with each of the seven EV isolates listed above (Fig. 1c). As expected, unlabeled human and rat IFN-γ readily displaced human [^{125}I]IFN-γ from its complexes with both VV and EV vIFN-γRs (Fig. 1b and c).

Inhibition of IFN-γ biological activity by the EV vIFN-γR. Although the binding of mouse IFN-γ by the EV vIFN-γR has been demonstrated, this evidence alone does not constitute proof that the viral factor inhibits the biological activity of mouse IFN-γ by preventing its binding to its cellular IFN-γR. Therefore, we examined the ability of EV vIFN-γRs to prevent the induction of an antiviral state in cultured cell lines by human or mouse IFN-γ (Fig. 2a). Cocal virus plaque formation in human HeLa and mouse L-929 cells was almost completely inhibited by treatment of the cells with either 50 U of human IFN-γ (HeLa) or 10 U of mouse IFN-γ (L-929) per ml. However, little or no inhibition of viral plaque formation was observed when cells were treated with the same concentration of IFN-γ and supernatants from BSC-1 cells that had been infected with each of 12 distinct EV strains and isolates. All of the EVs therefore express a secreted vIFN-γR that can inhibit the biological activity of human and, uniquely, mouse IFN-γ. As reported previously (6), supernatants from VV WR- and CPV BR-infected BSC-1 cells but not from mock-infected cells inhibited human but not mouse IFN-γ in these experiments (Fig. 2a).

In further experiments we compared the potency of VV WR- and EV Hampstead-infected BSC-1 supernatants as inhibitors of human, mouse, and rat IFN-γ (Fig. 1b, c, and d). Similar quantities of EV and VV supernatants (equivalent to 3×10^3 to 6×10^3 cells) were required to completely inhibit the antiviral effects induced in HeLa cells by 10 U of human IFN-γ per ml (Fig. 2b). Interestingly, EV Hampstead supernatants blocked the activity of mouse IFN-γ, while VV WR supernatants had no activity in this assay (Fig. 2c). The requirement for larger quantities of EV supernatant (equivalent to 10^5 cells) to completely block the biological activity of 10 U of mouse IFN-γ per ml compared to human IFN-γ suggested a lower affinity for mouse IFN-γ, since the concentration of EV IFN-γR in the supernatants is constant. Notably, EV Hampstead supernatants inhibited rat IFN-γ at lower doses than did VV WR supernatants.

The antiviral effects induced in L-929 cells by 40 U of rat

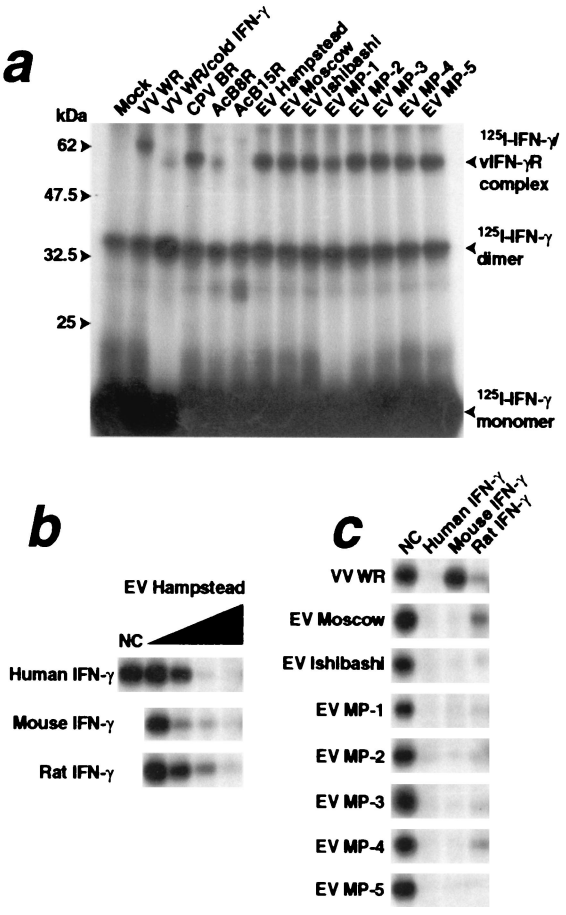

FIG. 1. Cross-linking of human [^{125}I]IFN-γ to the vIFN-γR of eight different EV isolates. (a) Medium from 10^4 BSC-1 or Sf-21 cells either mock infected or infected with the indicated viruses was cross-linked to 1.51 nM human [^{125}I]IFN-γ with EDC and analyzed by SDS-PAGE and autoradiography. Unlabeled (cold) human IFN-γ was used in 1,000-fold molar excess over [^{125}I]IFN-γ to compete with its binding to the VV WR vIFN-γR. AcB8R and AcB15R are recombinant baculoviruses expressing the secreted VV B8R (vIFN-γR) and B15R (vIL-1βR) gene products, respectively. (b) Competition of binding of human [^{125}I]IFN-γ to the vIFN-γR of the EV Hampstead isolate at a 10-, 50-, 100-, or 300-fold molar excess of human, mouse, or rat IFN-γ. (c) Competition of human [^{125}I]IFN-γ binding to the vIFN-γR of VV WR and seven EV isolates with a 300-fold molar excess of unlabeled IFN-γ from the species indicated. NC, no competitor.

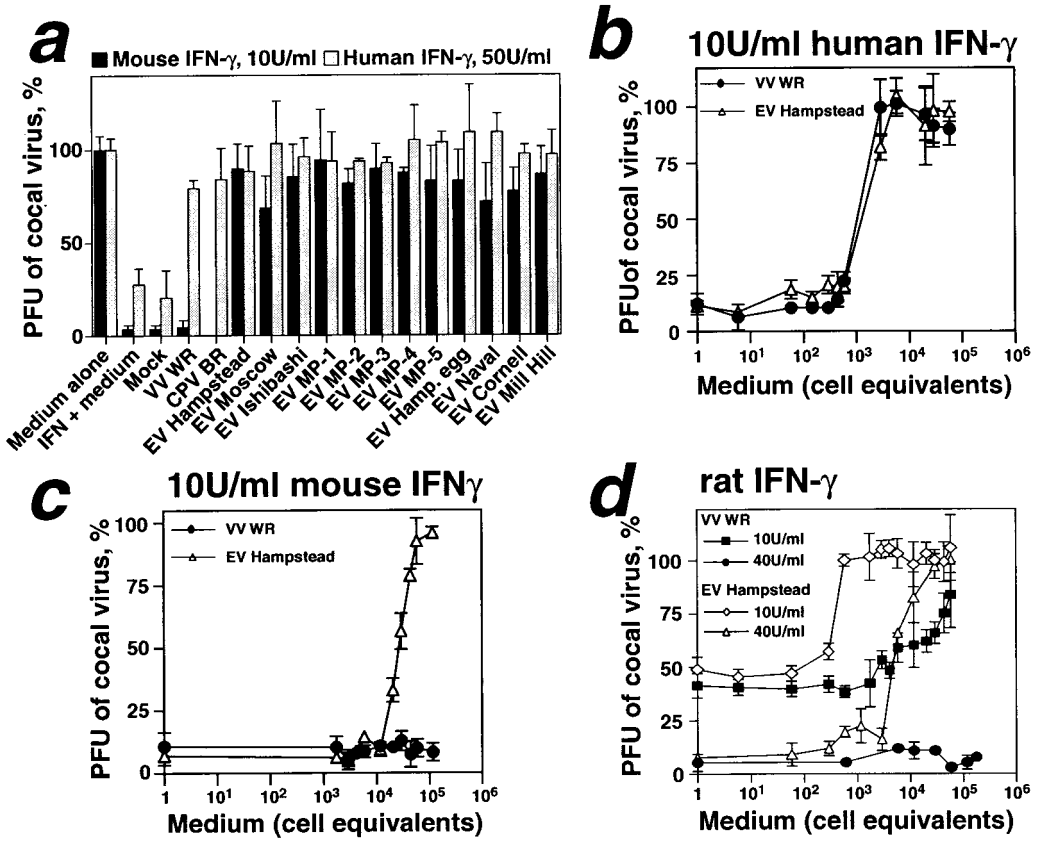

FIG. 2. Inhibition of IFN-γ by the EV vIFN-γR. (a) Expression of vIFN-γR by different EV strains and isolates. Monolayers of L-929 or HeLa cells were incubated with either medium alone or the indicated doses of mouse (L-929) or human (HeLa) IFN-γ, with or without medium from 2.5×10^4 BSC-1 cells that were either uninfected (mock) or infected with the indicated OPVs. After 24 h, monolayers were infected with approximately 100 PFU of cocal virus. Cocal virus plaque numbers are expressed as a percentage of the plaques formed in the absence of IFN or BSC-1 supernatant. (b, c, and d) Dose dependence of IFN-γ inhibition by vIFN-γRs from VV WR and EV Hampstead. Experiments were performed as in panel a but with a range of doses of VV WR- and EV Hampstead-infected BSC-1 supernatant. In d, L-929 cells were treated with either 10 or 40 U of rat IFN-γ per ml. Results shown are from representative experiments and are the means and standard deviation of four separate determinations.

IFN-γ per ml were prevented by equivalents of EV Hampstead supernatant from 6×10^4 cells, but were not inhibited at all by VV WR supernatant from up to 1.75×10^5 cells (Fig. 2d). At a lower concentration of rat IFN-γ (10 U/ml), inhibition of biological activity by the VV WR vIFN-γR was observed, as reported previously (6), but the supernatant dose required was greater than that of EV Hampstead (Fig. 2d).

Inhibition of IFN-α/β by the EV vIFN-α/βR. A previous report provided evidence for the existence of an EV vIFN-α/βR by showing that EV Moscow-infected L-929 cells produced a secreted and cell surface-bound activity that could be cross-linked to human [^{125}I]IFN-α2 (23). More recently, sequencing of the vIFN-α/βR gene from EV Moscow revealed that a full-length open reading frame (ORF) that is predicted to be functional is present in this virus (22). However, to date there have been no published analyses of the ability of the EV vIFN-α/βR to inhibit the biological activities of IFN-α/β.

Using a cocal virus plaque formation assay similar to that employed for IFN-γ, we tested 12 EV strains and isolates for expression of secreted vIFN-α/βRs. Medium from BSC-1 cells infected with each of the 12 EVs prevented the induction of antiviral effects in HeLa cells by either a mixture of IFN-α subtypes or recombinant human IFN-β (both at 50 U/ml) (Fig. 3a). Similar results were obtained with VV WR and CPV BR, as reported previously (71). Supernatants from mock-infected cells and cells infected with the vIFN-α/βR deletion mutant VV ΔB18R (71) did not affect IFN bioactivity. Therefore, all of the EV isolates and strains that we tested expressed a functional vIFN-α/βR.

EV is likely to be a natural pathogen of the mouse, so it was essential to determine whether its vIFN-α/βR effectively inhibits mouse IFN-α/β. Like the VV and CPV vIFN-α/βRs, the EV vIFN-α/βR completely inhibited the antiviral effects induced in L-929 cells by 50 U of recombinant mouse IFN-αA per ml (Fig. 3b). We were surprised to find, however, that none of the OPV supernatants tested contained measurable inhibitory activity against 50 U of recombinant mouse IFN-β per ml.

Comparison of the dose dependence of IFN-α/β inhibition by VV WR and EV Hampstead supernatants revealed no difference between the vIFN-α/βRs of the two viruses (Fig. 4). For both viruses, medium from 10^2 to 10^3 infected BSC-1 cells was sufficient to block the biological activity of 10 U of either

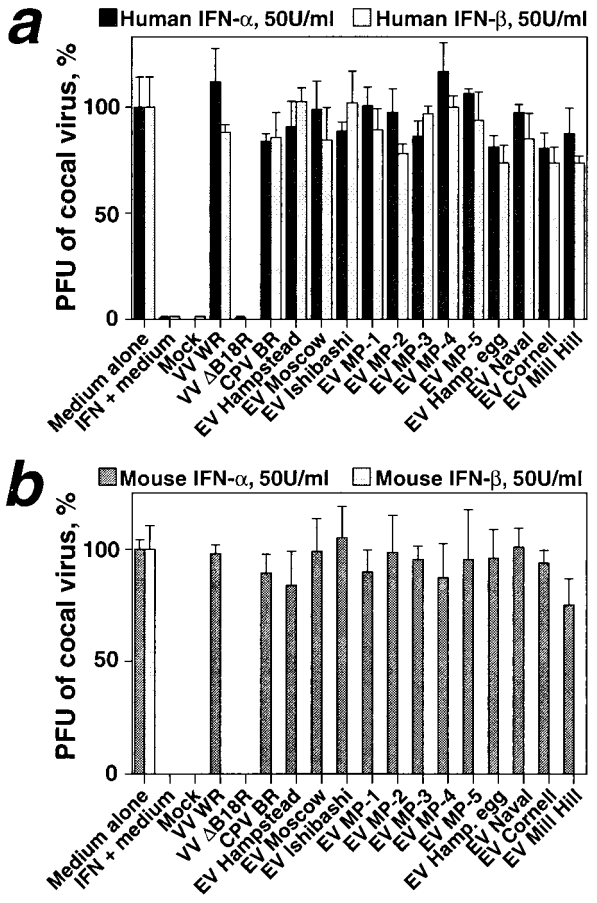

FIG. 3. Expression of vIFN-α/βRs by different EV strains and isolates. HeLa (a) and L-929 (b) cells were treated with 50 U of human natural IFN-α or recombinant IFN-β (a) or recombinant mouse IFN-α or -β (b) per ml with or without medium from 2.5×10^4 uninfected or OPV-infected BSC-1 cells. Plaque numbers determined after cocal virus infection are expressed as in Fig. 1. VV ΔB18R is a recombinant VV WR lacking the vIFN-α/βR gene B18R (71). Results shown are from representative experiments and are the means and standard deviation of four separate determinations.

human IFN-α (Fig. 4a), human IFN-β (Fig. 4b), or mouse IFN-α (Fig. 4c) per ml. In contrast, medium from 10^6 EV- or VV-infected cells did not inhibit the antiviral activity of 10 U of mouse IFN-β per ml (Fig. 4d).

Comparison of EV vIFN-α/βR gene sequences with those of other OPVs. We amplified the region of the EV Hampstead and EV Naval genomes containing the EV vIFN-α/βR gene by PCR and sequenced the PCR products using flanking and internal oligonucleotide primers. The predicted amino acid sequences of the EV Hampstead and Naval vIFN-α/βRs were compared with published vIFN-α/βR sequences from EV Moscow (22), VaV (strain Bangladesh 1975 [BSH]) (49), CPV strain GRI-90 (64), and VV WR (67) (Fig. 5). The three EV sequences were identical except for the conservative substitution of a glutamate for an aspartate residues at position 144 in EV Naval. The three EV genes encode a 358-amino-acid product that is 88.6, 89.7, and 94.6% identical to the corresponding gene products from VaV BSH, VV WR and CPV GRI-90, respectively.

There are 15 residues in the predicted mature EV gene product that differ from those in the corresponding positions in all of the other OPV sequences. Nine of these residues occur within a stretch of 18 amino acids in the center of the second immunoglobulin domain. It is not known whether these mutations have a subtle influence on vIFN-α/βR function in EV, since they do not affect its binding specificity in our assays.

Sequence of the EV dsRNA-binding protein and veIF-2α genes. Numerous studies have demonstrated the importance of the VV E3L (dsRBP) and K3L (veIF-2α) gene products in promoting the resistance of the virus to IFN-induced antiviral effects in in vitro culture. Therefore we examined whether EV encodes homologs of these important VV virulence factors.

A search of the sequence database for homologs of the 88-amino-acid VV K3L ORF revealed that the corresponding EV locus has been sequenced in the Moscow strain (accession no. U67964 [77]). The ORF of the EV gene has been truncated by the insertion of a T residue in position 1 of codon 32, which creates a TGA stop codon (Fig. 6a). The reading frame that codes for the final 57 amino acids of the ancestral ORF is further disrupted by the deletion of two G residues spanning codons 58 and 59 of the full veIF-2α amino acid sequence. Finally, there is a 25-bp deletion towards the end of the gene which would have resulted in the replacement of the final 13 amino acids of the intact veIF-2α ORF with 17 unrelated residues (data not shown).

Examination of the VV veIF-2α gene sequence revealed that the 25 bases deleted from the EV gene form a tandem repeat

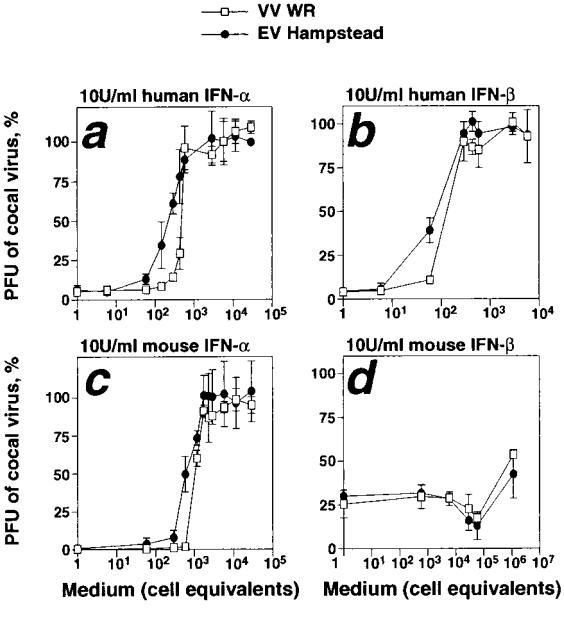

FIG. 4. Comparison of IFN-α/β inhibitory activities of VV WR and EV Hampstead vIFN-α/βRs. Experiments were performed as in Fig. 2, with the indicated concentrations of human and mouse IFN-α and IFN-β and a range of doses of supernatants from VV WR- and EV Hampstead-infected BSC-1 cells. Results shown are from representative experiments and are the means and standard deviation of four separate determinations.

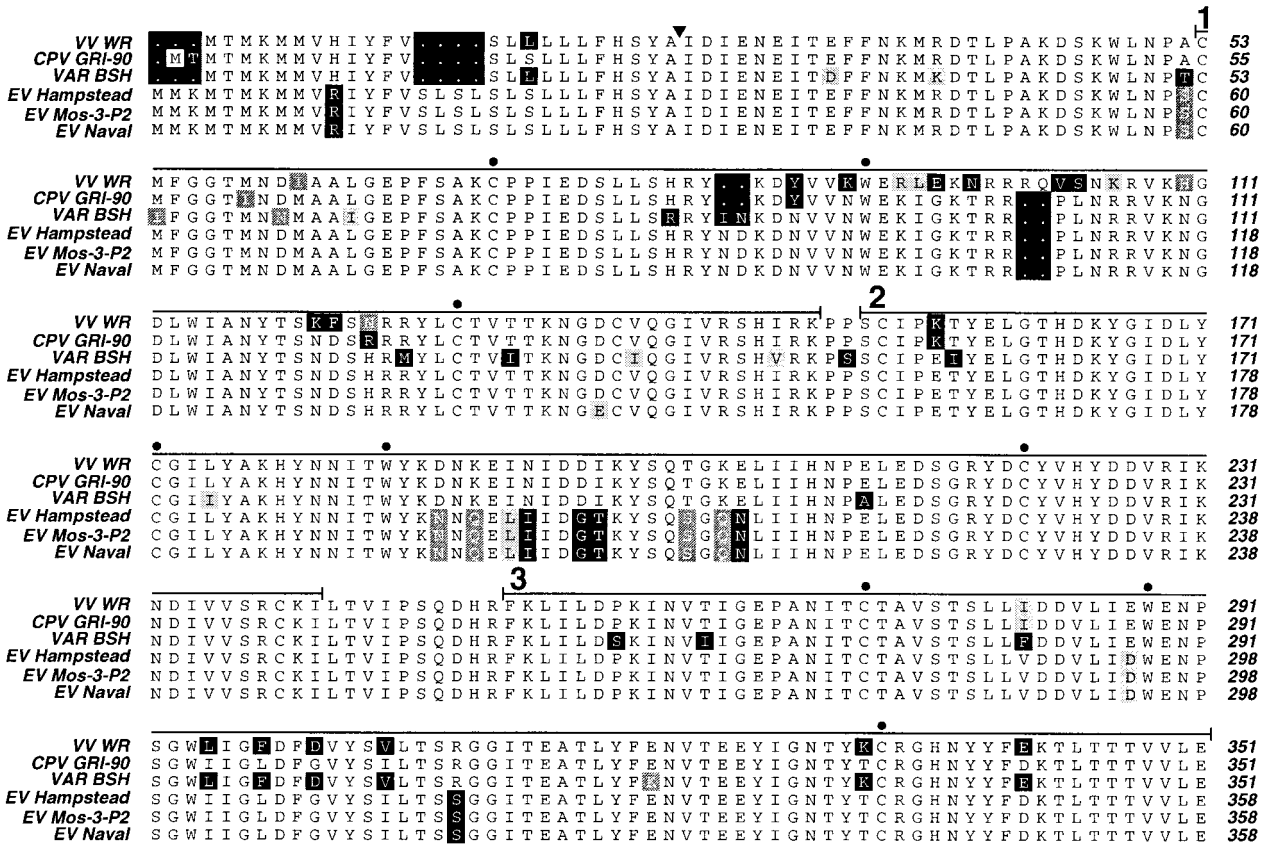

FIG. 5. Comparison of the predicted amino acid sequences of OPV vIFN-α/βRs. Multiple alignment of predicted amino acid sequences from the indicated OPV vIFN-α/βR genes (accession no. D01019, Y15035, P33795, AJ319804, AAC99571, and AJ319805). In this alignment, residues which differ from the consensus sequence are highlighted. Identical amino acids are shown black on white, nonconservative substitutions are white on black, semiconservative substitutions are white on dark grey, and conservative substitutions are black on pale grey. The predicted site of N-terminal secretory signal peptide cleavage is indicated by an arrowhead. Solid circles indicate the positions of invariant cysteine residues. The immunoglobulin domains (1, 2, and 3) are indicated by an unbroken line above the sequences, and their characteristic cysteine and tryptophan residues are indicated by solid circles. Dots indicate amino acid deletions.

with the 25 bases immediately downstream of them (data not shown). The deletion therefore probably occurred as a result of recombination between the two short, highly homologous sequences in the repeat and may indeed have been the lesion which originally inactivated the functional ancestral gene. Sequencing of the veIF-2α locus in the genomes of EV Hampstead and CPV BR revealed that the EV Hampstead DNA sequence is 100% identical to that of EV Moscow, while the CPV BR sequence encodes an intact veIF-2α ORF (Fig. 6a).

The EV and CPV homologs of the VV E3L gene have not been identified and sequenced previously. We therefore PCR amplified and sequenced fragments of the EV Hampstead and CPV BR genomes that are homologous to the VV E3L gene using oligonucleotide primers based on the VV DNA sequence. The predicted amino acid sequences of the two genes revealed that they are likely to be functional (Fig. 6b). Both are over 90% identical to their counterparts in VV and VaV BSH. The EV sequence is most divergent, being 91 to 93% identical to the CPV and VV sequences and 95.2% identical to VaV BSH E3L. The CPV sequence is over 95% identical to those of VV Copenhagen and VaV BSH and 93.7% identical to VV WR. Unsurprisingly, almost all of the variability in the OPV E3L amino acid sequences occurs towards the N terminus, outside the highly conserved dsRNA-binding domain (18) that is essential for the inhibition of IFN-induced antiviral pathways (Fig. 6b).

Resistance of EV to IFN antiviral effects. A recombinant VV lacking the K3L gene was markedly more susceptible to the effects of mouse IFN in L-929 cells than was a wild-type VV expressing a functional veIF-2α (11). Since EV is predicted not to express a functional veIF-2α, it was of interest to assess the resistance of this virus to IFN in a similar experimental system. We examined the effect of treatment with various doses of IFN on virus production by L-929 cells infected with either EV Hampstead or VV WR (Fig. 7a). Treatment of the monolayer with mouse IFN-α/β or IFN-γ (667 U/ml) reduced the production of VV WR from the cells by 99.8%. A 10-fold reduction in virus production was observed with doses of 17 U of IFN-α/β and 170 U of IFN-γ per ml. In contrast, 667 U of IFN-α/β or IFN-γ did not significantly reduce the production of EV Hampstead from the cells.

In a further experiment, the effect of treatment with 167 U

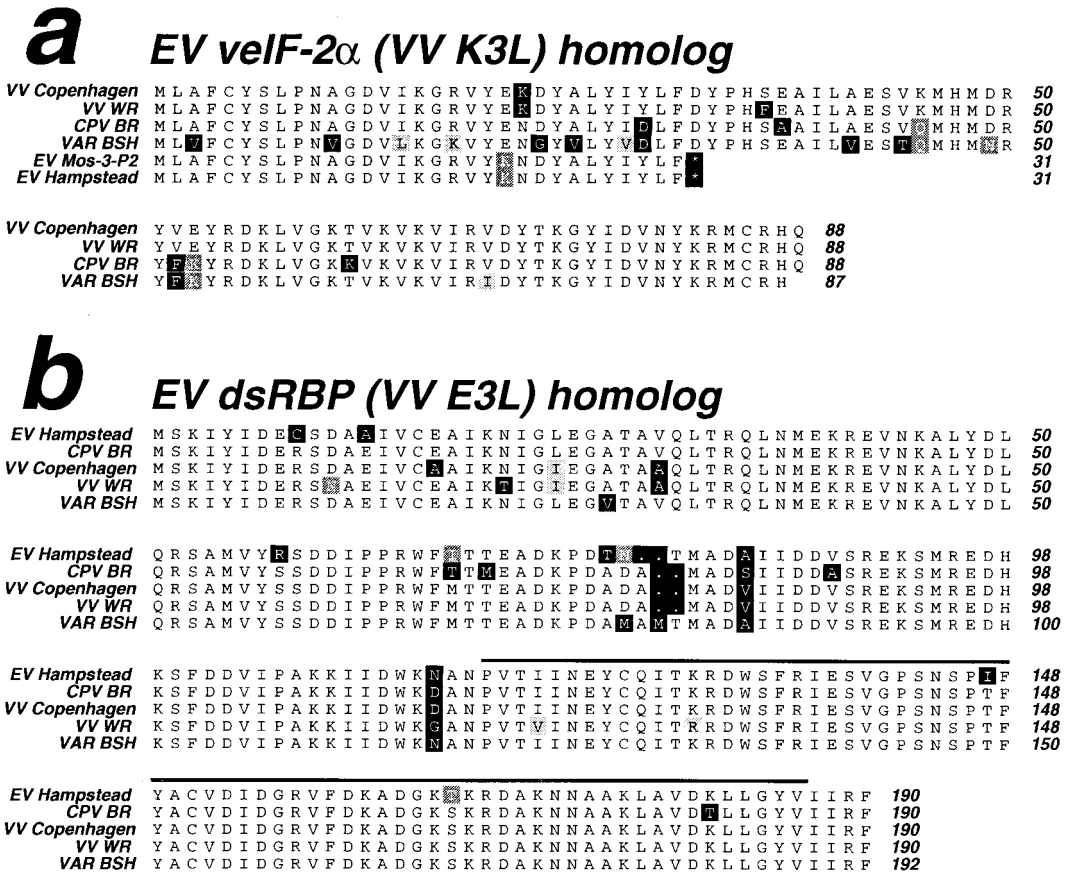

FIG. 6. Comparison of the predicted amino acid sequences of OPV veIF-2α and dsRBP genes. (a) Multiple alignment of predicted amino acid sequences of veIF-2α genes from the indicated OPVs (accession no. M35027, U67964, P18378, L22579, AJ312291, and AJ312292). The two EV ORFs are truncated by a frameshift mutation that has converted codon 32 to a stop codon (indicated by asterisks). (b) Comparison of predicted amino acid sequences of dsRBP genes from the indicated OPVs (accession no. AJ312294, AJ312293, M35027, P21605, and Q85372). The C-terminal dsRNA-binding domain is indicated by an unbroken line above the sequence. In both panels, identical residues are shown black on white, conservative substitutions are black on pale gray, semiconservative substitutions are white on dark gray, and nonconservative substitutions are white on black.

of IFN-α/β or 667 U of IFN-γ per ml on virus replication in cells infected with each of our 12 EV strains and isolates as well as with CPV BR and VV WR was assessed. Again, a large IFN-induced reduction in VV WR production was observed, while neither the EVs nor CPV BR showed a reduction in virus output of greater than 10-fold.

DISCUSSION

We have demonstrated that 12 strains and isolates of EV that were isolated from a wide geographical and temporal range of laboratory mousepox outbreaks (68) express functional homologs of the OPV vIFN-γR and vIFN-α/βR. For the first time, we have established that the binding of mouse IFN-γ by the EV vIFN-γR is biologically relevant, since it results in a blockade of the antiviral activity of this cytokine. This finding is important because it is not possible to accurately predict the biological activities of viral cytokine inhibitors by using binding assay data alone. For example, early studies showed that the myxomavirus vIFN-γR binds to human and mouse IFN-γ (73), yet later work demonstrated that this vIFN-γR specifically blocks only rabbit IFN-γ (53).

In further experiments, we found that the EV vIFN-α/βR blocks the biological activity of mouse IFN-α. Surprisingly, since the binding of mouse IFN-β by the VV vIFN-α/βR has been demonstrated (71), neither the EV nor the VV vIFN-α/βR inhibited mouse IFN-β. The replication of EV was also highly resistant to the antiviral pathways induced in a mouse cell line by IFN-α/β and IFN-γ. Sequencing studies indicated that this resistance is partly mediated by an EV dsRBP, a homolog of the VV E3L protein, but not by a homolog of the VV K3L (veIF-2α) protein, since the EV veIF-2α gene is nonfunctional.

These and earlier studies (14, 22, 23, 47, 52, 68, 69) have now established that EV encodes soluble, secreted receptors and binding proteins for IL-1β (vIL-1βR), TNF (vTNFR), CC-chemokines (vCKBP), IFN-γ (vIFN-γR), IFN-α/β (vIFN-α/βR) and IL-18 (vIL-18BP), all of which inhibit mouse cytokines. This subset of EV gene products includes all of the known OPV secreted cytokine inhibitors except the vTNFRs

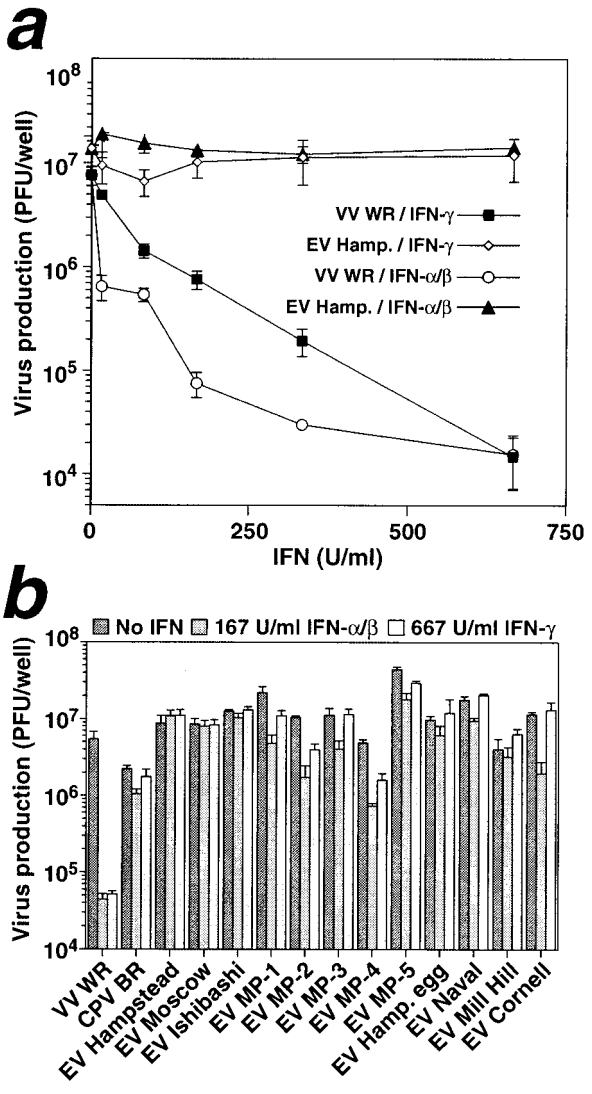

FIG. 7. Replication of EV in IFN-treated L-929 cells. (a) Effect of a range of concentrations of IFN-α/β and IFN-γ on infectious OPV production by L-929 cells infected with either VV WR or EV Hampstead. (b) Production of infectious OPVs from BSC-1 cells after treatment of the cells with either no IFN, 167 U of IFN-α/β, or 667 U of IFN-γ per ml. Data, which are representative of several experiments, are the means and standard deviation from two samples that were each titrated in duplicate on BSC-1 cell monolayers.

cytokine response modifier B (CrmB) (38), CrmC (65), and CrmE (62), whose activities overlap to some extent those of CrmD, the sole vTNFR identified in EV (47, 62). All of the factors are expressed by all of the EV isolates examined to date (68, 69), with the exception of EV Mill Hill, a laboratory-passaged derivative of EV Hampstead that lacks a functional vCKBP (68).

The expression of functional cytokine receptors by all EV isolates implies their importance in establishing a successful EV-host interaction that guarantees persistence of EV in its natural host. Indeed, studies using VVs lacking specific cytokine receptor genes have demonstrated their significant contribution to OPV virulence in the mouse (5, 71, 75). However, unlike EV, VV is not a natural mouse virus; only one EV cytokine inhibitor, the vIL-18BP, has been studied in this way using the mousepox model (14). In vivo studies of the other EV cytokine inhibitors will provide information on their roles in a natural OPV infection.

The uniform expression of cytokine inhibitors by different EVs is interesting because early studies demonstrated clear differences in their virulence. For example, the EV Hampstead Egg and Ishibashi I-111 strains are both attenuated in mice. The highly virulent EV Moscow and EV Hampstead isolates also differ subtly from each other in their pathogenesis (32, 33). The polymorphisms responsible for these differences must therefore lie within other virulence genes. In contrast, the expression of vTNFRs and the vCKBP is not uniform among different VV strains (2, 7).

Consideration of the properties of IFN inhibitors expressed by EV provides an insight into the selective pressures imposed by the mouse IFN system. Via its expression of the vIFN-γR and vIFN-α/βR and predicted expression of the intracellular dsRBP, EV sequesters mouse IFN-γ and IFN-α in biologically inactive forms and directly blocks their antiviral effects. The blockade of IL-18 by the EV vIL-18BP is also relevant because this cytokine synergizes with IL-12 to induce IFN-γ production by NK and T cells (28). The balance between the activities of EV IFN inhibitors and the ability of the host to produce IFN will influence viral replication and spread. It is therefore instructive to compare our data with earlier studies of the role of IFNs in mousepox.

Previous studies showed that IFN-γ production is essential for the recovery of mousepox-resistant C57BL/6 mice from infection with EV Moscow (43). Antibody-mediated depletion of IFN-γ from these mice converted inapparent mousepox into a severe disease resembling that seen in susceptible strains of mice such as BALB/c. Susceptibility to mousepox correlates with weak production of the T helper 1 (Th1) cytokines IL-12 and IFN-γ (42). Furthermore, IFN-γ inhibits EV replication in mouse macrophages via its induction of nitric oxide synthase; inhibitors of this enzyme converted resolving into fulminant mousepox in resistant C57BL/6 mice (44).

Indirect evidence for the importance of IFN-γ in the response to EV infection comes from two recent studies. In the first, a recombinant EV overexpressing mouse IL-4 suppressed IFN-γ production and caused severe mousepox in C57BL/6 mice (40). In the second study, BALB/c mice deficient in the STAT6 transcription factor, which is essential in the development of T helper 2 (Th2) responses, were more resistant to EV infection than wild-type mice. Increased resistance to EV correlated with enhanced expression of Th1 cytokines, including IFN-γ (48). Additionally, the attenuation of an EV with an inactivated vIL-18BP gene correlated with increased IFN-γ production in infected mice (14).

The vIFN-γRs from VV and CPV inhibit human, bovine, rat, and chicken IFN-γ (6, 58) but fail to neutralize mouse IFN-γ (6). In contrast, EV has acquired the unique ability to inhibit mouse IFN-γ (52). Mousepox resistance must therefore depend partly on the production of sufficient IFN-γ to saturate the EV vIFN-γR. The evidence for the role of IFN-γ in recovery from EV infection suggests that this occurs in resistant mice, because if the virus completely inhibited host IFN-γ, then experimental IFN-γ depletion would not affect pathogen-

esis (14, 40, 43, 44). Conversely, weaker IFN-γ production by susceptible mouse strains (42) may allow the virus to neutralize it completely, with the result that severe disease occurs.

The structural and biochemical basis for the inhibition of mouse IFN-γ by the EV vIFN-γR is unknown but results from a small number of changes in its amino acid sequence relative to the highly similar vIFN-γRs of VV and CPV. A previously published comparison of vIFN-γRs from EV Moscow, VV Copenhagen, and VaV India 1967 (52) revealed that the EV gene product differed from the VV and VaV proteins at 19 and 23 positions, respectively. Similarly, the EV amino acid sequence differs from those of VV WR and CPV GRI-90 at 26 and 21 positions, respectively (data not shown). Unfortunately, it is not obvious which of the EV mutations confer improved binding and, hence, inhibition of mouse IFN-γ. Several point mutations occur within the predicted secretory signal sequence at the N terminus of the EV vIFN-γR and can be discounted. Within the rest of the EV ORF, there are no large amino acid insertions, deletions, or small, highly divergent domains relative to other OPV vIFN-γRs. In fact, amino acid substitutions are distributed fairly evenly throughout the EV ORF. The high degree of conservation means that future mutagenesis studies aiming to identify those residues that are important in the inhibition of mouse IFN-γ by the EV vIFN-γR need not be especially extensive.

Considering the differences between the vIFN-γRs of EV and VV, it was interesting to find that their vIFN-α/βRs were functionally indistinguishable in our biological assays. The conservation of this factor by all of the EVs tested implies an important contribution to EV pathogenesis. Most surprisingly, neither the VV nor the EV vIFN-α/βR could inhibit mouse IFN-β. This is consistent with previous data on the role of IFN-α/β in the resistance of C57BL/6 mice to mousepox (41, 43). Footpad inoculation of mice with EV Moscow after depletion of either IFN-α and β or IFN-β alone revealed that IFN-β but not IFN-α played a role in limiting viral disease. This is compatible with the view that mouse IFN-α is neutralized by the EV vIFN-α/βR regardless of antibody-mediated effects, while IFN-β activity is not restricted by the virus. Furthermore, mice with a disrupted IFN-β gene are more susceptible to VV infection, consistent with this IFN having antiviral activity that is not inhibited by the VV vIFN-α/βR (26).

Regardless of its inability to inhibit mouse IFN-β, the vIFN-α/βR makes a significant contribution to VV virulence (71). One biochemical function of IFN-β is as an inducer of IFN-α production by mouse fibroblasts (26, 29), but this may not be important in EV or VV infection, since the vIFN-α/βR should inhibit IFN-α produced as a result of IFN-β stimulation at sites of viral infection.

The VV K3L gene encodes a veIF-2α that promotes viral mRNA translation via its competitive inhibition of PKR-mediated eIF-2α phosphorylation (11, 16, 25). Homologous ORFs encoding presumably functional veIF-2α gene products have been sequenced in CPV and VaV (63, 64), as well as in *Swinepox virus* (50). Surprisingly, the EV K3L gene homolog is truncated by multiple mutations. The predicted 31-amino-acid product is highly unlikely to have biological activity, since deletion of 6 amino acids from the C terminus of the VV veIF-2α destroys PKR inhibitory activity (45). Inactivation of the K3L gene of VV Copenhagen resulted in decreased viral replication in IFN-treated L-929 cells (11). Nevertheless, we observed that veIF-2α-negative EV strains and isolates were unaffected by IFN concentrations in excess of 600 U/ml in L-929 cells and that they were more IFN resistant than wild-type VV WR. CPV BR, which encodes a full-length veIF-2α, was as IFN resistant as EV, suggesting that veIF-2α does not contribute to the observed IFN resistance of EV and CPV in this cell line.

The retention of intact dsRBP genes by EV and CPV is not surprising, since the dsRBP contributes to IFN resistance (20) and inactivation of the E3L gene in VV renders the virus apathogenic in a mouse model (15). However, it is unlikely that the dsRBP is entirely responsible for the observed IFN resistance of EV and CPV. One possibility is that mutations in the EV and CPV genes increase dsRBP activity in mouse cells. However, the EV and CPV dsRBP genes encode dsRNA-binding domains which are necessary and sufficient for IFN resistance in vitro that differ from those of other OPVs at only two (EV) and one (CPV) amino acid residue, which are different for the two viruses. Of these, only the S166T mutation in EV occurs in a residue that is conserved between dsRNA-binding domains of different proteins, but mutagenesis studies have not identified a role for any of these residues in dsRNA binding (18, 19, 36).

Recent work has shown that the N terminus of the dsRBP, while dispensable for in vitro IFN resistance (19), is essential for the full pathogenicity of VV in vivo (15). It is unclear whether this unexpected in vivo role relates to IFN resistance in any way, but it is known that the dsRBP N terminus contains domains responsible for nuclear localization, Z-DNA binding, interaction with PKR, and oligomerization (15, 19, 37, 61, 81). Both the EV and CPV dsRBPs show greatest divergence from the VV and VaV dsRBPs in this region of the protein, but none of the EV or CPV mutations are identical in the two viruses. It is therefore probable that another gene or complex of genes that is either absent or inactive in VV is responsible for the enhanced IFN resistance of EV and CPV observed in our experiments. This enhanced resistance perhaps results from the prolonged coevolution of these viruses with rodent hosts (21, 32).

In conclusion, we have demonstrated that EV, the OPV which causes mousepox, deploys a number of anti-IFN strategies, all of which are likely to contribute to disease pathogenesis in mice. EV has specifically evolved to evade the mouse IFN system, particularly by acquiring the ability to sequester mouse IFN-γ outside the cell and to resist the intracellular antiviral effects of mouse IFNs. Like other OPVs, EV cannot inhibit mouse IFN-β via its vIFN-α/βR, even though this cytokine has a role in host defense against the virus. Study of the in vivo function of EV IFN inhibitors in the mousepox model should lead to a better understanding of the interactions between this virus and the IFN system of its natural host.

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