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Orf Virus Encodes a Novel Secreted Protein Inhibitor of Granulocyte-Macrophage Colony-Stimulating Factor and Interleukin-2

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The parapoxvirus orf virus encodes a novel soluble protein inhibitor of ovine granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-2 (IL-2). The GM-CSF- and IL-2-inhibitory factor (GIF) gene was expressed as an intermediate-late viral gene in orf virus-infected cells. GIF formed homodimers and tetramers in solution, and it bound ovine GM-CSF with a K_d of 369 pM and ovine IL-2 with a K_d of 1.04 nM. GIF did not bind human GM-CSF or IL-2 in spite of the fact that orf virus is a human pathogen. GIF was detected in afferent lymph plasma draining the skin site of orf virus reinfection and was associated with reduced levels of lymph GM-CSF. GIF expression by orf virus indicates that GM-CSF and IL-2 are important in host antiviral immunity.

Poxviruses stimulate a vigorous immune response in their hosts. In spite of this, these viruses can replicate and induce lesions. A possible explanation for this is that poxviruses, along with other large DNA viruses, express immunomodulatory virulence proteins that inhibit or mimic key effector molecules of the host immune and inflammatory response to infection (35, 56, 57). A common general mechanism is the production of viral proteins inhibiting early events in the host response to infection, including inflammatory cytokine, interferon, chemokine, and complement function and apoptosis. Many of the immunomodulatory genes are orthologues of host cellular genes that have been acquired and modified by the viruses. For example, the orthopoxviruses vaccinia virus and cowpox virus encode soluble receptor proteins that bind to and inactivate the host cytokines interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α), and interferons (IFNs) as well as complement components (1, 2, 8, 31, 50, 58, 62). Viral proteins that do not bind directly to IFNs but instead interfere with downstream signalling molecules following ligand-receptor coupling also inhibit the antiviral activity of interferons (10, 27, 44). By studying these viral immunomodulator proteins, insight into the mechanisms of not only virus virulence but also host protective immunity to virus infection is gained.

We have been studying the mechanisms of immune system evasion by the prototype parapoxvirus orf virus (contagious ecthyma virus). Orf virus is a ~140-kb double-stranded DNA (dsDNA) parapoxvirus that has a worldwide distribution and infects sheep, goats, and man (reviewed in references 26 and 49). Infections are acute, giving rise to pustular lesions that turn to scabs. Virus is contained locally and shed with the scab. The virus infects via broken or scarified skin and replicates in regenerating epidermal keratinocytes. The immune response to orf virus is characterized by a local accumulation of $CD4^+$ and $CD8^+$ T cells, B cells, neutrophils, and a dense network of dermal dendritic cells (32, 33, 38). Immune system evasion by orf virus is implicated because the virus can repeatedly infect previously exposed lambs in spite of an apparently normal host antivirus immune and inflammatory response (21–24, 64, 65). Host immunity has some effect, since the size of the lesions and the time to resolution in reinfections are diminished compared to those of the initial infection.

Most of the orf virus genome of 140 kbp has been sequenced. However, only 31 gene sequences (or partial gene sequences) spanning the genome are presently in the databases. Several putative immunomodulating genes have been discovered: a viral orthologue of mammalian vascular endothelial growth factor (VEGF) (40), a viral orthologue of IL-10 (16), and an orf virus orthologue of the vaccinia virus E3L gene, which codes for an interferon resistance protein (27, 44). In a study of cytokine production in orf virus-infected keratinocytes, IL-8, TNF-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNAs and IL-8 and TNF-α protein, but not GM-CSF protein, were detected (37). In this article, we describe the isolation and characterization of a novel protein, GM-CSF-inhibitory factor (GIF), derived from a gene within the right terminal quarter of the orf virus genome, that binds to and inhibits the ovine cytokines GM-CSF and IL-2.

MATERIALS AND METHODS

Viruses. The orf virus strains NZ-2 (47), orf 11 (generated at the Moredun Research Institute [unpublished]), and scabbymouth (52) were tissue culture adapted from field isolates and were maintained by passage in primary bovine testis or fetal lamb muscle (FLM) cells. Semliki Forest virus was used as an unrelated virus control; it was maintained by passage in ST-6 ovine fibroblasts (12). MRI scab virus (45) was obtained by infection of sheep and harvesting of virus from the resultant scabs; it has not been adapted to grow in cell culture. Ovine primary keratinocytes were obtained, cultured, and characterized as described previously (37). Vaccinia virus-orf virus recombinants (VVOVs) containing approximately 95% of the orf virus genome in overlapping DNA fragments have been described previously (48) and were propagated in CV-1 cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Lymph samples were from previous experiments (21, 23). In these experiments, scabbymouth virus was used to infect Suffolk cross sheep in the hind flank (i.e., the prefemoral lymph node drainage region) by scarification with a needle

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and topical application of orf virus (10^6 50% tissue culture infective doses [$TCID_{50}$]). For controls, the virus was inactivated by UV irradiation (21) and the equivalent of ~10^6 $TCID_{50}$ of virus was injected intradermally into the hind flank. Cannulated pseudo-afferent lymph (herein referred to as afferent lymph) and efferent lymph samples were obtained from reinfected sheep as described previously (21, 23).

DNA and RNA techniques. dsDNA templates were sequenced by using a LI-COR 4200 automated DNA sequencing system and manufacturer-recommended procedures. Single-stranded DNA (ssDNA) templates were sequenced by using a T7 DNA Sequencing Kit (Amersham Pharmacia Biotechnology [APB], St. Albans, United Kingdom). Viral RNA was prepared from FLM cells, infected for 5 or 18 h with NZ2 orf virus at a multiplicity of infection (MOI) of 20 $TCID_{50}$, by an acid phenol-guanidine hydrochloride method (6). Cells were grown in the presence and absence of cytosine arabinoside (CA) (40 μg/ml), which inhibits viral DNA replication but not the expression of early viral genes (27). Preparation of dsDNA and ssRNA probes and Northern analysis were performed as described previously (44).

Expression and purification of recombinant GIF. A 908-bp DNA fragment containing the entire open reading frame (ORF) of the GIF gene was amplified by PCR with oligonucleotides 5′-GGAAAGCTTGCGCCGGCTCTAGGAAAGAT-3′ and 5′-GGGGAATTCAAGGATAAGGTCCACGGCGT-3′. The PCR product was ligated into the pEE14 expression vector (Celltech, Slough, United Kingdom) (7, 13) in tandem with a glutamine synthetase selectable marker gene and verified by sequencing prior to transfection into CHO cells by the use of Superfect transfection reagent (Qiagen, Crawley, United Kingdom) in accordance with the manufacturer's recommended procedures. The stable transfected CHO cells were maintained in glutamine-free Glasgow's modified Eagle's medium (Gibco BRL, Paisley, United Kingdom) supplemented with 7.5% heat-inactivated dialyzed FBS (PAA Laboratories, Kingston upon Thames, United Kingdom) and methionine sulfoxamine (an inhibitor of glutamine synthetase) (Sigma, Poole, United Kingdom) to select cells with high levels of production of GIF. GIF was purified from CHO cell-free supernatants (CFSs) (serum-free medium) by affinity chromatography with purified recombinant ovine GM-CSF (rovGM-CSF) bound to CNBr-Sepharose (APB) followed by gel filtration on a Sephacryl S-200 column (APB). The rabbit anti-GIF immunoglobulin G (IgG) was prepared by injecting affinity-purified GIF (10 μg) and 20 μg of Quil-A saponin adjuvant intramuscularly followed by two booster injections. An IgG fraction was prepared by protein A-Sepharose affinity chromatography. Western blot analysis of proteins was performed with phosphate-buffered saline (PBS) plus 4% nonfat milk (blocking buffer) and PBS (0.5 M NaCl) plus 0.5% Tween 80 (antibody dilution and blot wash buffer) by the enhanced chemiluminescence technique (ECL; APB). Proteins were electrotransferred (Schleicher & Schuell) to BA 83 nitrocellulose membranes (Anderman, Kingston upon Thames, United Kingdom).

N-terminal amino acid sequencing of the soluble recombinant protein was achieved by liquid-phase Edman degradation chemistry on a model 492 Procise Protein Sequencer (Perkin-Elmer Applied Biosystems, Warrington, Cheshire, United Kingdom). The purified protein was applied to a Polybrene-treated glass fiber filter prior to sequencing. The N-terminal 20 amino acids of two separate samples were analyzed.

Cytokines and GIF-binding assays. The recombinant ovine cytokines IL-1β, IL-2, IL-3, IL-4, IL-5, IL-8, GM-CSF, MCP-1, macrophage inflammatory protein 1α, RANTES, TNF-α, and IFN-γ were prepared by transfection of the cytokine cDNAs (from Ian Colditz, T. Yoshimura, Heng-Fong Seow, Paul Wood, J.-P. Scheerlink, and Paul Chaplin, CSIRO, Melbourne, Australia, and Gary Entrican, Moredun Research Institute) into CHO cells and purification of the recombinant proteins by fast protein liquid chromatography, anion-exchange chromatography, and/or gel filtration chromatography. Purified human (hu) and murine (mu) GM-CSF, huIL-2, and huIL-4 recombinant proteins produced in *Escherichia coli* were purchased from R&D Systems (Abington, United Kingdom). Heparin was purchased from Sigma.

For GIF-cytokine ligand blots, purified GIF was radioiodinated (^{125}I labelled) by the chloramine T method (42) and further purified on Sephadex G-25 and Sephacryl 200 HR (APB) columns in PBS with 0.15% (vol/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) buffer, pH 7.2. Two hundred to 350 ng of each cytokine was separated by sodium dodecyl sulfate (SDS)–15% polyacrylamide gel electrophoresis (PAGE) under reducing and nonreducing conditions and then transferred to nitrocellulose membranes. The membranes were blocked in blocking buffer, washed in PBS plus 0.05% Tween 20 (wash buffer), and then incubated with ^{125}I-GIF (20 pM) for 2 h at room temperature. After the membranes were washed three times in wash buffer, cytokine-GIF binding was detected by autoradiography, using Hyperfilm MP X-ray film (APB). A 100 nM concentration of unlabeled ("cold") GIF was used for competitive inhibition of binding of ^{125}I-GIF to the cytokines.

GIF activity was assayed by inhibition of ovine (ov) GM-CSF detection in an ovGM-CSF capture enzyme-linked immunosorbent assay (ELISA) using two monoclonal murine antibodies: 8D8 as the capture antibody and horseradish peroxidase-conjugated 3C2 as the detection antibody (13). 1,2 Diaminobenzene and tetramethylbenzidine peroxidase substrates were used (determined by optical densities at 450 and 492 nm [OD_{450} and OD_{492}], respectively). Samples containing GIF and control samples were incubated (spiked) with either 4 or 8 ng of ovGM-CSF/ml (in 20-μl volumes) for 1 h at 37°C. These samples were then assayed by ovGM-CSF ELISA. Binding of GIF to ovGM-CSF results in the loss of antibody binding to ovGM-CSF and a reduction in ELISA optical density (OD). The specificity of the ovGM-CSF inhibition ELISA was demonstrated by preincubation of samples for 1 h at room temperature with the rabbit anti-GIF IgG prior to spiking of the mixture with ovGM-CSF and proceeding as described above. The antibody-GIF complex inhibits the binding of GIF to exogenously added ovGM-CSF, which is detected in the ELISA. This is not a quantitative assay for GIF. Cytokines were assayed for GIF binding by competition with ovGM-CSF in the ovGM-CSF ELISA. Cytokines (10 to 20 ng/ml) were added to GIF prior to the addition of 4 ng of ovGM-CSF/ml and subsequent ELISA.

Scatchard analysis of GIF reactivity. Purified cytokines were radioiodinated by the chloramine T method. A soluble-ligand-binding assay was performed for each cytokine as described by Symons et al. (61) with some modifications. Briefly, 50-μl volumes of a range of ^{125}I-GM-CSF and ^{125}I-IL-2 concentrations (2 to 20 nmol) were incubated with 100 μl of GIF (100 ng) containing 5% FBS for 2 h at room temperature. Bound proteins were precipitated by the addition of 300 μl of 20% polyethylene glycol (PEG 6000; Sigma) in PBS and incubation on ice for 30 min. The material precipitated from each sample was collected by filtering through GF/C filter discs (Whatman, Maidstone, United Kingdom) under a vacuum and washing with four 10-ml volumes of ice-cold 10% PEG 6000 in PBS. Radiolabelled complex was detected and quantified in a gamma scintillation counter (Cobra II Auto-Gamma Counter; Packard, Pangbourne, United Kingdom). The levels of nonspecific binding of ^{125}I-cytokines to the filters were obtained in the absence of GIF, and the data were adjusted accordingly. Scatchard analysis was performed on best-fit plots (bound counts per minute/free counts per minute [*y* axis] versus bound counts per minute [*x* axis]) generated by using Origin software (Microcal, Northampton, Mass.).

GIF bioassays. The soft-agar hematopoietic cell clonogenic assay has been described in detail elsewhere (25, 43). Briefly, sternal bone marrow cells (5×10^4/ml of culture) in Iscove's modification of DMEM (Gibco BRL) containing 20% FCS and 3% (vol/vol) Bacto Agar (Difco) were set up in 35-mm-diameter petri dishes. Dilutions of rovGM-CSF and rovIL-3 with and without dilutions of GIF were added, and the cultures were incubated in a highly humidified atmosphere of 5% CO_2 in air. Cell colonies (>40 cells) were analyzed on day 14 of culture.

GIF inhibition of ovIL-2 activity was measured in a T-cell proliferation assay. Briefly, mesenteric lymph node cells were enriched (>85%) for $CD4^+$ T cells by magnetic activated cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) depletion of $CD8^+$, γδ T-cell receptor-positive, and B lymphocytes, using 7C2, 86D, and VPM8 (anti-light chain) antibodies, respectively (22). Lymphoblasts that developed after stimulation with 5 μg of concanavalin A (Sigma)/ml for 3 days were expanded in ovIL-2 or huIL-2 for 3 days. After the lymphoblasts were thoroughly washed with medium containing 2% FBS to remove IL-2, 100 μl of T-cell blasts at a density of 5×10^5/ml were added to each well of a 96-well plate (Costar). Additional 100-μl volumes containing a range of ovIL-2 dilutions (final concentration range, 10 pg/ml to 100 ng/ml) in Iscove's modification of DMEM with and without GIF were added to quadruplicate wells. After 24 h, the cells were pulsed with [^{3}H]thymidine (18.5 MBq/well) for a further 24 h before being harvested onto glass fiber sheets in a Micromate 196 cell harvester (Packard). Incorporated [^{3}H]thymidine in each sample was measured with a Matrix 96 direct beta counter (Packard).

Statistical analysis. Where appropriate, Student's *t* test was applied to data normalized by $\log_{10}$ transformation.

Nucleotide sequence accession number. The nucleotide sequence of the GIF cDNA has been deposited in the GenBank database under accession no. AF 192803.

RESULTS

Identification and isolation of the GIF gene. In preliminary studies, orf virus infection of ovine skin keratinocytes prior to a cytopathic effect occurring at around 20 to 24 h after infection stimulated ovGM-CSF mRNA expression. However, ovGM-CSF was at a low or undetectable level in cell lysates or CFSs as measured by ovGM-CSF ELISA. In contrast, the ovIL-8 and ovTNF-α cytokine concentrations increased in the same cultures. The ovGM-CSF inhibition was not due to proteolytic activity, since the addition of proteinase inhibitors to the CFSs did not result in ovGM-CSF detection (37).

To determine whether the ovGM-CSF inhibition was due to the product of a viral gene(s), FLM cells were infected with 18 VVOVs containing among them >95% of the NZ2 orf virus genome in overlapping fragments. CFSs were analyzed for ovGM-CSF inhibition by adding 4 ng of ovGM-CSF/ml and then testing for ovGM-CSF clearance by ELISA. VVOV 85, containing a ~10-kb DNA fragment from within the right terminal quarter of the orf virus genome, expressed ovGM-CSF-inhibitory activity. Subclones of this fragment in the

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TCGGGTAAAAACGTACACCGTCGCCTTGTCGCGGCGGTGTACGCTTTTTTCACGCCCTTT   60

                                          M  A  C  L  R  V  F
TTGCAAAATTTAAATTGTACCCGCGCCGGCTCTAGGAAAGATGGCGTGCCTCAGAGTGTT  120

 L  A  V  L  A  L  C  G  S  V  H  S  A  Q  W  I  G  E  R  D
CCTGGCGGTGCTCGCGCTGTGCGGGAGCGTGCACTCGGCGCAATGGATCGGCGAGCGCGA  180

 F  C  T  A  H  A  Q  D  V  F  A  R  L  Q  V  W  M  R  I  D
CTTCTGCACGGCCCACGCGCAGGACGTCTTCGCGCGGCTGCAGGTGTGGATGCGCATTGA  240

 R  N  V  T  A  A  D  N  S  S  A  C  A  L  A  I  E  T  P  P
CCGGAACGTGACCGCCGCGGACAACAGCTCGGCCTGCGCGCTGGCGATAGAGACGCCGCC  300

 S  N  F  D  A  D  V  Y  V  A  A  A  G  I  N  V  S  V  S  A
GAGCAACTTCGACGCGGACGTCTACGTCGCCGCGGCCGGCATAAACGTCAGCGTGTCCGC  360

 I  N  C  G  F  F  N  M  R  Q  V  E  T  T  Y  N  T  A  R  R
GATCAACTGCGGCTTCTTCAACATGCGCCAGGTAGAGACGACGTACAACACGGCACGCCG  420

 Q  M  Y  V  Y  M  D  S  W  D  P  W  V  I  D  D  P  Q  P  L
GCAGATGTACGTGTACATGGACTCCTGGGACCCTTGGGTGATCGACGACCCCCAGCCGCT  480

 F  S  Q  E  Y  E  N  E  T  L  P  Y  L  L  E  V  L  E  L  A
CTTCAGCCAGGAGTACGAAAACGAAACGCTTCCGTACCTGCTGGAGGTTCTGGAGCTAGC  540

 R  L  Y  I  R  V  G  C  T  V  P  G  E  Q  P  F  E  V  I  P
GAGGCTGTACATTCGCGTGGGCTGCACGGTGCCCGGAGAGCAGCCCTTCGAGGTGATCCC  600

 G  I  D  Y  P  H  T  G  M  E  F  L  Q  H  V  L  R  P  N  R
GGGGATCGACTACCCGCACACCGGCATGGAGTTTCTCCAGCACGTTCTACGGCCGAACCG  660

 R  F  A  P  A  K  L  H  M  D  L  E  V  D  H  R  C  V  S  A
CCGGTTCGCTCCGGCGAAGCTGCACATGGACCTCGAGGTGGACCACCGGTGCGTGAGCGC  720

 V  H  V  K  A  F  L  Q  D  A  C  S  A  R  K  A  R  T  P  L
CGTCCACGTGAAGGCGTTCCTGCAGGACGCCTGTAGCGCCCGCAAGGCGCGGACGCCACT  780

 Y  F  A  G  H  G  C  N  H  P  D  R  R  P  K  N  P  V  P  R
CTACTTCGCGGGGCATGGCTGCAACCATCCAGATCGCCGGCCAAAAAACCCAGTACCGCG  840

 P  Q  H  V  S  S  P  I  S  R  K  C  S  M  Q  T  A  R
CCCTCAGCACGTATCGTCGCCGATCTCCAGGAAGTGCAGCATGCAGACGGCGCGCTAAGG  900

GCGCTCACCGCGCTGACGGCGGCCGTGGTGTGCGCGATCGCCATCGCGCTCGAGCGCGGG  960

GCGGAGGCCGACGCCGTGGACCTTATCCTTATAAAATT  998
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FIG. 1. DNA sequence of, and predicted amino acid sequence encoded by, the NZ2 orf virus GIF cDNA. The entire GIF ORF together with 100 bases of flanking sequence on either side of the ORF is shown. The initiator methionine is 20.1 kb from the right terminus of the orf virus genome. The gene is transcribed toward this terminus. The mature secreted protein starts with Ala^{20}. The region containing potential transcription promoter and/or initiator sequences is underlined.

pEE14 mammalian expression vector were assayed as described above. A single ORF, located 20.1 kb from the right terminus of the viral genome that encoded GIF was identified. The sequence of the ORF plus 100 bases of flanking sequence on either side, as well as the predicted amino acid sequence of GIF, is shown in Fig. 1. The GIF gene is predicted to encode a 265-amino-acid (27.9-kDa) protein, the first 19 amino acids of which constitute a putative signal peptide. A comparison of the predicted GIF protein sequence with that of the vaccinia virus A41L gene product by the use of the Swiss-Prot (release 37.0) and TREMBL (release 10.0) databases revealed 32% amino acid similarity (over the entire protein) (17, 18, 55). Although the vaccinia virus A41L protein has sequence similarity to the T1 secreted chemokine-binding proteins of leporipoxviruses, its function is not known. Furthermore, a comparison of the GIF amino acid sequence with that of the Shope fibroma virus T1 protein did not reveal any similarity. The GIF

TABLE 1. GIF activity in ovine keratinocytes infected with tissue culture-adapted orf virus strains[a]

Sample	OD_{492} (SEM)
NZ2 virus	0.032 (0.008)
Orf 11 virus	0.05 (0.002)
Scabbymouth virus	0.012 (0.004)
SFV control	0.62 (0.02)
Mock infection	0.86 (0.004)
ovGM-CSF, 4 ng/ml[b]	0.71 (0.03)

[a] Ovine primary keratinocytes were infected with each of the viruses at an MOI of 2 $TCID_{50}$/cell or were mock infected (control). Twenty-four hours later, CFSs were harvested. GIF activity was detected by adding 4 ng of ovGM-CSF/ml to the CFS samples and analyzing them by ovGM-CSF ELISA. Results are expressed as ELISA OD_{492} units. Semliki forest virus (SFV) was used as a control.

[b] ovGM-CSF in culture medium (not keratinocyte CFS).

gene sequences from orf 11 and MRI scab viruses were also obtained. These were predicted to encode proteins that were 98% identical to the NZ2 sequence. The GIFs of orf 11 and MRI scab viruses, expressed in CV-1 cells transfected with the GIF cDNAs, inhibited ovGM-CSF as detected by ELISA. Furthermore, NZ2, orf 11, and scabbymouth virus GIF activities were demonstrated in CFSs of virus-infected ovine keratinocyte cultures (Table 1).

The NZ2 GIF cDNA was expressed as a secreted protein in CHO cells. Recombinant GIF was purified by ovGM-CSF affinity chromatography and Sephacryl S-200 gel filtration (Fig. 2A). Sequence analysis of the 20 N-terminal amino acids of the secreted recombinant GIF revealed that the mature protein started with Ala^{20} (Fig. 1).

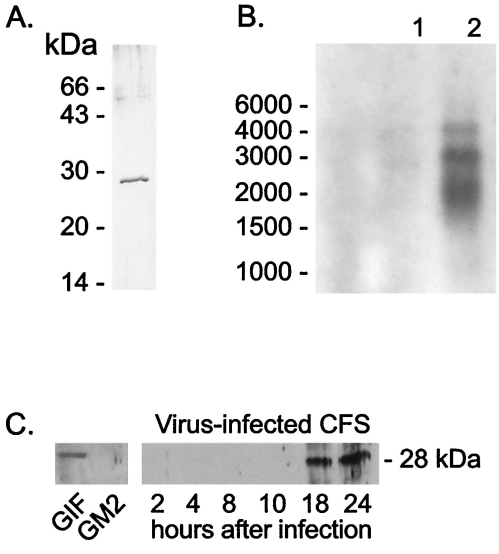

FIG. 2. Expression of recombinant and nonrecombinant GIF in virus-infected FLM cells. (A) Silver stain of recombinant GIF produced in CHO cells and purified by GM-CSF affinity chromatography followed by Sephacryl S-200 gel filtration. The positions of molecular mass markers are shown on the left. (B) Northern analysis of GIF mRNA expression in FLM cells at 18 h after infection with NZ2 virus at an MOI of 20 $TCID_{50}$ in the presence (lane 2) or absence (lane 1) of CA. Positions of RNA size markers (in bases) are indicated. (C) GIF expression in FLM CFSs at various times after infection with NZ2 (MOI = 2 $TCID_{50}$) in the absence of CA. Shown is a Western blot, prepared with rabbit anti-GIF, of samples subjected to SDS–15% PAGE. Recombinant GIF and GM2 (the product of an uncharacterized ORF adjacent to the GIF gene) were included as a positive and negative control, respectively. Both the recombinant and native GIFs are 28-kDa proteins.

GIF is an intermediate-late viral gene. In orf virus-infected cells in culture, progeny virions are detected at approximately 12 h after infection. Maximum titers are obtained between 24 and 72 h, concomitant with the virus-induced cytopathic effect (37). Using a DNA probe derived from sequence entirely within the GIF ORF, GIF mRNA was detected at 18 h after infection of FLM cells with orf virus in the absence of CA, an inhibitor of late viral gene expression and viral DNA replication (Fig. 2B). GIF mRNA was not detected at 5 h after infection of FLM cells in either the presence or the absence of CA. This demonstrated that the GIF gene was expressed as an intermediate or late, but not early, viral gene. The detection of multiple bands rather than a single mRNA also supports this conclusion, since the point at which intermediate and late poxvirus gene transcription stops can be heterogeneous (9, 41, 63). An equivalent ssRNA probe gave the same result as the DNA probe. Confirmation that the GIF gene was expressed as an intermediate or late viral gene was obtained by Western blot analysis of GIF protein production in FLM cells after virus infection, using the rabbit anti-GIF IgG. GIF was detected at 18 and 24 h after infection in CFSs (Fig. 2C).

GIF forms functional dimers and tetramers. During GIF purification it was observed that two peaks of GIF activity were separated by Sephacryl S-200 gel filtration. To determine whether GIF forms dimers and/or oligomers, purified recombinant ^{125}I-GIF was separated by S-200 gel filtration. Two ^{125}I-GIF peaks eluted from the column, at approximately 56- and 112-kDa-equivalent volumes (Fig. 3A). Both of these ^{125}I-GIF peaks contained ovGM-CSF-binding activity, as determined by ligand blot analysis (Fig. 3B). The 56- and 112-kDa GIF moieties correspond in mass to homodimers and tetramers, respectively, of 28-kDa GIF. There was no S-200 ^{125}I-GIF (or protein) peak at the elution point predicted for a GIF monomer mass of 28 kDa. Furthermore, each of the 56- and 112-kDa GIF moieties dissociated to the 28-kDa monomer form in the presence of SDS in PAGE performed under nonreducing conditions (Fig. 3C).

GIF binds ovGM-CSF and ovIL-2. Currently available ovine cytokines expressed in CHO cells, a selection of human and murine cytokines, and heparin were screened for the ability to bind GIF by the competitive ovGM-CSF inhibition ELISA. Only ovIL-2 inhibited the binding of GIF to ovGM-CSF (data not shown). Binding of GIF to ovGM-CSF and ovIL-2 was confirmed by a direct cytokine-GIF ligand blot assay (Fig. 4). Unlabelled (cold) GIF inhibited the binding of ^{125}I-GIF to ovGM-CSF and ovIL-2 (data not shown). The affinity binding of GIF to ovGM-CSF and ovIL-2 was determined by Scatchard analysis. GIF bound to ovGM-CSF with a K_d of 369 pM (range, 317 to 421 pM) and to ovIL-2 with a K_d of 1.04 nM (range, 0.961 to 1.124 nM). GIF therefore binds to ovGM-CSF with a higher affinity than it binds to ovIL-2. GIF did not bind to human or murine GM-CSF (Fig. 4a) or to human IL-2 (Fig. 4b). In the presence of SDS, GIF dimers and tetramers dissociated to the monomer form, which did not bind ^{125}I-ovGM-CSF or ^{125}I-ovIL-2 in SDS-PAGE ligand blot assays (data not shown).

GIF inhibits GM-CSF and IL-2 biological activities. Figure 5 shows that GIF inhibited the hematopoietic activity of ovGM-CSF, but not that of the control, IL-3, in the soft-agar bone marrow cell colony assay and that GIF inhibited the activity of ovIL-2 in the T-cell proliferation assay. Neutralization of GIF by rabbit anti-GIF prevented the inhibition of each of the cytokines in the assays.

GIF is produced in vivo during orf virus reinfection. To determine whether orf virus produces GIF in vivo, samples of cannulated afferent and efferent lymph draining the skin site

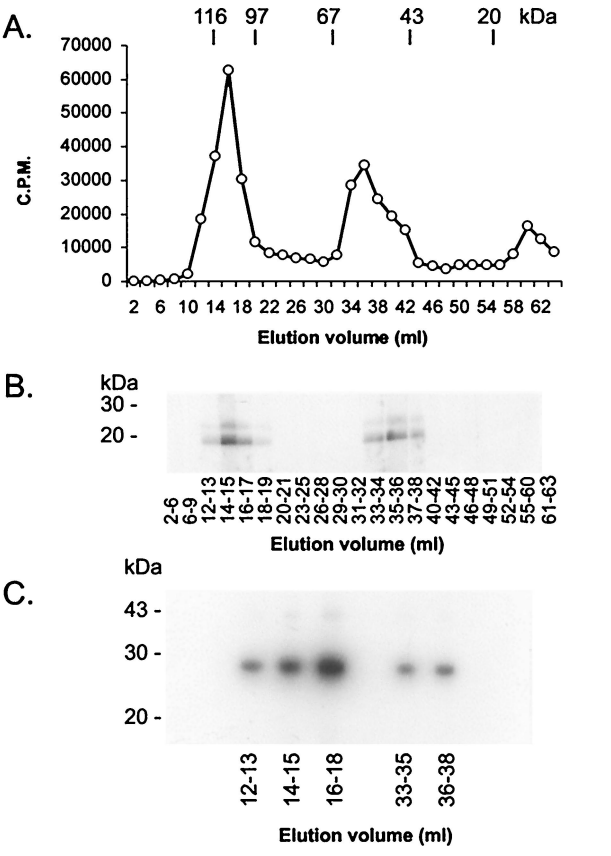

FIG. 3. GIF forms dimers and tetramers under physiological conditions. (A) Separation of ^{125}I-GIF on a Sephacryl S-200 column in 0.15% (vol/vol) CHAPS buffer at pH 7.4. Each fraction contained 1 ml of eluate. Two peaks of GIF eluted, with apparent molecular masses of ~112 and ~56 kDa. The arrows indicate the elution positions of protein standards (β-galactosidase, phosphorylase B, bovine serum albumin, ovalbumin, and chymotrypsinogen A) used to construct the molecular mass-versus-elution volume standard curve. (B) ovGM-CSF–GIF ligand blot showing the pooled and concentrated (10-fold) Sephacryl S-200 fractions of ^{125}I-GIF that bound to ovGM-CSF (19–21-kDa dimer), subjected to SDS–15% PAGE under nonreducing conditions and electrotransferred onto nitrocellulose strips. (C) Pooled and concentrated ^{125}I-GIF fractions from the S-200 column, subjected to SDS–15% PAGE under nonreducing conditions and revealed by autoradiography. Fractions 12 to 18 contained the 112-kDa GIF peak from the S-200 column, and fractions 33 to 38 were from the 56-kDa peak. All fractions showed a 28-kDa protein band. To the left of panels B and C are shown the positions of molecular mass markers.

(prefemoral lymph node drainage area) of orf virus reinfection from previous studies (21, 23) were analyzed for GIF activity by GM-CSF inhibition ELISA. Afferent lymph plasma from virus-infected sheep contained GIF (Fig. 6b). The presence of GIF was associated with reduced levels of GM-CSF in the lymph plasma (Fig. 6a). GIF was detected only in afferent lymph plasma samples from infected animals, not in those from control animals injected intradermally with UV-inactivated virus (data not shown). GIF was not detected in efferent lymph plasma or in CFSs from cultured afferent or efferent lymph cells. IL-2 was not tested for clearance by GIF because an IL-2-specific antibody was not available.

DISCUSSION

Poxviruses as a group encode a large number of immunomodulatory proteins that interfere with host immune and inflammatory responses to infection and, consequently, aid virus

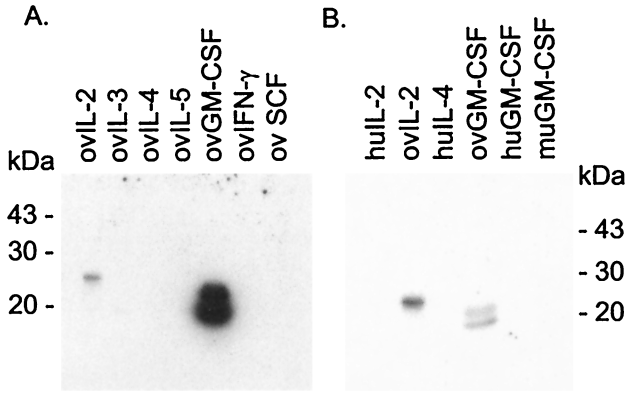

FIG. 4. GIF binding of ovGM-CSF and ovIL-2: ^{125}I-GIF–cytokine ligand blot analysis. ^{125}I-GIF was used to probe a range of ovine cytokines (A) and ovine, human, and murine cytokines (B) which were separated by SDS–15% PAGE and transferred to nitrocellulose membranes. ^{125}I-GIF bound to ovGM-CSF and ovIL-2. As a specificity control, 100 nM of unlabelled GIF was used to compete for ^{125}I-GIF binding to the cytokines. This inhibited ^{125}I-GIF binding to ovGM-CSF and ovIL-2. The positions of molecular mass markers are indicated.

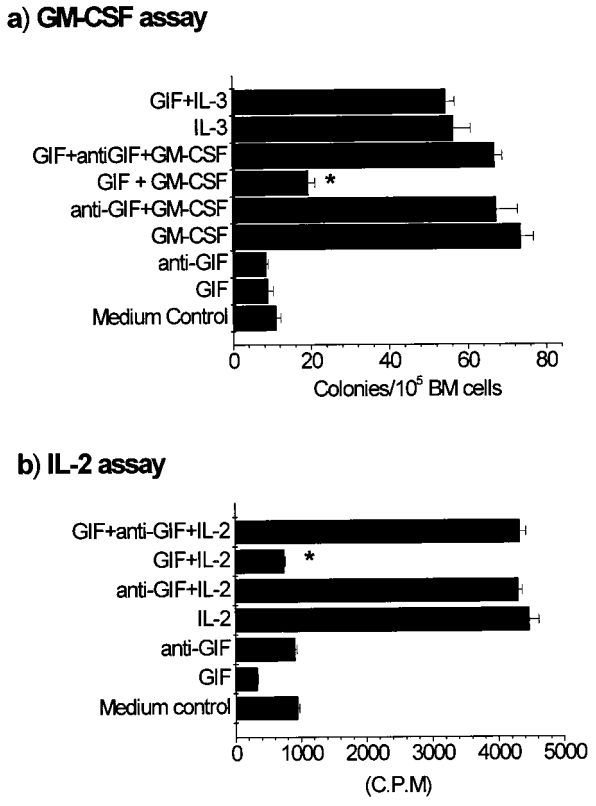

FIG. 5. GIF inhibits ovGM-CSF and ovIL-2 activities. (a) GIF inhibition of ovGM-CSF activity in the soft-agar bone marrow cell clonogenic assay. Colonies in triplicate cultures were counted on day 14 of culture, and the numbers were adjusted to represent the mean number of colonies, ± the standard error of the mean, that developed from 10^5 bone marrow cells plated on day zero. ovGM-CSF was used at 460 pg/ml; ovIL-3 and GIF were used at 520 and 50 ng/ml, respectively, based on dose-response experiments. (b) GIF inhibition of ovIL-2 activity in the 48-h T-cell proliferation assay. C.P.M., mean counts per minute of [^{3}H]thymidine incorporated into dividing cells in quadruplicate wells ± standard error of the mean. ovIL-2 and GIF were used at 40 and 100 ng/ml, respectively, based on dose-response experiments. *, $P < 0.002$ versus ovGM-CSF-stimulated colonies, (a) or IL-2-stimulated T-cell proliferation counts per minute (b).

replication. A proportion of these proteins bind to and inhibit cytokines that regulate the host response to infection. In this study, we isolated and characterized GIF, a novel cytokine-inhibitory protein encoded by several strains of the parapoxvirus orf virus.

GIF bound to and inhibited the biological function of ovGM-CSF and ovIL-2. GM-CSF is produced by a variety of cell types, including T cells. It stimulates neutrophil, monocyte, and eosinophil myelopoiesis and the recruitment and/or activation of these cell types in the tissues (46). GM-CSF is also involved in early events in immune responses, regulating the differentiation and function of antigen-presenting dendritic cells. IL-2 is a T-cell-derived lymphokine that stimulates T-cell and NK cell activation and proliferation and activated-B-cell proliferation (15). This is the first description, to our knowledge, of a microbial inhibitory protein for GM-CSF or for both GM-CSF and IL-2. huIL-2 was bound by a 38-kDa protein, encoded by tanapox virus, a poxvirus pathogen of primates, which was secreted from infected cells and bound huIL-5 and huIFN-γ (14). The ability to bind and inactivate multiple, sometimes apparently unrelated cytokines is a property of some poxvirus proteins and represents an economical way of controlling host immunity. Another example of such a protein is the M-T7 gene product of myxoma virus, which inhibited rabbit IFN-γ and CXC, CC, and C family chemokines (36). The chemokine binding was via a conserved chemokine heparin-binding domain. The binding of GIF to both GM-CSF and IL-2 indicates the existence of a binding domain(s) shared by both cytokines. A comparison of the ovGM-CSF and ovIL-2 sequences did not reveal any obvious common feature other than the fact that both GM-CSF and IL-2 are members of the short-chain, four-α-helical-bundle family of cytokines that also includes IL-4. However, ovIL-4 did not bind to GIF. Interestingly, GM-CSF has been shown to compete with IL-2 for binding to IL-2 receptors on the myeloid leukemia cell line MO7E (34), indicating that there may be a common receptor-binding domain in huGM-CSF and huIL-2.

Many of the viral cytokine-binding proteins are orthologues of host cytokine receptor molecules. GIF has no counterpart in the amino acid sequence databases. This may be because the ovGM-CSF receptor proteins have not been characterized. The human and murine cellular GM-CSF receptors consist of a low-affinity-binding α subunit that is specific for GM-CSF and a β subunit that is common to GM-CSF, IL-3, and IL-5 receptors (51). Cross-linking of the α and β subunits gives rise to a high-affinity binding site for GM-CSF. The IL-2 receptor (IL2R) consists of α, β, and γ chains. The α subunit (p55) has a low affinity for IL-2 but forms a high-affinity IL-2-binding site when cross-linked with the β and γ subunits (60). The ovIL-2R α chain has been cloned and sequenced (5), but there are no regions of homology between this sequence and GIF. There is a natural secreted form of the IL-2R α subunit and evidence of secreted forms of the IL-2R β and γ subunits (11, 30). All of these subunits have a low affinity for IL-2.

The only other natural inhibitor of GM-CSF described to date is a posttranslationally modified secreted form of the GM-CSF receptor alpha chain (GMRα) (3, 29, 53). The secreted form of GMRα was produced by myeloid but not lymphoid cell lines, and it bound ligand with a K_d of 3.8 nM (3, 29). Both GIF and GMRα form oligomers in solution in the absence of ligand (this study and reference 4). However, GIF bound ovGM-CSF with a 10-fold-higher affinity than GMRα bound huGM-CSF (K_d, 369 and 3.8 nM, respectively). In addition, GIF dimers and tetramers bound ovGM-CSF, whereas the monomer of the multimeric forms of GMRα exhibited the

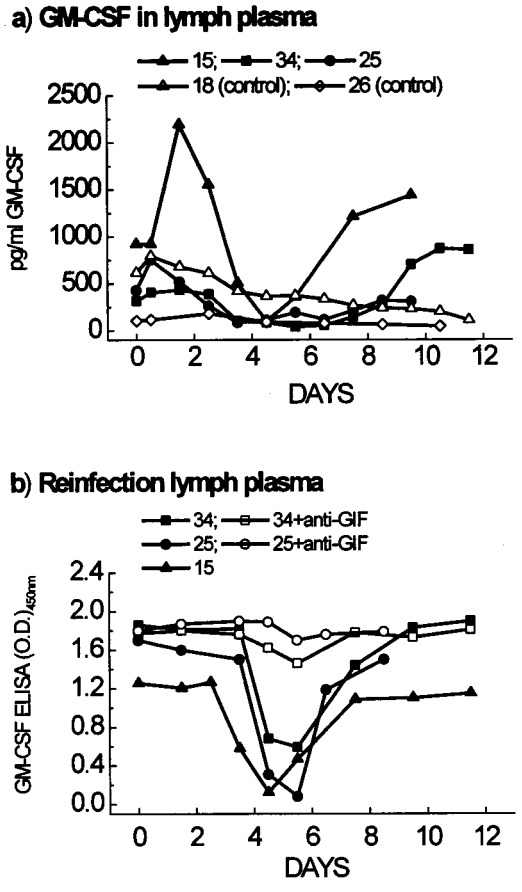

FIG. 6. GIF and ovGM-CSF in virus reinfection and control afferent lymph plasma. (a) GM-CSF concentrations in afferent lymph plasma samples from reinfected and control sheep. Control sheep (no. 18 and 26) received UV-inactivated virus intradermally. (b) Detection of GIF, by ovGM-CSF inhibition ELISA, in afferent lymph plasma samples obtained at various times after orf virus reinfection of sheep 15, 25, and 34 on day zero. Lymph plasma samples were spiked with 8 ng of GM-CSF/ml (4 ng/ml for sheep 15 samples, hence the lower OD) and analyzed for GM-CSF by ELISA. Rabbit anti-GIF was used as a specificity control to neutralize GIF in the assay.

highest-affinity binding of huGM-CSF (4). We did not observe binding of ^{125}I-ovGM-CSF or ^{125}I-ovIL-2 to the 28-kDa GIF monomer in SDS-PAGE ligand blot assays.

However, the lack of homology to host cytokine receptors by viral cytokine-binding proteins is not without precedent. The 35-kDa virulence proteins of variola and cowpox viruses inhibit β-chemokines but have no sequence homology to known proteins (54). The B18R gene product of vaccinia virus binds IFN-α/β but is in a protein superfamily different from that of known host IFN-α/β receptor proteins (62). It is possible that in the coevolution of virus and host, radical modification of the acquired host gene occurred such that the host and virus proteins no longer resemble each other except for short stretches of sequence that are important for ligand binding. Viral genome sizes for different families of viruses tend to be critically controlled. The ability to acquire host genes and modify them to produce minimally sized immunomodulatory proteins that inhibit one, or preferably several, important immune effector molecules has a clear advantage with regard to survival of the virus.

After orf virus reinfection of sheep, GIF was detected in afferent lymph plasma samples that had low levels of ovGM-CSF. Coupled with the fact that GIF was detected only in the afferent lymph plasma and not in cultured afferent lymph CFS, this demonstrated that GIF is produced from virus-infected epidermal cells and has in vivo relevance. The inability to detect GIF in efferent lymph plasma indicates that GIF either does not pass through the lymph node into efferent lymph or is diluted out by the large volume of efferent lymph plasma carrying lymphocytes derived from the blood via high endothelial venules (for a review of lymph, refer to reference 28). The time at which GIF was detected in afferent lymph corresponded to the period of maximum viral replication in the epidermis of the cannulated sheep, which occurred between 5 and 7 days after reinfection (21).

We can only speculate the function of GIF in orf virus infection. ovGM-CSF and ovIL-2 mRNAs have been detected in skin biopsy specimens obtained during orf virus reinfection (24). ovGM-CSF and ovIL-2 have been detected in afferent and efferent lymph after orf virus reinfection (21, 22). The principal source of these cytokines in both lymph compartments is the $CD4^+$ T cell. $CD4^+$ T cells, in particular of the lymphocyte subsets, accumulate in large numbers adjacent to and underlying orf virus-infected cells in sheep skin. IL-2 and IFN-γ have been implicated in protective immunity to orf virus reinfection (21, 24, 37, 39). The role of GM-CSF is less clear. GM-CSF is involved in the activation of neutrophils and macrophages, both of which are present in orf lesions. Macrophage colony-stimulating factor (M-CSF; CSF-1), granulocyte colony-stimulating factor, and IL-3 are also hematopoietic growth factors that support the development and activation of neutrophils and macrophages. Recently, an M-CSF-inhibitory protein encoded by the Epstein-Barr virus *BARF1* gene was identified (59). The *BARF1* product has sequence homology to the M-CSF receptor protein (c-fms). The function of macrophages in orf or Epstein-Barr virus infection is not clear. However, GM-CSF also regulates antigen presentation by dendritic cells, and this would be a useful point of intervention for viral immunomodulator proteins. We have previously shown that ovGM-CSF and ovTNF-α are involved in the recruitment of dendritic cells to the ovine dermis (19) and in supporting the survival and proliferation of afferent lymph veiled dendritic cells in culture (20). The inhibition of GM-CSF in the vicinity of orf virus-infected epidermal keratinocytes could affect dendritic cell function. Ovine keratinocytes in culture produce ovGM-CSF both constitutively and after stimulation with phorbol ester and calcium ionophore (37). A role for GIF in aiding virus replication in infected keratinocytes must also be considered.

ovGM-CSF and ovIL-2 were bound by GIF, whereas huGM-CSF, muGM-CSF, and huIL-2 were not. In the broader context, the orf virus IL-10 and VEGF protein sequences are most homologous to those of ovIL-10 and ovVEGF, respectively (16, 40). Taken together, these data demonstrate that orf virus has adapted to sheep, rather than man, as its principal host. Orf virus lesions in man are grossly similar to those in sheep, but they have not been studied in the same detail. The consequences (if any) of orf virus immunomodulatory proteins that are active in sheep but not active in man are of interest but are not known.

Except for GIF, the parapoxvirus immunomodulatory proteins discovered so far are all the products of early viral genes. GIF was expressed as an intermediate-late viral gene. orf virus expression of vIL-10 (16), the orf virus interferon resistance protein (27, 44), and GIF represents a coordinated interference with host inflammatory and type 1 immune responses to virus infection. This suggests that GM-CSF, along with IFN-γ,

IFN-α/β, and IL-2, are important in host immunity to orf virus (24).

GIF, a protein with unusual properties, is part of a growing number of pathogen immunomodulators that will be useful not only in determining mechanisms of viral pathogenesis and the nature of host antipathogen immunity but also as templates for potentially therapeutic proteins or peptides.

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