Orf virus-encoded interleukin-10 stimulates the proliferation of murine mast cells and inhibits cytokine synthesis in murine peritoneal macrophages

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Orf virus (ORFV) is the type species of the parapoxvirus genus and produces cutaneous pustular lesions in sheep, goats and humans. The genome encodes a polypeptide with remarkable homology to interleukin-10 (IL-10), particularly ovine IL-10, and also to IL-10-like proteins encoded by Epstein–Barr virus (EBV) and equine herpesvirus. IL-10 is a pleiotropic cytokine that can exert either immunostimulatory or immunosuppressive effects on many cell types. We have expressed and purified C-terminal FLAG and His6-tagged versions of ORFV-IL-10 and shown that ORFV-IL-10 costimulates murine mast cells (MC/9) and inhibits tumour necrosis factor-α synthesis in activated mouse peritoneal macrophages. Our results demonstrate that although ORFV-IL-10 is structurally similar to EBV-IL-10 it has evolved a different spectrum of activities. EBV-IL-10 does not stimulate the proliferation of thymocytes or mast cells whereas ORFV-IL-10 has both of these activities. Recent studies show that the critical difference in molecular structure of human IL-10 and EBV-IL-10, which may be the basis of their functional differences, is linked to a single amino acid substitution. Consistent with the activity spectrum reported here for ORFV-IL-10, the viral gene encodes the critical amino acid seen in human IL-10. Although the ORFV-IL-10 gene has clearly undergone significant evolutionary change at the nucleotide level compared with ovine IL-10, it has largely retained the polypeptide structure and functional characteristics of its ovine counterpart, suggesting that mutations of the gene to a potentially more potent immunosuppressive form may compromise the co-existence of host and virus.

Introduction

Interleukin-10 (IL-10) is a multifunctional cytokine that has both immunostimulatory and immunosuppressive activities. IL-10 inhibits the induction of a number of proinflammatory cytokines in activated monocytes and macrophages but is a costimulator for immature and mature lymphocytes, mast cells and B cells (reviewed in Mosmann, 1994; Moore et al., 2001). IL-10 has been captured by a few viruses and is thought to play a critical role in protecting cells infected by these viruses from the host immune defences and thereby enhancing viral survival. Homologues of cellular IL-10 (cIL-10) have been reported in three herpesviruses, Epstein–Barr virus (EBV) (Moore et al., 1990), equine herpesvirus (EHV) (Rode et al., 1993) and cytomegalovirus (CMV) (Kotenko et al., 2000), and our studies have revealed a homologue of IL-10 in orf virus (ORFV) (Fleming et al., 1997, 2000).

Studies on virus-encoded IL-10s are providing important insights into the structural and functional relationships of this cytokine. Although the secreted form of EBV-IL-10 is approximately 85% identical to cIL-10, with most differences found in the N-terminal 20 amino acids (aa), EBV-IL-10 has only a subset of activities of cIL-10. EBV-IL-10 does not costimulate lymphocytes, mast cells or B cell MHC class II expression (MacNeil et al., 1990; Fei et al., 1990; Vieira et al., 1991) whereas cIL-10 exhibits all these stimulatory activities. Furthermore, transduction of tumours with a retroviral vector encoding mouse IL-10 results in enhanced antitumour immunity and rejection, whereas transduction of tumour cells with EBV-IL-10 results in immune suppression and tumour growth (Suzuki et al., 1995). This observation suggested that the enhanced immunosuppressive effects of EBV-IL-10 are related to the reduced immunostimulatory functions of this...
virokine. In addition, there are differences in receptor binding affinity. EBV-IL-10 has a 1000-fold lower affinity for the human IL-10 receptor than does human IL-10 (Liu et al., 1997).

An explanation at the molecular level for the multiple activities of cIL-10 and the differences with EBV-IL-10 has still to be fully elucidated. Vieira et al. (1991) proposed that the differences in activity between cIL-10 and EBV-IL-10 may be due to a degree of heterogeneity in IL-10 receptors, variations in receptor number or differences in the threshold of activation. However, molecular analysis of the mouse IL-10 receptor has revealed that there is only one form of the receptor and that different regions within the cytoplasmic domain of the receptor mediate different signalling pathways (Ho et al., 1995). It was subsequently shown that these regions recruit different signal transducer and activator of transcription (STAT) proteins in different cell types and that the various biological activities induced by IL-10 are mediated by these proteins (Weber-Nordt et al., 1996). The exact nature of the interaction of IL-10 with its receptor has not been defined. Crystal structure models of IL-10 and IL-10 ligand–receptor interactions have suggested residues and domains that are likely to be involved in binding but have not identified structural elements of EBV-IL-10 which could account for the functional differences between the viral IL-10 and cIL-10 (Zdanov et al., 1996; Kotenko et al., 1997). Gessler et al. (1997) proposed that cIL-10 has different functional domains; an immunostimulatory domain near the N terminus and an immunoinhibitory domain at the C terminus. The lack of the immunostimulatory domain in the case of EBV-IL-10 was thought to account for the functional differences. However, studies by Ding et al. (2000) using cIL-10/viral IL-10 hybrid molecules have revealed that subtle changes in the structure of EBV-IL-10 within the centre of the molecule appear to underlie these functional differences.

ORFV is the type species in the parapoxvirus genus of the family Poxviridae (Francki et al., 1991) and infects sheep, goats and humans. In sheep, the virus often infects through a break in the skin around the mouth and causes a disease called contagious pustular dermatitis or scabby mouth. The disease results in cutaneous pustular lesions that last for 4–6 weeks after which time they resolve (Robinson & Lyttle, 1992). The ORFV-IL-10-like protein shows high levels of amino acid identity to the IL-10 of sheep (80%), cattle (75%), humans (67%), mice (64%), and to EBV-IL-10 (63%) and EHV2-IL-10 (67%). The C-terminal region of ORFV-IL-10, which constitutes two-thirds of the ORFV protein, is identical to ovine IL-10, which suggests that this gene has been captured from its host (sheep) during the evolution of ORFV.

As part of the further characterization of ORFV-IL-10, we wished to determine whether this virokine has immunosuppressive effects, as this activity might subvert the host immune response to ORFV infection. A well-characterized function of IL-10 is the regulation of macrophage function by the inhibition of cytokine synthesis. IL-10 has been shown to inhibit such products as IL-1, IL-6 and tumour necrosis factor (TNF-α) in activated macrophages in vitro (Fiorentino et al., 1991). In order to investigate the immunosuppressive activity of ORFV-IL-10 we examined its effect on the synthesis of the proinflammatory cytokine TNF-α in activated macrophages. We also explored further the immunostimulatory activities of ORFV-IL-10. We have previously shown that ORFV-IL-10 has a different activity spectrum to EBV-IL-10 in that it stimulates the proliferation of thymocytes (Fleming et al., 1997). In this study we set out to determine whether or not this difference extended to the stimulation of mast cells since this activity also distinguishes EBV-IL-10 from cIL-10 and appears to be linked to the enhanced immunosuppressive effects of EBV-IL-10 in vivo. Furthermore these assays, in particular mast cell proliferation, may reveal important structural and functional relationships of cIL-10 and viral IL-10s. Here, we report that ORFV-IL-10 inhibits TNF-α synthesis in murine peritoneal macrophages and stimulates the proliferation of the murine mast cell line MC/9, which strongly suggests that ORFV-IL-10 has the same range of activities as its cellular counterpart. The significance of the activities of ORFV-IL-10 is discussed in relation to the structure of the molecule.

Methods

Expression and purification of orf virus IL-10 and ovine IL-10. The coding sequence of the IL-10-like gene of ORFV strain NZ-2 (Robinson et al., 1982) was amplified by PCR using the recombinant plasmid pUV89 as a template (Mercer et al., 1995). The primers that were used to amplify ORFV-IL-10 and incorporate a sequence encoding a FLAG octapeptide at the C terminus of the polypeptide were 5′ CGCTTCTAGAGCCACCATGTCGAGAGAAGAAAAATCT (orf1) and 5′ CGCTTCTAGATTACGTCTACGTCGTCTCTGGTAGCGTTTATT TTGATGTCATGTAGT. The primers that were used to amplify ORFV-IL-10 and incorporate a sequence encoding a 6 × histidine (His6) tag at the C terminus of the polypeptide were orf1 and 5′ CGCTTCTAGATTTAGTGGTGGTTGCTGTGCTGCTTATATT GTAGCATGTGATGTG. The primers that were used to amplify EBV-IL-10 and incorporate a sequence encoding a 6 × histidine (His6) tag at the C terminus of the polypeptide were 5′ CGCTTCTAGATTAGTGGTGTGTGTGCTGTGCTGCTTATATT GTAGCATGTGATGTG. The primers that were used to amplify ovine IL-10 and incorporate a His6 tag at the C terminus were orf1 and 5′ CGCTTCTAGATTAGTGGTGTGTGTGCTGTGCTGCTTATATT GTAGCATGTGATGTG and which incorporated a FLAG tag sequence at the C terminus. The primers that were used to amplify ovine IL-10 and incorporate a His6 tag at the C terminus were ovine1 and 5′ CGCTTCTAGATTAGTGGTGTGTGTGCTGTGCTGCTTATATT GTAGCATGTGATGTG and which incorporated a FLAG tag sequence at the C terminus. The primers that were used to amplify ovine IL-10 and incorporate a His6 tag at the C terminus were ovine1 and 5′ CGCTTCTAGATTAGTGGTGTGTGTGCTGTGCTGCTTATATT GTAGCATGTGATGTG and which incorporated a FLAG tag sequence at the C terminus. The primers that were used to amplify ovine IL-10 and incorporate a His6 tag at the C terminus were ovine1 and 5′ CGCTTCTAGATTAGTGGTGTGTGTGCTGTGCTGCTTATATT GTAGCATGTGATGTG and which incorporated a FLAG tag sequence at the C terminus. The primers that were used to amplify ovine IL-10 and incorporate a His6 tag at the C terminus were ovine1 and 5′ CGCTTCTAGATTAGTGGTGTGTGTGCTGTGCTGCTTATATT GTAGCATGTGATGTG and which incorporated a FLAG tag sequence at the C terminus. The primers that were used to amplify ovine IL-10 and incorporate a His6 tag at the C terminus were ovine1 and 5′ CGCTTCTAGATTAGTGGTGTGTGTGCTGTGCTGCTTATATT GTAGCATGTGATGTG and which incorporated a FLAG tag sequence at the C terminus. The primers that were used to amplify ovine IL-10 and incorporate a His6 tag at the C terminus were ovine1 and 5′ CGCTTCTAGATTAGTGGTGTGTGTGCTGTGCTGCTTATATT GTAGCATGTGATGTG and which incorporated a FLAG tag sequence at the C terminus. The primers that were used to amplify ovine IL-10 and incorporate a His6 tag at the C terminus were ovine1 and 5′ CGCTTCTAGATTAGTGGTGTGTGTGCTGTGCTGCTTATATT GTAGCATGTGATGTG and which incorporated a FLAG tag sequence at the C terminus. The primers that were used to amplify ovine IL-10 and incorporate a His6 tag at the C terminus were ovine1 and 5′ CGCTTCTAGATTAGTGGTGTGTGTGCTGTGCTGCTTATATT GTAGCATGTGATGTG and which incorporated a FLAG tag sequence at the C terminus. The primers that were used to amplify ovine IL-10 and incorporate a His6 tag at the C terminus were ovine1 and 5′ CGCTTCTAGATTAGTGGTGTGTGTGCTGTGCTGCTTATATT GTAGCATGTGATGTG and which incorporated a FLAG tag sequence at the C terminus.
**Functional characterization of ORFV-IL-10**

- **SDS–PAGE and immunoblotting.** Purified proteins, diluted in SDS–PAGE sample buffer containing 5% 2-mercaptoethanol, were boiled and then resolved by SDS–PAGE (Laemmli, 1970). Proteins were visualized by Coomassie blue or silver staining. Proteins that were analysed by Western blotting were transferred to nitrocellulose and reacted with anti-FLAG Bio-M2 antibody (FLAG-tagged proteins) or India His probe HRP (His-tagged proteins) (Achen et al., 1998). IL-10 polypeptides separated by SDS–PAGE were confirmed by N-terminal sequencing analysis (Edman degradation).

- **Cells.** The MC/9 cell line (murine mast cells, ATCC CRL-8306) was maintained in RPMI 1640 (Gibco BRL) supplemented with 10% FCS, 50 mM 2-mercaptoethanol and 5% supernatant from Concanavalin A (ConA)-activated mouse spleen cells.

- **Cytokines.** Recombinant mouse IL-3 (cat. no. 19221T), recombinant mouse IL-4 (cat. no. 19231V) and recombinant mouse tumour necrosis factor-α (TNF-α) (cat. no. 19321T) were purchased from Phamingen. Recombinant murine IL-10 (product no. 13019) and recombinant murine interferon (IFN)-γ (product no. 15517) were purchased from Sigma.

- **Production of anti-ORFV-IL-10 antiserum.** Anti-ORFV-IL-10 antiserum was produced in rabbits by immunizing with a synthetic peptide immunogen. The peptide sequence was based on the C-terminal end of ORFV-IL-10, Y-I-E-S-Y-M-T-T-K-M (Molecular Resources), and was conjugated to the carrier molecule keyhole limpet haemocyanin (KLH) using glutaraldehyde. Rabbits were immunized with the immuno-gen emulsified in Freund’s complete adjuvant for primary immunization and Freund’s incomplete adjuvant for booster immunizations. The levels of anti-ORF-IL-10 peptide antibodies were determined by ELISA using Covalink covalent binding ELISA plates (Nunc) and methods described by the manufacturer.

- **MC/9 cell assay.** The immunostimulatory activity of ORFV-IL-10 fusion proteins and ovine IL-10 fusion proteins was determined by their ability to stimulate MC/9 cell proliferation as described by Thompson-Snipes et al. (1991). MC/9 cells were cultured in RPMI (Gibco) containing 2.0 g/l sodium bicarbonate, 0.11 g/l sodium pyruvate, 50 μM 2-mercaptoethanol, 0.081 g/l MEM non-essential amino acids (Sigma), 10% FCS and 10% conditioned medium. Conditioned medium was prepared from the supernatant of ConA-activated mouse spleen cells cultured in RPMI containing 5 × 10^5 M 2-mercaptoethanol, 2 mM glutamine, 2 μg/ml ConA, 5% FCS after 45 h incubation at 37 °C. Recombinant murine IL-3 (500 U/ml) and recombinant murine IL-4 (200 U/ml) were added with either recombinant ovine IL-10, recombinant ORFV-IL-10 or recombinant murine IL-10. MC/9 cells were stimulated with cytokines for 3–5 days and proliferation was determined by the addition of methyl[^H]thymidine (Amersharm Pharma) for the last 12 h of incubation. Cells were harvested with a Tom Tech Harvester 96 and [^H]thymidine counted in a 1450 Microbeta Plus liquid scintillation counter.

- **Cytokine synthesis inhibition assay.** The cytokine synthesis inhibition assay was carried out using mouse peritoneal macrophages. Cells were obtained from the peritoneal cavity of specific pathogen-free BALB/c mice (6–10 weeks) by firstly inducing inflammation in the cavity with proteose peptone brain heart infusion and then harvesting the cells 4 days later by peritoneal lavage with cold DMEM. Cells were centrifuged at 4 °C for 10 min and maintained in six-well plates (Nunc) in high glucose (4500 mg/ml) DMEM containing 3.7 g/l sodium bicarbonate, 4.77 g/l HEPES, 15 μl/l 2-mercaptoethanol, 110 mg/l sodium pyruvate and 10% FCS. After 1 h incubation at 37 °C non-adherent cells were removed by washing with DMEM and adherent cells were primed for 1 h with murine IFN-γ followed by activation with lipopolysaccharide (LPS) for 1 h. Cell medium was replaced with fresh...
Fig. 2. Effect of ORFV-IL-10 on MC/9 cell proliferation. (a) Purified ORFV-IL-10–His$_6$ and –FLAG-tagged proteins, ovine IL-10–His$_6$ and –FLAG-tagged proteins and murine IL-10 were assayed at 50 ng/ml (black shading), 5 ng/ml (dark-grey diagonal shading) and 0.5 ng/ml (light-grey dot shading) final concentration. All assays containing IL-10 were supplemented with 500 U/ml IL-3 and 200 U/ml IL-4. Controls included medium only, medium containing 500 U/ml IL-3 and 200 U/ml IL-4, His$_6$ and FLAG purification controls containing supernatants of 293 cells transfected with the pAPEX plasmid only (see Fig. 1) and 500 U/ml IL-3 and 200 U/ml IL-4. Cell proliferation was determined by $[^{3}H]$thymidine uptake over the final 12 h of a 3–5 day incubation and expressed as c.p.m.. All assays were performed in triplicate and error bars represent 3 standard deviations from the mean. This assay was performed three times and the results shown are representative of these assays. (b) MC/9 cells were incubated with either 50 ng/ml of ORFV-IL-10–FLAG, 50 ng/ml ovine IL-10–FLAG or 10 U/ml of murine IL-10 final concentration and with 2% anti-ORFV-IL-10 polyclonal antiserum (final concentration) or 2% preimmune antiserum or without antiserum. The cells were supplemented with 500 U/ml IL-3 and 200 U/ml IL-4. Controls included medium only and medium containing 500 U/ml IL-3 and 200 U/ml IL-4. Cell proliferation was determined by $[^{3}H]$thymidine uptake over the final 12 h of a 3–5 day incubation and expressed as c.p.m.. All assays were performed in triplicate and error bars represent 3 standard deviations from the mean.
DMEM and IL-10 added and the plates incubated for 24 h. The cell supernatant was removed and assayed for TNF-α levels by ELISA. Total RNA was recovered from cells by an extraction procedure using guanidinium thiocyanate and phenol (Chomczynski & Sacchi, 1987) for real-time quantitative PCR analysis of TNF-α mRNA.

ELISA. The levels of murine TNF-α were determined in cell culture supernatants by ELISA following methods described in the Pharmingen Cytokine ELISA Handbook. Purified rat anti-mouse TNF-α mAb (MP6-XT22, Pharmingen) was used as a capture antibody in Maxisorb ELISA plates (Nunc). Recombinant mouse TNF-α was used to generate a standard curve: dilutions were made in PBS containing 10% FCS. Similarly, cell culture supernatants that were assayed for TNF-α were diluted in PBS containing 10% FCS. Biotinylated rat anti-mouse TNF-α mAb (MP6-XT3, Pharmingen) and streptavidin-biotinylated horseradish peroxidase were used to detect TNF-α. Bound enzymatic activity was measured with the substrate p-phenylenediamine dihydrochloride.

RT–PCR and quantitative PCR. cDNA synthesis was carried out with oligo(dT)$_{12}$ primer, total RNA isolated from murine peritoneal macrophages and Superscript II (Gibco BRL) using methods described by Matathias et al. (1999). The PCR reaction for murine β-actin was carried out with primers 5’-CTTCATGAGGTAGTCAAGGTC and 5’-ATGGGTCAAGAAGGATTCCTATGTG (Bongers et al., 1999) using Amplitaq Gold DNA polymerase (Perkin-Elmer) and methods described by PE Applied Biosystems. PCR amplifications were performed in an Omigene PCR machine (Hybaid). Real-time quantitative PCR was carried out in an ABI PRISM 7700 Sequence Detection System by methods described in Lee et al. (1993) and the PE Applied Biosystems User Bulletin #2, 1997. Murine TNF-α primers and a TNF-α-specific fluorogenic probe were designed by PE Biosystems as a pre-developed Taqman assay reagent kit. Analyses of real-time quantitative PCR data are described in PE Applied Biosystems User Bulletin #2, 1997.

Results

Expression and purification of ORFV-IL-10

In order to express and purify ORFV-IL-10 and ovine IL-10 we cloned the coding sequences into the eukaryotic expression vector pAPEX-3 incorporating either a His$_6$ or FLAG sequence at the C terminus and expressed the proteins in transfected 293 EBNA cells. Both the FLAG and His$_6$ versions of these proteins were made, since the FLAG tag has been shown to adversely affect the specific activity of EBV-IL-10 whereas it did not affect the activity of human IL-10. Furthermore, the specific activity of EBV-IL-10 and human IL-10 were not affected by a C-terminal His$_6$ tag. (Kevin Moore, personal communication; Liu et al., 1997). The secreted proteins were purified from cell culture supernatants by affinity chromatography using either anti-FLAG M2 affinity gel in the case of ORFV-IL-10 fusions or Ni–NTA agarose in the case of IL-10–His$_6$ fusions. The proteins were characterized by SDS–PAGE. Fig. 1(a) shows that ORFV-IL-10–FLAG and ovine IL-10–FLAG migrate with a molecular mass of 22 kDa and 20 kDa, respectively. To confirm the identity of these proteins, the electrophoretically separated molecules were transferred to a nitrocellulose matrix by Western blotting and probed with anti-FLAG mAb M2. The results in Fig. 1(b) show that the proteins identified by SDS–PAGE match the size of the proteins detected by Western blotting. Similarly, purified ORFV-IL-10–His$_6$ and ovine IL-10–His$_6$ proteins were separated by SDS–PAGE and Western blotted (not shown). The molecular masses determined for the His$_6$-tagged proteins were the same as those determined for the FLAG-tagged proteins. Further confirmation that we had correctly expressed and purified the viral and ovine IL-10 polypeptides was obtained by N-terminal sequence analysis by Edman degradation. The N-terminal sequence obtained for ORFV-IL-10 (Y-C-V-E) showed that the ORFV-IL-10 immature polypeptide is cleaved between aa 22 and 23 to produce the mature secreted protein of 164 aa (see Fig. 5). The N-terminal sequence obtained for ovine-IL-10 (A-S-R-D) showed that this protein is cleaved between aa 18 and 19, which fits with the predicted cleavage site determined by Martin et al. (1995).

ORFV-IL-10 costimulates the growth of mast cell line MC/9

Optimal growth of mast cells is dependent on multiple factor interactions. Of these, only IL-3 has been shown to support the growth of both long-term mast cell lines and freshly isolated mast cell progenitors. IL-4 is weakly stimulatory for most mast cell lines, but potentiates their IL-3-dependent growth. Similarly, IL-10 by itself is not capable of stimulating the growth of mast cells but will enhance their growth in the presence of suboptimal levels of IL-3 or saturating or suboptimal concentrations of IL-4 (Thompson-Snipes et al., 1991).

In order to examine the effect of ORFV-IL-10–FLAG and ORFV-IL-10–His$_6$ on MC/9 cell proliferation, optimal concentrations of conditioned medium derived from ConA-activated spleen cells were determined in the first instance. We then established the optimal levels of murine IL-3 and IL-4 that maximized the costimulatory effect of viral IL-10 on mast cell proliferation. The optimal concentration of conditioned medium in the assay was approximately 5% final volume, which represents approximately one-quarter of the concentration that gave maximal proliferation with 200 U/ml of IL-3 and 200 U/ml IL-4. Optimal amounts IL-3 and IL-4 and an optimal incubation period for the assay was determined by titrating each cytokine with 5% conditioned medium and measuring the proliferative response as [H]$^3$Thymidine uptake at 12, 24, 48, 72 and 96 h. [H]$^3$Thymidine was added over the final 12 h of incubation. Maximum proliferation of mast cells was observed between 72 and 96 h for both cytokines with IL-3 producing a greater effect on growth than IL-4 (data not shown).

The conditions established to determine the effect of ORFV-IL-10 on MC/9 mast cell growth were 5% conditioned medium, 500 units of IL-3 (which gave half-maximal mast cell proliferation) and 200 U/ml of IL-4 (which gave maximal mast cell proliferation). These conditions closely approximated those used by Thompson-Snipes et al. (1991). Significant costimulation of mast cells was found for both ORFV-IL-10–FLAG and ORFV-IL-10–His$_6$ at concentrations of 0.5–50
ng/ml (Fig. 2a), with the lower concentrations showing a reduced effect on mast cell growth. There was no significant difference in costimulation between ORFV-IL-10–FLAG and ORFV-IL–10–His6. The level of proliferation observed for viral IL-10 and ovine IL-10 was similar at all concentrations and only slightly less than that for recombinant murine IL-10.

The effect of viral IL-10 on mast cell growth was also examined in the presence of anti-ORFV-IL-10 antisera (made by immunizing rabbits with an immunogen, in which a synthetic peptide corresponding to the C-terminal region of ORFV-IL-10 was conjugated to KLH). Previously Reineke et al. (1998) had shown that mAb CB/RS/10 neutralized human IL-10 via a linear epitope which mapped to the C terminus of the molecule. Similarly, polyclonal antisera made to a synthetic peptide–KLH conjugate, where the peptide corresponded to the last 9 aa at the C terminus of human IL-10, neutralized IL-10 activity (Gesser et al., 1997). Fig. 2(b) shows that anti-ORFV-IL-10 antiserum significantly reduced the effect of viral IL-10 on mast cell proliferation, indicating that the costimulatory effect was due to ORFV-IL-10.

**ORFV-IL-10 inhibits the expression of TNF-α in mouse peritoneal macrophages**

cIL-10 is a potent anti-inflammatory cytokine and this response is mostly regulated via macrophage function and T cell activation. Proinflammatory factors such as IL-1, IL-6 and TNF-α are expressed in macrophages in culture in response to IFN-γ and LPS and their expression can be inhibited by cIL-10 (Fiorentino et al., 1991).

Prior to examining the effect of ORFV-IL-10 on TNF-α synthesis in activated mouse peritoneal macrophages, we optimized the amount of LPS required to induce TNF-α expression. Fig. 3(a) shows that the optimal concentration of LPS required to induce TNF-α was approximately 10 μg/ml. Higher levels of LPS (100 μg/ml) produced lower expression while 1 μg/ml gave only 30% of that obtained with 10 μg/ml.

The effect of ORFV-IL-10 on the synthesis of TNF-α was examined by firstly incubating the cells in the presence of IL-10 followed by priming with IFN-γ and finally activating the cells with LPS. Sequential stimulation of bovine alveolar macrophages with IFN-γ and LPS has been associated with increased levels of TNF-α synthesis compared with stimulation of macrophages with LPS alone (Aldwell et al., 1997).

We examined the levels of TNF-α in cell culture supernatants. We found that when cells were incubated in the presence of ORFV-IL-10 prior to activation, viral IL-10 strongly inhibited the expression of TNF-α (Fig. 3b). Furthermore, ORFV-IL-10 was as effective at inhibiting TNF-α synthesis in murine peritoneal macrophages as ovine IL-10 or murine IL-10. Approximately 10 U/ml of ORFV-IL-10 produced maximal inhibition of TNF-α synthesis (Fig. 3c), where 1 U has been defined as the amount that stimulates 50% maximal proliferation of MC/9 cells. In addition, these assays were also performed with ORFV-IL-10–His6 and ovine IL-10–His6 and produced similar results to their FLAG-tagged counterparts (data not shown). We also demonstrated that the addition of anti-ORFV-IL-10 antiserum abrogated the inhibitory effect of viral IL-10 in this assay (Fig. 3b).

We examined the effect of ORFV-IL-10 on TNF-α mRNA transcription in LPS-activated murine peritoneal macrophages by real-time quantitative PCR amplification. RNA was isolated from cells immediately following the removal of cell culture medium for TNF-α protein analysis as described above. The relative levels of total RNA extracted from cells were determined by cDNA synthesis and PCR amplification of a segment of β-actin mRNA. Relative levels of cDNA were compared by the band strength of PCR-amplified β-actin separated on 1% agarose (Fig. 4a). cDNA levels were then standardized prior to analysis of TNF-α by real-time quantitative PCR. Quantitative PCR amplifications for murine TNF-α were carried out over 40 cycles using 10-fold dilutions of cDNA and the data analysed as described in Methods. A standard curve for TNF-α mRNA produced in murine peritoneal macrophages in response to LPS, was used to calculate the relative levels of TNF-α mRNA produced in cells preincubated with ORFV-IL-10 prior to stimulation with LPS. We showed in this assay that ORFV-IL-10 inhibited the expression of TNF-α mRNA synthesis by approximately 80% (Fig. 4b).

**Discussion**

We have shown that ORFV-IL-10 costimulates the growth of murine mast cells in culture. We found little difference between virus-encoded IL-10 and ovine IL-10 with both producing equivalent stimulatory effects on MC/9 cells. In addition, we have shown that ORFV-IL-10 suppresses macrophage activation. We found that viral IL-10 and ovine IL-10 were only slightly less potent than mouse IL-10 in inhibiting the expression of TNF-α in LPS-activated murine peritoneal macrophages.

We also observed no significant difference in specific activity between the FLAG-tagged and His6-tagged versions of ORFV-IL-10, which is contrary to the effects that these tags have been shown to have on the activity of EBV-IL-10. Moreover, neither of these tags affect the specific activity of human IL-10. These differences may be related to the binding affinity of the ligand for its receptor, since human IL-10 has a 1000-fold higher binding affinity for its receptor than EBV-IL-10. Structural analysis of ORFV-IL-10 suggests that this ligand may have a relatively high binding affinity for its receptor, since it is almost structurally identical to ovine IL-10 over those regions which are predicted to interact with the receptor (see below) and, like human IL-10, its activity is unaffected by the addition of small peptide sequences to the C terminus.

It is apparent that ORFV-IL-10 has evolved a different set of functional activities to EBV-IL-10, although both have...
Effect of ORFV-IL-10 on TNF-α expression in murine peritoneal macrophages. (a) Effect of LPS concentration on TNF-α expression in mouse peritoneal macrophages. Murine peritoneal macrophages were primed with IFN-γ prior to activation with LPS. Cells were activated for 1 h with the following concentrations (µg/ml) of LPS: 100, 10, 1, and 0. The levels of TNF-α produced in cell culture supernatants were determined 24 h post-activation by ELISA. The assay was carried out in duplicate. Quantification of TNF-α in all samples was performed in triplicate. Error bars represent 3 standard deviations from the mean. (b) Effect of ORFV-IL-10 on the synthesis of TNF-α in activated murine peritoneal macrophages. The latter were incubated in the presence of 10 µg/ml final concentration of LPS. Recombinant murine IL-10 was used at 10 µg/ml final concentration of LPS. Recombinant murine IL-10 was used at 10 µg/ml. FLAG purification control represents the products of FLAG purifications from supernatants of 293 cells transfected with the pAPEX plasmid only (see Fig. 1). ORFV-IL-10 activity was neutralized with anti-ORFV-IL-10 antiserum at 5% final concentration. The levels of TNF-α in cell culture supernatants were determined by ELISA 18 h post-activation with LPS. Amplifications were performed over 40 cycles and the data analysed as described in Methods. The relative levels of TNF-α mRNA are shown for assays in which PCR amplifications were carried out with a dilution of cDNA of 1 in 100. The error bars represent 3 standard deviations from the mean and the numbers in parentheses indicate the relative levels of TNF-α mRNA.

Fig. 3. Effect of ORFV-IL-10 on TNF-α expression in murine peritoneal macrophages. (a) Effect of LPS concentration on TNF-α expression in mouse peritoneal macrophages. Murine peritoneal macrophages were primed with IFN-γ prior to activation with LPS. Cells were activated for 1 h with the following concentrations (µg/ml) of LPS: 100, 10, 1, and 0. The levels of TNF-α produced in cell culture supernatants were determined 24 h post-activation by ELISA. The assay was carried out in duplicate. Quantification of TNF-α in all samples was performed in triplicate. Error bars represent 3 standard deviations from the mean. (b) Effect of ORFV-IL-10 on the synthesis of TNF-α in activated murine peritoneal macrophages. The latter were incubated in the presence of 10 µg/ml final concentration of ORFV-IL-10-FLAG and ovine IL-10-FLAG for 1 h before priming the cells with IFN-γ and activation with 10 µg/ml final concentration of LPS. Recombinant murine IL-10 was used at 10 µg/ml. FLAG purification control represents the products of FLAG purifications from supernatants of 293 cells transfected with the pAPEX plasmid only (see Fig. 1). ORFV-IL-10 activity was neutralized with anti-ORFV-IL-10 antiserum at 5% final concentration. The levels of TNF-α in cell culture supernatants were determined by ELISA 18 h post-activation with LPS. The assays were carried out in duplicate. Quantification of TNF-α in all samples was performed in triplicate. Error bars represent 3 standard deviations from the mean. (c) Effect of ORFV-IL-10-FLAG concentration on the synthesis of TNF-α in activated murine peritoneal macrophages. The latter were incubated in the presence of 1, 10 and 20 µg/ml final concentration of ORFV-IL-10-FLAG and ovine IL-10-FLAG for 1 h before priming the cells with IFN-γ and activation with 10 µg/ml final concentration of LPS. The levels of TNF-α in culture supernatants were determined by ELISA 18 h post-activation with LPS. ORFV-IL-10-FLAG (○); ovine IL-10-FLAG (×); murine IL-10-FLAG (□); FLAG purification control (△).
activity of this virokine. Similarly, we were not able to find a significant match between oligomer IT9403 and the polypeptide sequence at the near N terminus of ORFV-IL-10. The findings of Gesser et al. (1997) have not been confirmed by others and a recent report by Ding et al. (2000) has shown that residues in cIL-10 which are critical for stimulating the proliferation of MC/9 cells map towards the centre of the molecule. Moreover, it would appear that there exists a common domain in cIL-10 involved in costimulation of murine mast cells, thymocytes and MHC class II expression on B cells. Such findings fit with our observations that (a) the central region of ORFV is highly conserved when compared with cIL-10 and (b) ORFV-IL-10 stimulates the proliferation of murine thymocytes and mast cells. Furthermore, Ding et al. (2000) were able to demonstrate that an isoleucine residue located at position 87 (see Fig. 5) was critical for the immunostimulatory effects of cIL-10 and if this was replaced by the equivalent residue in EBV-IL-10, namely alanine, cIL-10 lost its immunostimulatory activities. Conversely, if this particular alanine of EBV-IL-10 was replaced with isoleucine the virokine acquired the immunostimulatory activities of its cellular counterpart. Perhaps significantly ORFV-IL-10 has an isoleucine located at this position (see Fig. 5).

Residues of IL-10 that interact with the IL-10 receptor have also been predicted from crystal structure models and these predictions fit with our observations that ORFV-IL-10 and ovine IL-10 have functions that are indistinguishable in the assays that we have done. The interaction of IL-10 with its receptor is based on the IFN-γ–IFN-γR1 complex (Walter & Nagabhushan, 1995). The crystal structures of IL-10 and IFN-γ share similar topological features (Ealick et al., 1991; Zdanov et al., 1995, 1996; Walter & Nagabhushan, 1995). Asterisk indicates residues of human IL-10 that are predicted to interact with the IL-10R1 (Zdanov et al., 1996). # indicates an isoleucine residue identified in human IL-10 that is critical for immunostimulation of thymocytes and mast cells (Ding et al., 2000). The functional domains identified by Gesser et al. (1997) for human IL-10 are underlined in bold: the sequence underlined near the N terminus corresponds to octapeptide IT9403 and the sequence underlined at the C terminus corresponds to octapeptide IT9302.
γ and IL-10 receptors belong to the same class II cytokine receptor family (Bazan, 1990; Thoreau et al., 1991). It has been proposed that the IL-10 receptor complex consists of two chains, R1 and R2, and that signal transduction occurs only if both chains are present in the complex (Kotenko et al., 1997). IL-10 is predominantly α-helical with six helices forming approximately 67% of the structure. It is predicted that residues of IL-10 within helices A and B, the AB loop and helix F interact with R1 and that highly conserved residues within helices C and D and the AE loop may interact with R2 since this middle part of the molecule does not interact with R1 (Zdanov et al., 1996; Kotenko et al., 1997). These regions are shown in Fig. 5. The primary structures of ovine IL-10 and ORFV-IL-10 are almost identical over the regions that are predicted to interact with R1, except for two residues within helix A of ovine IL-10 (aspartic acid and valine at positions 44 and 45 respectively), which are substituted with glutamic acid and leucine in ORFV-IL-10 (Fig. 5). These substitutions, however, are unlikely to alter the interaction of ORFV-IL-10 with R1 appreciably since they represent structurally conservative changes and are present in bovine and mouse IL-10 at the equivalent positions. Furthermore, the primary structures of ORFV-IL-10 and ovine IL-10 are identical over those regions predicted to interact with R2 (Kotenko et al., 2000). In addition, we have compared the predicted tertiary structure of ORFV-IL-10 with human IL-10 using the structural prediction program Swiss Model (Guex et al., 1999; Guex & Peitsch, 1997; Peitsch, 1995) and have shown that the only significant difference exists at the N terminus within the first 20 aa of the ORFV-IL-10 mature peptide. The ligand–receptor models based on crystal structure strongly support the view that only the predicted alpha-helical components of the ORFV-IL-10 homodimer are likely to interact with the IL-10 receptor complex and that the unstructured N-terminal parts of the homodimer will have little affect on this interaction.

It is unclear why ORFV-IL-10 has retained the immunostimulatory activities of cIL-10 since this activity appears to play no role in immune evasion. Furthermore, it is apparent that only very minor changes to ORFV-IL-10 could diminish or eliminate this activity. Studies by Suzuki et al. (1995) suggest that such a mutation towards an EBV-like IL-10 would favour a strain with enhanced immunosuppressive potential and viral pathology. Sheep with large persisting ORFV lesions that resemble tumour-like growths have been observed (Greig et al., 1984; McKeever, 1986), but it is thought that this phenomenon is due to a defective host immune system rather than a virus strain with enhanced virulence. We and others have sequenced the IL-10-like gene from various ORFV isolates from Britain, Europe and New Zealand and have found that the critical residues that are thought to be involved in receptor/ligand interactions are highly conserved (Fleming et al., 2000). Furthermore, the nucleotide sequence of ORFV-IL-10 is only 67% identical to ovine IL-10, which suggests that major changes to the viral gene have taken place during the course of evolution, but the proteins encoded by these genes have remained remarkably similar. This may suggest that mutations of the ORFV-IL-10 gene to a potentially more potent immunosuppressive form may compromise the coexistence of host and virus.

In conclusion, we have shown that ORFV-IL-10 costimulates the proliferation of murine mast cells in addition to murine thymocytes and inhibits cytokine synthesis in murine peritoneal macrophages. Functional analysis and molecular structure analysis of ORFV-IL-10 strongly suggest that this virekine has the full spectrum of activities of cIL-10. These activities suggest that ORFV-IL-10 is likely to play a critical role in protecting virus-infected cells from the immune defences, in particular suppressing the inflammatory response and innate responses early in infection.

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References


Inhibitory factor (IL-10) to the Epstein–Barr virus gene BCRF1.


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