Vaccinia virus strains Lister, USSR and Evans express soluble and cell-surface tumour necrosis factor receptors

Antonio Alcamí†, Anu Khanna, Nina L. Paul and Geoffrey L. Smith

Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK

Poxviruses encode a broad range of proteins that interfere with host immune functions such as soluble versions of cytokine receptors. Soluble virus tumour necrosis factor receptors (vTNFRs) were described originally in myxoma and Shope fibroma viruses. Cowpox virus (CPV) encodes three vTNFRs (CrmB, CrmC and CrmD). The genes equivalent to CrmB and CrmC in vaccinia virus (VV) Copenhagen are mutated and are named B28R/C22L and A53R, respectively. CrmD was identified recently in CPV and ectromelia virus but the gene is absent in VV Copenhagen. We have tested for expression of soluble binding activity for human TNF in cultures infected with 18 orthopoxviruses and have found that TNFRs are mostly absent but are produced by VV strains Lister, USSR and Evans, by the CPV elephantpox and by camelpox virus. Interestingly, we also found TNFR activity on the surface of cells infected with VV Lister, USSR and Evans. Sequence analysis of the relevant regions in VV Lister identified an intact A53R gene and an inactive B28R gene. Expression of VV Lister A53R in baculovirus and VV Western Reserve demonstrated that gene A53R encodes an active soluble vTNFR of 22 kDa. Expression and characterization of recombinant vTNFRs from VV Lister (A53R) and CPV (CrmB and CrmC) showed a similar binding specificity, with each receptor binding TNF from man, mouse and rat, but not human lymphotoxin-α. Lastly, the VV Lister and CPV vTNFRs bind human TNF with high affinity and prevent the binding of TNF to cellular receptors.

Introduction

Viruses have evolved strategies to evade the host defence mechanisms which otherwise prevent virus replication. Poxviruses, a family of large cytoplasmic DNA viruses, express several secreted proteins that function as soluble cytokine receptors, or binding proteins, and block cytokine activity and modulate virus virulence. Poxvirus proteins that bind tumour necrosis factor (TNF), interleukin (IL)-1β, interferon (IFN)-γ, IFN-α/β and CC chemokines have been described; for review see Smith et al. (1997).

TNF (TNFα) and lymphotoxin-α (LTα or TNFβ) are important cytokines in orchestrating the early defence against virus infection and are cytotoxic to virus-infected cells (Beutler, 1992). Both forms of TNF are trimers, bind to the p55 and p75 cellular TNF receptors (TNFRs), and induce receptor oligomerization and biological responses. LTα is also found at the cell surface complexed with LTβ and this complex binds a different receptor (Browning et al., 1993; Crowe et al., 1994).

Poxvirus TNFR (vTNFR) genes were identified by sequence similarity to cellular TNFRs but lack the membrane anchor and cytoplasmic signalling domains (Howard et al., 1991; Smith et al., 1991; Upton et al., 1991). Shope fibroma and myxoma virus vTNFRs are secreted from cells and bind TNF (Upton et al., 1991; Schreiber & McFadden, 1994). Cowpox virus (CPV) encodes three vTNFRs (CrmB, CrmC and CrmD). CrmB is a 48 kDa secreted protein that is transcribed early during infection and binds TNF and LTα (Hu et al., 1994). CrmC is a 25 kDa protein that is expressed late during infection and binds TNF but not LTα (Smith et al., 1996). CrmD was identified recently in CPV and ectromelia virus and is a late, 46 kDa protein that is secreted as disulphide-linked complexes and binds TNF and LTα (Loparev et al., 1998). vTNFRs from

Author for correspondence: Geoffrey L. Smith.
Fax +44 1865 275501. e-mail glsmith@molbiol.ox.ac.uk
† Present address: Division of Virology, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, UK.

The nucleotide sequences of the VV strain Lister A53R and B28R/C22L genes and the VV Western Reserve B28R/C22L gene have been submitted to the EMBL nucleotide sequence database and assigned the accession numbers Y17728, Y17729 and Y17730, respectively.
myxoma virus (M-T2) and CPV (CrmC) bind TNF with high affinity and block TNF-mediated cytolysis in vitro (Schreiber et al., 1996; Smith et al., 1996). Myxoma virus M-T2 prevents TNF binding to cellular receptors (Schreiber et al., 1996).

Myxoma virus vTNFR affected virus virulence in a rabbit model (Upton et al., 1991). CPV CrmB or CrmC did not affect pox morphology in chick embryos and their role in virus virulence in mammals is unknown (Hu et al., 1994; Smith et al., 1996).

Two vTNFR genes, termed A53R and B28R/C22L in VV Copenhagen (equivalent to CPV genes CrmB and CrmC, respectively), were identified in vaccinia virus (VV) Copenhagen, but both are fragmented by mutations and predicted to be inactive (Goebel et al., 1990; Upton et al., 1991). Similarly, the VV Western Reserve (WR) strain A53R gene is also mutated and predicted to be inactive (Howard et al., 1991). Interestingly, although B28R/C22L is predicted to be active in variola virus, the A53R gene is deleted from all variola virus strains sequenced (Aguado et al., 1992; Alcamí & Smith, 1993; Shchelkunov et al., 1993; Massung et al., 1994).

Here the expression of vTNFRs by 15 VV strains, CPV and camelpox virus has been investigated. Only three VV strains predicted to be inactive (Goebel et al., 1991). CPV CrmB or CrmC did not affect pock morphology in chick embryos and their role in virus virulence in mammals is unknown (Hu et al., 1994; Smith et al., 1996).

Construction of recombinant baculoviruses. VV Lister and CPV vTNFR genes were amplified by PCR using Pyrococcus furiosus DNA polymerase, virus DNA as template and oligonucleotides corresponding to the 5' and 3' ends of the open reading frames (ORFs) which provided BanH1 and HindIII sites, respectively. VV Lister A53R and CPV CrmC were amplified by PCR with oligonucleotides A53R4 (5' TATGGATGCATCGGATATTTAAAGATT 3') and A53R11 (5' CCTTGCCCGAAGCCTTTAATACATTAGATGTT 3'), and CPV CrmB with oligonucleotides B28R11 (5' CCGGGATCCGAATGTCGATTATATGCTA 3') and B28R12 (5' CCGGATCCGACACCTACTATAAAAATTGGGTGG 3'). Resultant fragments were cloned into BamHI/HindIII-digested pACCL29 (Livingstone & Jones, 1989) creating plasmids pAA24 (VV Lister A53R), pAA25 (CPV CrmC) and pAA30 (CPV CrmB). The DNA sequence of the inserts was confirmed not to contain mutations. The plasmids were used to produce recombinant baculoviruses as described (Alcamí & Smith, 1995b), and were termed AcA53 (VV Lister A53R), AcAA7 (CPV CrmC) and AcA8 (CPV CrmB) but are referred to hereafter as AcA53Lister, AcCrmC and AcCrmB, respectively.

Construction of rVVs. vTNFR genes from VV Lister and CPV were amplified by PCR with virus DNA as template and oligonucleotides as described above for baculovirus expression. Resultant fragments were cloned into BanH1/HindIII-digested pmL601 (Davison & Moss, 1990) creating plasmids pAA26 (VV Lister A53R), pAA27 (CPV CrmC) and pAA29 (CPV CrmB). The DNA sequence of the inserts was confirmed not to contain mutations. Plasmids were transfected into VV WR-infected cells and thymidine kinase-negative (TK−) rVVs were screened for β-galactosidase expression and plaque-purified three times (Davison & Moss, 1990; Smith, 1993). These rVVs were termed vAA16 (VV Lister A53R), vAA17 (CPV CrmC) and vAA18 (CPV CrmB) but are referred to hereafter as vA53Lister, vCrmC and vCrmB, respectively. For insertion of the VV Lister B28R gene and flanking sequences into the VV WR TK locus, a 1–8 kb fragment was amplified by PCR using VV Lister DNA as template and oligonucleotides TNFR3 (5' CCGGGATCCGATATGTCATTACATGCTC 3') and TNFR4 (5' CCGGGATCCGATATGTCATTACATGCTC 3'), providing BanH1 and EcoRI restriction sites, respectively. The PCR fragment was cloned into BanH1/EcoRI-digested pRK19 (Kent, 1988; Smith, 1993), creating pAA28. Five plasmid clones were transfected independently into VV WR-infected cells and TK− rVVs selected and named vAA19 (clones 1–5), but are referred to hereafter as vB28Lister (clones 1–5).

Metabolic labelling of proteins and electrophoretic analysis. TK−143B or SF cells were infected with orthopoxviruses or baculoviruses, respectively, at 10 p.f.u. per cell. At the indicated time post-infection (p.i.), cultures were labelled with 150 µCi/ml [35S]Promix (Amersham; 1200 Ci/mmol) and 150 µCi/ml [35S]cysteine (DuPont–New England Nuclear, 600 Ci/mmol) in methionine- and cysteine-free medium in the absence of serum. Cells or media were dissociated in sample buffer and analysed by SDS–PAGE in 12% gels and fluorography with Amplify (Amersham).

Sucrose gradient centrifugation. Samples were layered onto a 5–25% sucrose gradient in PBS and centrifuged in a Beckman 54Ti rotor (40 000 r.p.m., 17 h, 4 °C). Gradient fractions were mixed with 5 µg BSA and proteins were precipitated with 10% TCA (10 min, 4 °C) and collected by centrifugation (15 min, microfuge). Pellets were dissolved in SDS–PAGE sample buffer and their pH adjusted by addition of ammonium vapours before analysis by SDS–PAGE. Prestained size markers were analysed in parallel and the sucrose concentration in the fractions was confirmed to be similar by determination of the refractive index.
Results
vTNF binding activity in the supernatants of cultures infected with orthopoxviruses

vTNFR genes in VV WR (A53R) and Copenhagen (A53R and B28R/C22L) were predicted to be inactive because of mutations that introduce stop codons or frameshifts (Goebel et al., 1990; Howard et al., 1991; Upton et al., 1991). However, it was possible that these ORFs were intact in other VVs or orthopoxviruses, as for CPV Brighton Red (Hu et al., 1994; Smith et al., 1996). Soluble vTNFRs were sought in a binding assay that quantifies bound 125I-TNF by precipitating the ligand–receptor complex with PEG (Alcamì & Smith, 1992). Fig. 1(a) shows that a soluble vTNFR is produced by VV Lister, USSR and Evans, but not by 12 other VVs, including VV isolated from animals (rabbitpox, buffalopox), consistent with sequence data for VV WR and Copenhagen. vTNFR activity was also detected in CPV Brighton Red, as previously described, elephantpox virus (a CPV strain) and camelpox virus.

Most VV strains downregulated the TNF binding activity found in uninfected TK-143B cells, but enhanced cell-surface TNFR activity was found after infection with VV strains Lister, USSR and Evans (Fig. 1b) – those VV strains encoding soluble vTNFR activity (Fig. 1a). Notably, although CPV Brighton Red, elephantpox virus and camelpox virus encode soluble TNFRs, no cell-surface binding activity was detected (Fig. 1b). Although the level of vTNFR expression is difficult to compare, the cell-surface TNFR activity was less abundant. For example, 1·5 × 10⁶ cells infected by VV Lister bound approximately 6000 c.p.m. of 125I-TNF at the cell surface (Fig. 1b), whereas medium from 1·5 × 10⁸ infected cells bound approximately 12 500 c.p.m. (Fig. 1a).

The possibility that the cell-surface TNFR activity of VV Lister was due to some soluble vTNFR being attached to the cell surface was investigated. Preincubation of mock-infected or VV WR-infected cells with medium from cultures infected with VV Lister or CPV, containing soluble vTNFR activity, did not confer TNFR activity at the cell surface (data not shown).

Kinetics of synthesis of the VV and CPV vTNFRs

Soluble vTNFR activity expressed by VV Lister was abundant 8 h p.i. (Fig. 2a) but absent in the presence of cytosine arabinoside (AraC) (an inhibitor of DNA synthesis and late protein synthesis). With CPV, large amounts of vTNFR were detected 8 h p.i. in the presence or absence of AraC. This was consistent with early and late expression of CPV vTNFRs (Hu et al., 1994; Smith et al., 1996). Fig. 2(a) did not determine whether vTNFRs continued to be synthesized later during infection, because maximum binding was already observed by 8 h. Note the significant vTNFR activity in the VV Lister inoculum, which was removed by washing the monolayer with medium after the adsorption period (Fig. 2a). Like VV Lister, vTNFR activity was produced late after infection by VV USSR and Evans (Fig. 2b).

The kinetics of cell-surface TNFR synthesis by cells infected with VV Lister was also examined (Fig. 2c). The absence of the
Fig. 2. Kinetics of synthesis of soluble and cell-surface TNF binding activity. (a) Soluble TNF binding activity. TK−143B cells were infected with VV Lister or CPV in the absence (open symbols) or in the presence (filled symbols) of 40 µg/ml AraC. After adsorption for 1 h, cells were rinsed twice with medium and incubated in fresh medium. At the indicated time p.i., supernatants were harvested and an aliquot (equivalent to 2 × 10⁵ cells) was tested for its human 125I-TNF binding activity. The TNF binding activity of the VV Lister inoculum was also determined. Specific 125I-TNF binding of duplicate samples (mean ± SEM) after subtraction of background with medium is shown. (b) TK−143B cells were infected with the indicated viruses in the absence (ﬁ) or presence (›) of 40 µg/ml AraC, and rinsed twice with medium after 1 h of adsorption. Supernatants were harvested 24 h p.i. and the presence of human 125I-TNF binding activity was determined in a soluble binding assay. Specific 125I-TNF binding of duplicate samples (mean ± SEM) after subtraction of background with medium is shown. (c) TK−143B cells were mock-infected (*) or infected with VV Lister in the presence (●) or absence (○) of 40 µg/ml AraC. After adsorption for 1 h, cells were rinsed twice with medium and incubated in fresh medium. At the indicated time p.i., cells were detached from the plate and tested for their capacity to bind 125I-TNF. Bound 125I-TNF of duplicate samples (mean ± SEM) was determined by phthalate oil centrifugation. (d) TK−143B cells were infected with the indicated viruses in the presence (+) or absence (−) of 40 µg/ml AraC, and rinsed twice with medium after 1 h of adsorption. At 24 h p.i. the presence of human 125I-TNF binding activity at the cell surface was determined. Bound 125I-TNF of duplicate samples (mean ± SEM) was determined by the phthalate centrifugation method.

activity in cultures infected in the presence of AraC with VV strains Lister (Fig. 2c), USSR and Evans (Fig. 2d) demonstrated protein expression at late times p.i., after virus DNA replication.

Analysis of A53R and B28R regions in VV WR and Lister

Sequence analysis of a ∼ 2 kb EcoRV genomic DNA fragment containing the VV Lister A53R gene revealed an A53R ORF of 186 amino acids, the same length as CPV CrmC (Smith et al., 1996). The predicted polypeptide contained an N-terminal hydrophobic sequence, a potential N-glycosylation site at position 85 and 24 cysteines, including all those forming the TNF-binding cysteine-rich domain characteristic of the TNFR superfamily. The protein shares 88, 34, 33, 31 and 32% identity to CPV CrmC, CPV CrmB, VV Copenhagen B28R/C22L, myxoma T2, Shope fibroma T2 and variola G4R proteins, respectively. Among the mammalian TNFRs, A53R protein was related most closely (37–8% identity) to human TNFR p75.

The B28R genes from VV Lister and WR were cloned as 2–1 kb AvaiI fragments and sequenced. Both genes were disrupted by multiple mutations so that functional vTNFRs were unlikely to be synthesized. In both cases, short ORFs were identified that when assembled together formed an ORF
Fig. 3. Comparison of vTNFRs. A diagram showing the A53R/CrmC and B28R/CrmB genes from different orthopoxviruses. The top panel shows the domain structure of the vTNFRs which include a signal sequence (S, hatched) followed by cysteine-rich domains (1–4, shaded) and a C-terminal region for B28R. N and C refer to the amino and carboxyl termini. Intact ORFs are indicated by a single horizontal arrow. A change in the arrow marks the position of a frameshift required to maintain the ORF. In the VV Copenhagen and WR A53R ORFs the tip of the arrowhead in the third domain indicates the position of a stop codon. The A53R and B28R sequences have been determined in CPV strain Brighton Red and GRI, and in variola virus strains India-1967, Bangladesh-1975 and Harvey-1947 (A53R only).

similar to the CPV CrmB. A comparison of the ORFs (including frameshifts) from VV WR and Lister with the equivalent ORFs from some other orthopoxviruses is summarized in Fig. 3.

Expression of VV and CPV vTNFRs in the baculovirus system

To characterize further the VV A53R and CPV CrmC and CrmB vTNFRs, recombinant baculoviruses expressing these genes were constructed. The VV Lister A53R, and CPV CrmC and CrmB were secreted from recombinant baculovirus-infected cells as polypeptides of 20, 23 and 48 kDa, respectively (Fig. 4a). The 23 kDa Lister A53R may represent a different glycosylated form. The sizes of the CPV vTNFRs were in agreement with previous reports (Hu et al., 1994; Smith et al., 1996). In cell extracts, several specific bands were observed, which probably represent polypeptides with different post-translational modifications. Other recombinant baculoviruses expressing the secreted VV IL-1β receptor (B15R), IFN-α/β receptor (B18R) and IFN-γ receptor (B8R) are shown as controls (Alcamí & Smith, 1992, 1995b).

The possible multimerization of vTNFRs was investigated in two ways. First, the electrophoretic mobility of 35S-labelled rVV Lister A53R and CPV CrmB (Fig. 4a) was identical with or without 2-mercaptoethanol (data not shown), suggesting no disulphide-linked multimerization. Second, the 35S-labelled recombinant A53R and CrmB proteins (Fig. 4a) sedimented as monomeric proteins during ultracentrifugation in sucrose gradients (Fig. 4b).

Expression of VV and CPV vTNFRs from VV WR

To characterize the VV and CPV vTNFRs in a mammalian expression system, these proteins were expressed from VV WR (which does not express vTNFR) using a strong VV promoter (Davison & Moss, 1990) (Fig. 1a). Cells infected with rVV expressions Lister A53R (vA53RLister), or CrmB (vCrmB) or CrmC (vCrmC) from CPV, were labelled with [35S]cysteine and [35S]Promix for 4 h from 24 h p.i. Proteins present in cells and media were analysed by SDS–PAGE and visualized by fluorography. The positions of the expressed proteins in supernatants (●) are indicated. Molecular masses in kDa are shown. (b) Supernatants from SI cells infected with AcA53RLister or AcCrmB and 35S-labelled as described in (a) were loaded onto sucrose gradients (5–25% sucrose in PBS) and ultracentrifuged. The original sample (S) and fractions collected from the bottom of the gradient and precipitated with TCA were analysed by SDS–PAGE. A fluorograph showing the distribution of radiolabelled VV Lister A53R and CPV CrmB is shown. The positions of the molecular size standards are indicated in kDa.

Fig. 4. Expression of TNFRs from VV Lister and CPV in baculovirus-infected insect cells. (a) SF cells infected with AcNPV or recombinant baculoviruses AcB15R, AcB18R, AcB8R, AcA53RLister, AcCrmC or AcCrmB were pulse-labelled with [35S]cysteine and [35S]Promix for 4 h from 24 h p.i. Proteins present in cells and media were analysed by SDS–PAGE and visualized by fluorography. The positions of the expressed proteins in supernatants (●) are indicated. Molecular masses in kDa are shown. (b) Supernatants from SF cells infected with AcA53RLister or AcCrmB and 35S-labelled as described in (a) were loaded onto sucrose gradients (5–25% sucrose in PBS) and ultracentrifuged. The original sample (S) and fractions collected from the bottom of the gradient and precipitated with TCA were analysed by SDS–PAGE. A fluorograph showing the distribution of radiolabelled VV Lister A53R and CPV CrmB is shown. The positions of the molecular size standards are indicated in kDa.
times (4–8 h p.i.) (CrmC) and at both early (1–5 h) and late (4–8 h) times p.i. (CrmB), as predicted from their transcriptional control (Hu et al., 1994; Smith et al., 1996). The different size of VV Lister A53R and CPV CrmC may be due to the absence of an N-glycosylation site at position 34 in VV Lister A53R.

The rVVs vB28RLister (clones 1–5), with the VV Lister B28R region inserted into the TK locus of VV WR, were also constructed. These rVVs contained the complete VV Lister B28R ORF and promoter. However, consistent with the sequencing data, no protein expression in labelling experiments nor vTNFR activity in cultures infected with vB28RLister viruses were observed (data not shown). In contrast, a similar rVV containing the CPV CrmB gene inserted into the TK locus of VV WR expresses an active vTNFR (Hu et al., 1994).

### TNF binding activity, specificity and affinity of vTNFRs produced by baculovirus

Secrated vTNFR activity from recombinant baculovirus-infected cells was determined in a solubile binding assay with human \(^{125}\text{I}\)-TNF. The baculoviruses expressing VV Lister A53R or CPV CrmB or CrmC produced high levels of vTNFR activity (7–11 \(\times\) 10^2 c.p.m. bound by medium equivalent to 2 \(\times\) 10^5 cells) that was not detected in SF cells uninfected or infected with AcB15R (data not shown).

To determine the binding specificity for human TNF and LTz, and for TNF from other species (mouse and rat), binding of human \(^{125}\text{I}\)-TNF to recombinant CPV and VV Lister vTNFRs was performed in the presence of excess unlabelled TNF or LTz. Fig. 6(a) shows that VV Lister A53R, CPV CrmC and CPV CrmB have very similar specificities, binding human, mouse and rat TNF but having a very low affinity for human LTz. The inhibition of binding of human \(^{125}\text{I}\)-TNF to AcA53Lister by a 1000-fold excess of unlabelled human TNF was normally higher, being 80±5, 98±8 and 99±0% inhibition in three other experiments. The almost undetectable binding of Lister A53R and CPV CrmB and CrmC to human LTz was corroborated in three experiments and by using recombinant human LTz from two different suppliers (data not shown). Similar binding experiments performed with VV Lister-infected cultures showed that TNFRs expressed both in supernatants and at the cell surface bound human, mouse and rat TNF, but not human LTz (data not shown). This confirmed that VV Lister A53R produced from VV Lister infected cells or from recombinant baculovirus has the same binding properties.

The binding affinity between vTNFRs produced in the baculovirus system and human TNF was determined in soluble binding assays with increasing doses of \(^{125}\text{I}\)-TNF. Scatchard analyses showed that high-affinity binding sites were detected in supernatants from insect cells infected with recombinant baculoviruses AcA53RLister, AcCrmC and AcCrmB (Fig. 6b). The number of binding sites for TNF secreted from infected cultures was very high, ranging from 7 \(\times\) 10^4 to 1 \(\times\) 10^5 sites per cell.

### TNF binding activity of vTNFRs produced from rVV

The binding activity for human TNF was higher from VV Lister than CPV in 15 experiments (not shown) and this is illustrated quantitatively using various doses of media from VV Lister- or CPV-infected cultures (Fig. 7a). This might be due to the secretion of higher quantities of vTNFRs from VV Lister-infected cells, or to a higher affinity of the VV Lister TNFR(s) for human TNF. Interestingly, expression of VV Lister A53R from VV WR under a strong late promoter did not produce as much vTNFR activity as that present in VV Lister supernatants. This was confirmed in more quantitative TNF binding assays with various doses of supernatant (Fig. 7a). Protein analysis had shown that VV Lister A53R is produced at much higher levels from vA53RLister than from VV Lister (Fig. 5). These results support the existence of an unidentified vTNFR in VV Lister encoding the greater vTNFR activity expressed by this VV strain.

Cells infected with VV WR, VV Lister, CPV, or rVVs expressing VV Lister A53R (vA53RLister) or CPV CrmB (vCrmB) were tested for expression of cell-surface vTNFRs. Whereas VV Lister, CPV, vA53RLister, and vCrmB produced soluble vTNFRs (Fig. 7a), cell-surface vTNFR activity was detected only with VV Lister (Fig. 7b). This showed that neither VV Lister A53R nor the other orthopoxvirus vTNFRs (CPV CrmB and CrmD) encode a membrane-bound vTNFR, and suggests strongly that a novel gene encodes a vTNFR in VV Lister.
Vaccinia tumour necrosis factor receptor

Fig. 6. TNF binding activity of vTNFRs expressed in the baculovirus system. (a) TNF binding specificity. Supernatants from insect cell cultures infected with recombinant baculoviruses expressing VV Lister A53R (AcA53RLister, equivalent to 10⁴ cells) or CPV CrmC (AcCrmC, equivalent to 10⁴ cells) or CrmB (AcCrmB, equivalent to 4 × 10³ cells) were incubated with human ¹²⁵I-TNF in the presence of the indicated fold excess of unlabelled human TNF (hTNF), human LTα (hLTα), mouse TNF (mTNF) or rat TNF (rTNF). Bound ¹²⁵I-TNF was determined by precipitation with PEG and filtration. The percentage of specific ¹²⁵I-TNF binding of triplicate samples (mean ± SEM) refers to binding in the absence of competitor. (b) Scatchard analysis of human TNF binding to vTNFRs. Medium from SF cell cultures infected with AcA53RLister (0–6 µl, equivalent to 10³ cells), AcCrmC (0–6 µl, equivalent to 10³ cells) or AcCrmB (0–15 µl, equivalent to 3 × 10² cells) was incubated with different concentrations (100–1200 pM) of radiolabelled human TNF for 2 h at room temperature, and the radioactivity bound was determined by the PEG precipitation method. Data were converted to the Scatchard co-ordinate system and analysed by the LIGAND program (Munson & Rodbard, 1980). Binding shown represents specific binding.

Blockade of TNF binding to cellular receptors by VV and CPV vTNFRs

The binding of human ¹²⁵I-TNF to U937 cells was specific and was inhibited by supernatants containing any of the vTNFRs from VV Lister or CPV (Fig. 8), although the VV Lister A53R vTNFR was less efficient than CPV CrmB in doing this. This difference was dose-independent and was not due to the expression of the proteins in insect cells since VV Lister A53R and CPV CrmB expressed from VV WR showed the same difference when blocking TNF binding to cellular receptors (Fig. 8).

Discussion

We report that soluble vTNFRs are encoded by VV Lister, USSR and Evans, by CPV elephantpox and by camelpox virus. The vTNFR activity in VV Lister is encoded by the A53R gene, the B28R gene being inactive due to fragmentation. These two vTNFR genes (B28R/CrmB and A53R/CrmC) are mutated in VV Copenhagen (Goebel et al., 1990; Howard et al., 1991; Upton et al., 1991) but active in CPV Brighton Red (Hu et al., 1994; Smith et al., 1996). VV WR has a mutated A53R gene (Howard et al., 1991), and here we report the sequence of the VV WR B28R gene which, consistent with the lack of TNF binding activity in VV WR, is also truncated.

Only three out of fifteen VV strains express a vTNFR whereas most other orthopoxviruses (CPV, variola, camelpox) are either known to, or predicted from sequence information to, express one or more vTNFR. This situation contrasts with the expression of other cytokine receptors by orthopoxviruses: soluble receptors for IFN-α/β and IFN-γ are expressed by almost all orthopoxviruses (Alcamí & Smith, 1995b; Symons et al., 1995) and the IL-1β receptor is expressed in most cases (Alcamí & Smith, 1992; Blanchard et al., 1998). The CC chemokine binding protein is expressed in approximately half of the viruses tested so far (Graham et al., 1997; Alcamí et al., 1995b; Symons et al., 1995).
Fig. 7. Soluble and membrane-bound TNFR activity expressed by rVV. (a) Soluble vTNFR activity produced by VV Lister, CPV and WR rVVs expressing VV Lister A53R or CPV CrmB. TK−143B cells were mock-infected or infected with the indicated viruses and supernatants were harvested 24 h p.i. The indicated volumes (µl) of supernatants, equivalent to 2 × 10^3 cells/µl, were tested for the presence of human 125I-TNF binding activity in a soluble binding assay. Specific 125I-TNF binding of duplicate samples (mean ± SEM) after subtraction of background with medium is shown. (b) Cell-surface TNFR activity. TK−143B cells were mock-infected or infected with the indicated viruses and tested for cell-surface TNF binding activity 24 h p.i. Bound human 125I-TNF of duplicate samples (mean ± SEM) was determined by phthalate oil centrifugation.

1998). On the other hand, CPV GRI-90 has four different TNFR-like genes (Shchelkunov et al., 1998), three of which express a vTNFR (Hu et al., 1994; Smith et al., 1996; Loparev et al., 1998). A soluble vTNFR has been predicted from sequence similarity in lymphocystis disease virus (Tidona & Darai, 1997), a large DNA iridovirus that infects fish, consistent with the conservation of TNF throughout evolution (Beck & Habicht, 1991).

This paper also reports TNFR activity at the surface of cells infected by three VV strains but not by CPV or camelpox virus. This must be encoded by the virus or be a cellular protein upregulated by virus infection. The latter is unlikely because poxvirus infections induce a potent inhibition of cellular protein synthesis and most orthopoxviruses not expressing membrane TNFRs downregulate TNFRs at the cell surface.

The identification of membrane-bound TNFRs in VV-infected cells was surprising because the vTNFR genes identified in VV are equivalent to those found in ortho- poxviruses which lack a transmembrane anchor sequence and are secreted from the cell. A probable explanation is the existence of an unidentified VV vTNFR gene that encodes cell-surface TNFRs. Several lines of evidence provided here support this proposal. First, the expression of the only known active vTNFR gene in VV Lister (A53R) from rVV WR (vA53RLister) produced a secreted protein with soluble but not cell-surface vTNFR activity. Second, expression of VV Lister A53R from vA53RLister does not account for the higher vTNFR activity found in supernatants of VV Lister-infected cultures, despite vA53RLister producing much higher levels of secreted A53R protein.

Recently, a third vTNFR was found in CPV Brighton Red (Loparev et al., 1998) that is expressed late during infection and binds human and rat TNF but not human LTα (Loparev et al., 1998). The expression late during infection is consistent with
the possibility that the putative novel VV vTNFR may be equivalent to CrmD. On the other hand, the ability of CrmD and the putative VV vTNFR to bind LTx differ, and our studies found no cell-surface vTNFR expressed by this strain of CPV. Another possibility is that the fourth ORF with sequence similarity to TNFR superfamily members that was identified in CPV strain GRI-90 (K3R) (Shchelkunov et al., 1998) represents the novel VV vTNFR, although no TNFR activity has been demonstrated for K3R. However, neither CrmD nor K3R were reported to have membrane-anchoring domains (Loparev et al., 1998). Conceivably, the novel cell-surface vTNFR of VV Lister may represent another uncloned poxvirus gene.

The VV A53R gene and CPV CrmB and CrmC genes were expressed in the same system to compare their properties. None of the three orthopoxvirus vTNFRs bound human LTx significantly, despite the fact that CPV CrmB was reported to bind both TNF and LTx (Hu et al., 1994). The different specificity of CrmB we have found may reflect the use of human 125I-TNF, whereas Hu et al. (1994) incubated CrmB with murine 125I-TNF in the presence of human LTx, or alternatively a different property of the baculovirus-produced CrmB. The binding properties of VV Lister A53R expressed in the baculovirus system were identical to those of TNFRs secreted from, and expressed at the surface of, VV Lister-infected cells. The broad species specificity of the orthopoxvirus vTNFRs, binding TNF from man, mouse and rat, is similar to the broad binding specificity of other orthopoxvirus cytokine receptors. By contrast, the myxoma virus TNFR binds TNF from rabbits, the natural host of the virus.

We found consistently that binding activity for human TNF is more abundant in supernatants from VV Lister-infected cells than in medium from cultures infected with CPV. The affinity constants for the interaction of human TNF with VV Lister A53R (Kd 7 nM), CPV CrmC (Kd 223 pM) and CPV CrmB (Kd 700 pM) are reported here for the first time. The affinity constant of CPV CrmC for murine TNF (Kd 200 pM) was reported by Smith et al. (1996), and is similar to that we found here for human TNF. The affinity of VV Lister A53R for human TNF is lower than that of the CPV vTNFRs (CrmB and CrmC) and thus does not account for the higher TNF binding activity present in VV Lister supernatants compared to CPV. The secretion of a vTNFR distinct from A53R and B28R by VV Lister may account for the high TNF activity.

The myxoma virus T2 protein is secreted both as a monomer and disulphide-linked dimers, and the latter is a more potent TNF inhibitor (Schreiber et al., 1996). Similarly CPV Brighton Red CrmD forms oligomers in solution (Loparev et al., 1998). TNF is produced as a trimer that induces oligomerization of cellular TNFRs; thus multimeric soluble receptors may be more effective in blocking TNF binding to cellular TNFRs. We have been unable to detect multimerization of recombinant Lister A53R or CPV CrmB by SDS–PAGE in the absence of reducing agents or by sucrose-gradient centrifugation.

The simultaneous expression of several vTNFRs in some poxviruses is intriguing and may reflect the complexity of the host TNF system. More than one vTNFR may be required to block TNF activity at different times of infection, to neutralize different TNF ligands or to interfere with TNF at different locations. The vTNFR CrmB is expressed early during CPV infection whereas CrmC/A53R and CrmD are expressed late (Hu et al., 1994; Smith et al., 1996; Loparev et al., 1998). The unique, longer C-terminal region of CrmB/B28R and CrmD has no sequence similarity to cellular TNFRs or CrmC/A53R, and may have functions distinct from TNF binding. The myxoma virus TNFR (M-T2) prevents apoptosis as a function independent from TNF binding, illustrating the adaptation of these virus proteins to perform different functions [see review by McFadden & Barry (1998)].

Additional complexity is added by our finding that some VV strains express TNFRs at the cell surface. TNF is a potent pro-inflammatory cytokine but at the same time has direct toxicity for virus-infected cells. Cell-surface TNFRs may enhance the ability of vTNFRs to protect infected cells from TNF. The poxvirus cytokine receptors identified so far are secreted proteins (Smith et al., 1997). The only exception is VV B18R, an IFN-α/β receptor that is both secreted and expressed at the cell surface (Colamonici et al., 1995; Symons et al., 1995), and expression at the surface protects from the anti-virus
effects of IFNs (A. Alcamí, J. A. Symons & G. L. Smith, unpublished). The VV surface TNFR may function in a similar way to efficiently protect infected cells from TNF.

Poxvirus cytokine receptors bind cytokines with high affinity and prevent the cytokines interacting with receptors at the surface of target cells. Here we show that VV Lister A53R, CPV CrmC and CPV CrmB prevent to a large extent the interaction of human TNF with TNFRs in U937 cells. A similar mechanism has been described for the inhibition of murine TNF by CPV CrmC and rabbit TNF by the myxoma virus vTNFR T2 (Schreiber et al., 1996; Smith et al., 1996).

Recombinant soluble versions of cellular TNFRs expressed as fusion proteins are potent inhibitors of TNF activity both in vitro and in vivo (Loetscher et al., 1991; Peppel et al., 1991). A role for poxvirus soluble vTNFRs as a virulence factor has been demonstrated for the myxoma virus M-T2 (Upton et al., 1991). Further characterization of the different poxvirus TNFRs and identification of their unique properties will help us to better understand the function of TNF and other members of the TNF family in defence against virus infections.

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References


as a monomer and dimer that each bind rabbit TNFα, but the dimer is a more potent TNF inhibitor. *Journal of Biological Chemistry* **271**, 13333–13341.


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