CLONING, SEQUENCING AND EXPRESSION OF THE OVINE INTERLEUKIN 6 GENE

B. Ebrahimi, D. J. Roy, P. Bird, D. R. Sargan

Gene amplification by reverse transcriptase PCR with heterologous primers has been used to obtain a cDNA clone encoding the structural sequences of ovine interleukin 6 from alveolar macrophages. This cDNA encodes a protein of M_r = 23 429, which is 53% homologous in amino acid sequence to human IL = 6. The clone hybridizes to an RNA of size 1260 nt in alveolar macrophages, expression of which is potentiated by LPS. The ovine IL-6 structural gene has been cloned into the yeast expression vector pOGS40, and used to produce a recombinant protein. This protein is capable of causing increased immunoglobulin production in pokeweed mitogen stimulated ovine peripheral blood mononuclear cells at concentrations of 10–100 ng/ml, but it only causes very limited replication of B9 cells, a murine IL-6 dependent cell line. This is in contrast to recombinant human IL-6, which is capable of stimulating B9 cell proliferation, but not immunoglobulin production by ovine PBMC.

Interleukin 6 (IL-6) is a multifunctional cytokine with important roles in the acute phase response,[1,2] haematopoesis[3] and multiple aspects of immune regulation.[4] IL-6 also has roles as an autocrine stimulator of growth in a number of tumours (most notably plasmacytomas and myelomas)[5,6] as well as for some normal cell types; it is inducible by poly(I).poly(C),[7] and has a number of functions in the endocrine and nervous systems. It has been suggested that it acts as a long range alarm signal in the body. In line with these functions, it has previously been known as B-cell stimulatory factor 2 (BSF-2), interferon β2 (IFN-β2), hepatocyte stimulatory factor (HSF)m 26 kD protein and hybridoma plasmacytoma growth factor (HPGF). Over-expression of IL-6 is known to be an important feature of the pathogenesis of a number of inflammatory diseases such as rheumatoid arthritis, glomerula nephritis and psoriasis, as well as the development of myeloma/plasmacytoma development. It may also be important in the development of pathology in a number of infectious diseases such as mycobacterial disease and AIDS.

IL-6 in the human is a 184 amino acid protein which is processed from a 212 amino acid preprotein. It is variably glycosylated and phosphorylated. The main transcript is 1.3 kb, although 2 further downstream polyadenylation sites have been mapped. Although the promoter regions of IL-6 genes are closely conserved between mouse and human, the structural sequences of the gene are less well conserved (42% at the amino acid level). Nonetheless, human IL-6 is able to support the growth of murine IL-6 dependent myeloma lines.

In order to study whether IL-6 mediates the pathogenesis of lentiviral disease in ruminants, we have cloned, sequenced and expressed in a yeast based system the structural regions of the ovine IL-6. Biological assays on the product show that there is a species barrier which prevents ovine IL-6 from supporting growth of murine IL-6 dependent cell lines, and similarly that human IL-6 is not recognized by ovine B-cells.

RESULTS

Cloning and sequence of ovine IL-6

To obtain the ovine IL-6 proprotein coding sequence, single stranded cDNA was made from total RNA of LPS stimulated alveolar macrophages and amplified using the polymerase chain reaction with primers derived from conserved regions of the 5′ and 3′ untranslated regions of IL-6 mRNA's of human and mouse. The product of this reaction was cloned into pTZ18R and sequenced (Fig. 1B). The derived sequence contains an open reading frame of 624 bp, which encodes a protein of predicted Mr = 23 429. This protein is 53% identical to human IL-6 in sequence, and 43% identical to mouse IL-6. By analogy with the known maturation pattern of human IL-6 it is likely that the mature ovine protein is cleaved from the pro-protein at the proline at position 29. A predicted N-linked

From the Department of Veterinary Pathology, University of Edinburgh, Edinburgh, UK
Correspondence to: D. R. Sargan, at present address, Department of Clinical Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, UK
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054R:  5'-GGAACGAAAGAGAGCTCCAT-3'

056R:  5'-GCCAGTGGACAGGTTTCTGA-3'

053R:  5'-CGGGATCCATTGAAGGTAGGATGAACTCCCGATTCACAA-3'
            BamHI   FactorXa          IL-6*

159R:  5'-CGGGATCCTACTTCATCCGAATAGCTCTC-3'
            BamHI          IL-6
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Figure 1A. Primers used in cloning the ovine IL-6 cDNA.

054R and 056R were derived from regions of consensus between murine and human genes, whilst 053R contains IL-6 sequence derived from the bovine gene (B. Collins, personal communication) and shows two changes from the sequence of the ovine gene in Figure 1B. Non IL-6 sequences used in the expression construct are underlined.

glycosylation site at amino acid position 38 is conserved in the bovine sequence but not in other sequenced IL-6 proteins.

Expression of ovine IL-6 in yeast

The same CDNA population was used as a substrate to amplify an expression cassette containing this open reading frame. This cassette was cloned into pTZ18R, and subsequently into the galactose inducible yeast Ty expression vector pOGS40. After resequencing, the resulting plasmid pOGIL6 was used to transform yeast strain BJ2168. After cloning, individual transformants were grown on in liquid culture, and lysed after growth in induction conditions. An inducible protein was produced by the transformed cells which

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ATGAACTCCCTCTTCACAAGCGCCTTCAGTCCACTCGCTGTCTCCCTGGGGCTGCTCCTG 60
M  N  S  L  F  T  S  A  F  S  P  L  A  V  S  L  G  L  L  L   20

GTGATGACTTCTGCTTTCCCTACCCCGGGTCCCCTGGGAGAAGATTTCAAAAATGACACC 120
V  M  T  S  A  F  P  T  P  G  P  L  G  E  D  F  K  N  D  T   40

ACCCCAAGCAGACTACTTCTGACCACTCCAGAAAAAACCGAAGCTCTCATTAAGCACATC 180
T  P  S  R  L  L  L  T  T  P  E  K  T  E  A  L  I  K  H  I   60

GTCGACAAAATCTCTGCAATAAGAAAGGAGATATGTGAGAAGAATGACGAGTGTGAAAAC 240
V  D  K  I  S  A  I  R  K  E  I  C  E  K  N  D  E  C  E  N   80

AGCAAGGAGACACTGGCAGAAAATAAGCTGAAACTTCCAAAAATGGAGGAAAAAGATGGA 300
S  K  E  T  L  A  E  N  K  L  K  L  P  K  M  E  E  K  D  G  100

TGCTTCCAATCTGGGTTCAATCAGGCGATTTGCTTGATCAAAACCACTGCTGGTCTTCTG 360
C  F  Q  S  G  F  N  Q  A  I  C  L  I  K  T  T  A  G  L  L  120

GAGTATCAGATATACCTGGACTTCCTCCAGAACGAGTTTGAGGGAAATCAGGAAACTGTC 420
E  Y  Q  I  Y  L  D  F  L  Q  N  E  F  E  G  N  Q  E  T  V  140

ATGGAGTTGCAGAGCAGTATCAGAACACTGATCCAGATCCTGAAGGAAAAGATCGCAGGT 480
M  E  L  Q  S  S  I  R  T  L  I  Q  I  L  K  E  K  I  A  G  160

CTAATAACCACTCCAGCCACACACACTGACATGCTGGAGAAGATGCAGTCCTCAAACGAG 540
L  I  T  T  P  A  T  H  T  D  M  L  E  K  M  Q  S  S  N  E  180

TGGGTAAAGAACGCAAAGGTTATCATCATCCTGAGAAGCCTTGAGAATTTCCTGCAGTTC 600
W  V  K  N  A  K  V  I  I  I  L  R  S  L  E  N  F  L  Q  F  200

AGCCTGAGAGCTATTCGGATGAAGT 627
S  L  R  A  I  R  M  K  * 208
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Figure 1B. Sequence of the coding region of ovine IL-6 cDNA.

The sequence of the PCR product of 054R and 056R was determined from both strands of three clones. The complete protein coding region is shown. Maturation of the protein leads to a probable amino-terminus at position 29 (underlined).

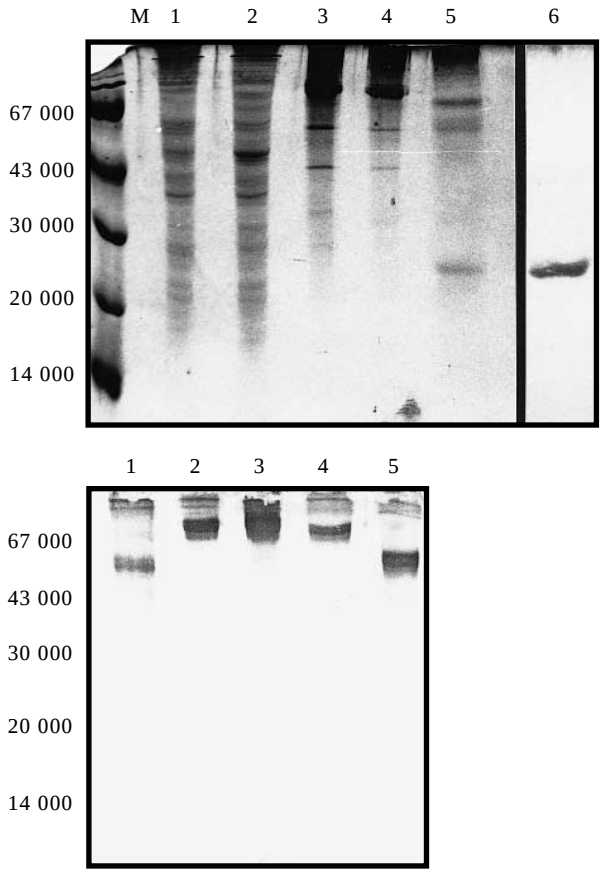

Figure 2. Expression of rovIL-6 protein in yeast.

(A) Proteins from various stages of IL-6 purification were analysed by electrophoresis on SDS-15% polyacrylamide gels, followed by staining with Coomassie blue R. Lane M: marker proteins. Lane 1: total proteins from a lysed control yeast expressing the TyP1 protein from the plasmid pOGS41 after 16 h induction with galactose. Lane 2: total proteins from a yeast strain transfected with pOGIL-6 after 16 h induction with galactose. Lane 3: partially purified virus-like particles of P1/IL-6 fusion protein from the 60% sucrose cushion after the first centrifugation step. Lane 4: virus-like particles of P1/IL-6 fusion protein with factor Xa added, prior to digestion. Lane 5: virus like particles after factor Xa cleavage. Lane 6: purified rov-IL-6 after removal of vlp's by centrifugation. This lane is a silver stained preparation, from a different gel. (B) A western blot of a similar gel (lanes 1–5) was probed with a polyclonal antiserum raised against P1 protein.

was of the predicted size for a TyP1-IL-6 fusion protein product, and blotted with an anti-P1 rabbit antiserum (Fig. 2). When purified by differential sucrose gradient centrifugation, this fusion protein could be digested with Factor Xa to yield a M_r = 24 000 protein, putatively ovine IL-6, as well as a M_r = 50 000 band which corresponds to P1 protein present as vlps. Further centrifugation to remove remaining vlps allows the recovery of the released IL-6 with a yield of *c*100 µg/litre of culture, at a purity of about 90% as estimated by silver stain staining of PAGE gels.

Biological activity of rov IL-6

The purified material was used in two assays of IL-6 activity. B9 cells offer a highly sensitive proliferation assay for human and murine IL-6, although these cells will also proliferate in the presence of murine (but not human) IL-4. Proliferation assays were performed using these cells and the yeast expressed rovIL-6 (Fig. 3). This material was able to support proliferation of these cells, but only relatively poorly, and at concentrations of 1–10 ng/ml and above, some four orders of magnitude higher than the concentrations at which rhuIL-6 is active. (In the assay shown, rhuIL-6 is already super-optimal for stimulation at 20 pg/ml. In other assays, optimal stimulation by rhuIL-6 was achieved in the range 0.1–1 pg/ml, in good agreement with the findings of other authors for this cell line.[8]) Mixing rovIL-6 with rhuIL-6 did not affect proliferation to rhuIL-6, showing that there was no toxicity from rovIL-6 (data not shown). P1 protein alone was unable to cause proliferation of B9 cells at any concentration measured.

To measure the activity of rovIL-6 on ovine cells, IL-6 was used to drive immunoglobulin production by peripheral blood mononuclear cells[9] (Fig. 4). RovIL-6 at concentrations above 10 ng/ml was able to potentiate total immunoglobulin production by these cells in the presence of pokeweed mitogen (measured in an ELISA assay) by anything from 2–8 fold in different sheep. (Production of Ig in the absence of IL-6 varied between 2 and 7 μg/ml.) These concentrations are comparable to those required to cause similar enhancements of Ig production by rhuIL-6 acting on human EBV transformed B-cells.[16] The same ovine cell populations were much less responsive to a control protein preparation consisting of the equivalent purification fractions from a factor Xa digested P1 expressing yeast. Nor were they sensitive to rhuIL-6 at these concentrations (data not shown). This suggests that there is a partial barrier in IL-6 recognition between sheep IL-6 and mouse IL-6 receptors and between human IL-6 and sheep IL-6 receptors which does not exist between human IL-6 and mouse IL-6 receptors.

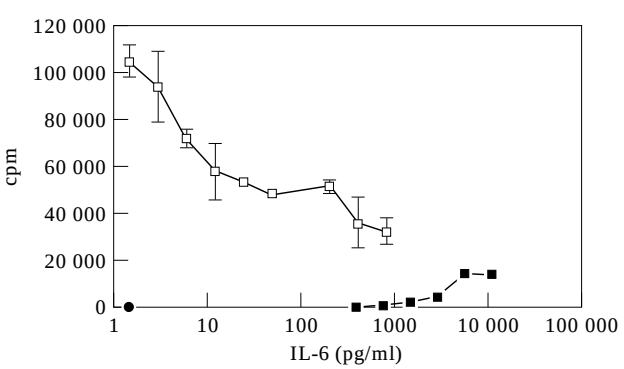

Figure 3. Proliferative response of B9 cells to recombinant IL-6.

Medium control (●), rhuIL-6 (□) or rovIL-6 (■) was used to stimulate proliferation of the murine myeloma cell line B9 for 36 h. ^{3}H-Thymidine incorporation was measured over the next 5 h. Means and ranges of incorporated counts are shown. Where no error bars are shown, they are within the diameter of the symbol.

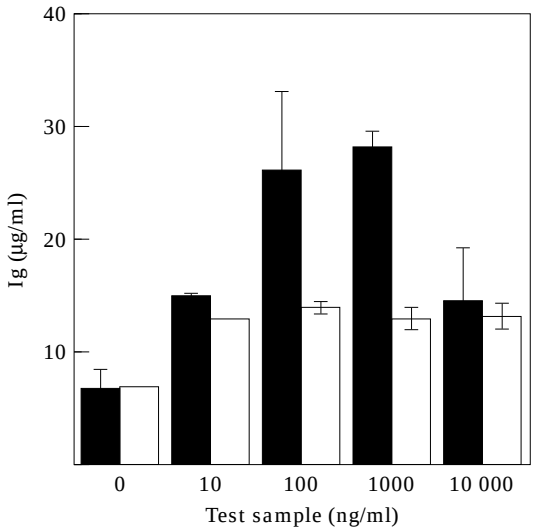

Figure 4. Immunoglobulin production by peripheral blood lymphocytes in response to IL-6.

Ig secretion from 10^5 cells over a period of 7 days in culture was measured in a light chain capture ELISA as stated. Test samples in a volume of 100 μl were purified rovIL-6 or a control preparation of supernatant after an insert-less P1vlp protein was digested with factor Xa and centrifuged. Means of Ig production and standard deviations are shown: (■) effect of addition of rovIL-6 to cells; (□) effect of addition of control preparation to cells.

DISCUSSION

Ovine recombinant IL-6 has been cloned and expressed in a yeast based system. The sequence is identical to that reported very recently by Andrews *et al.*,[10] although these authors did not express protein from their gene. On Northern blotting the clone described in the current study hybridizes to a rare transcript in sheep alveolar macrophages of 1.26 kb. RT PCR experiments showed that this manuscript was induced in the macrophages by LPS stimulation in vitro. Induction of the transcript occurs by 1 h post treatment, and continues for at least 3 h (data not shown).

The protein expressed in the current study is a proprotein, but it retains functional activity on ovine cells. However, there is a partial species barrier between ovine IL-6 and murine cells which is not present between human IL-6 and murine cells, preventing extensive proliferation of the B9 myeloma line in response to the ovine molecule. Similarly, human IL-6 is not able to potentiate immunoglobulin synthesis by ovine peripheral blood mononuclear cells. This type of species barrier has been noted before in other species.

Although human IL-6 will bind to the murine IL-6 receptor and activate murine IL-6 dependent cell lines, murine IL-6 is unable to activate human cells in the same way.[11]

IL-6 sequences are not extensively conserved between species, but four cysteine residues forming disulphide bridges at positions 72–78 and 101–111 are conserved across the ovine protein as well as those of other species.[12] (These cysteines define a structural family of cytokines including IL-2, IL-4, GM-CSF, G-CSF, to which LIF and erythropoietin are more distantly related.) The most closely conserved regions across species, within the IL-6 gene, are within the leader sequence and also between residues 68 and 126. These residues also form the best conserved region between the human and murine proteins and this region constitutes parts of two alpha helices, two turns defined by the cysteine bridges and a long loop, at least part of which is required for interaction between IL-6 and the IL-6 binding subunit of IL-6R.[19]

Most studies of IL-6 structure/activity relationships have used site directed mutagenesis of human IL-6 and have concentrated on the important role of the carboxyl end of the protein in receptor binding.[13–16] Residues 176–183 of the human mature protein and two sets of periodically arranged leucines (positions 167, 174 and 181; and positions 151, 158, 165 of the human sequence) have both been suggested to be important in this regard.[22] Of these leucines in the human sequence, only those at positions 167 and 174 have equivalents in the ovine sequence (positions 191 and 198 of the sequence in Fig. 2). That at position 181 (human) is not present in most other IL-6 sequences, and can be substituted with several other residues without effect.[23] However, the absence of the other spaced set of leucines may be important in the observed species barrier.

MATERIALS AND METHODS

Cloning and sequencing of ovine IL-6

Alveolar macrophages were obtained from gnotobiotic Finn × Blackface sheep by alveolar lavage and red blood cells removed by hypoosmotic shock as before.[17] 2×10^7 cells were plated into plastic tissue culture bottles in Iscove's serum free medium and allowed to adhere for 24 h. Adherent cells (>90% macrophages by non-specific esterase staining) were then stimulated with 10 μg/ml LPS from *Salmonella abortus equi* for 4 h.

Total RNA was purified by the acidified guanidine thiocyanate method[18] and used as a template for single stranded cDNA synthesis using oligo dT primers and AMV reverse transcriptase. Primers 054R and 056R for amplification of the ovine IL-6 gene were selected from well conserved regions of the 5′ and 3′ untranslated regions of the IL-6 gene in human and mouse (Fig. 1A). The cDNA was amplified through 35 cycles using these primers and the buffer conditions of Ohara and Gilbert.[19] The product of PCR was purified by agarose gel electrophoresis and electroelution. This product was ligated directly into the phosphorylated cloning site of the plasmid pCR2 according to the manufacturer's instructions (Invitrogen, Ca., USA). Clones containing inserts were sequenced on both strands using the di-deoxy nucleotide chain termination method.

Construction of a ruminant IL-6 cassette

A primer was designed containing the 5′ 22 bases of a ruminant IL-6 sequence (derived from an unpublished bovine IL-6 sequence, B Collins, personal communication) attached to a coagulation Factor Xa recognition site, a BamHI site and a GC clamp (Fig. 1A). This primer (053R) was used in combination with a second primer (159R) containing a GC clamp and BamHI site attached to the antisense of the last 22 bases of the ovine IL-6 structural sequence (up to and including the stop codon) in a PCR using the same cDNA population as before. The resulting product, after gel purification, was digested with BamHI and cloned into the BamHI site of pTZ18R (Pharmacia). A clone containing an insert of the expected size was sequenced, and the insert cleaved out with BamHI and purified by agarose gel electrophoresis. The insert was ligated into the BamHI site of the yeast Ty expression plasmid pOGS40. Plasmids containing the correct size of insert were sequenced across the junctions between insert and vector to determine the direction of cloning and make sure that no frame shifts had occurred during cloning. A plasmid pOGIL6 was selected for further work.

Expression and purification of IL-6 from yeast

The method used was essentially that of Gilmour *et al*.[20] with modifications as published previously.[21] Briefly, pOGIL6 was transfected into yeast BJ2168 by spheroplasting, plating in regeneration agar (0.67% yeast nitrogen base; 1M sorbitol; 1% glucose; 3.0% Difco agar) and incubating at 30°C. Colonies obtained, after re-streaking, were grown in liquid culture. For inducible expression they were grown at 30°C for 48 h in YNB (0.67% yeast nitrogen base) containing 1% glucose and 0.002% tryptophan, then induced for 16–24 h with 1% galactose + 0.3% glucose and and tryptophan. Cells were harvested, suspended in 10 mM Tris-HCl pH 7.4, 2 mM EDTA, 140 mM NaCl containing protease inhibitors and lysed by vortexing with acid washed glass beads. Supernatants were spun at 10 000 × ***g*** for 5 mins, the yeast debris discarded, and the vlp containing suspension partially purified and concentrated by spinning at 30 000 × ***g*** for 90 min onto a 60% sucrose cushion. The interface and cushion material were dialysed into 100 mM Tris/HCl pH 7.4, 10 mM $CaCl_2$ containing 0.1% DOC (Na deoxycholate). Expressed protein was released from the vlps by digestion with Factor Xa at a concentration of 1:50 w/w for 4 h at 25°C and further purified by centrifugation at 40 000 × ***g*** for 40 min to remove the P1, vlps present post cleavage as well as any remaining yeast proteins. Samples were dialysed into PBS and stored at −70°C.

Analysis of ovine IL-6 RNA and protein

Alveolar macrophages were plated in Iscoves medium and stimulated with 10 μg/ml LPS for various times. Total

RNA extracted using RNAzol B (Cinna Biotix) was analysed by Northern blotting followed standard methods. For RT PCR, cDNA was prepared from 1 μg total RNA, using the method of Rolfs *et al*.[22] Primers 053R and 159R were then used in a 40 cycle PCR (otherwise as before) with 1/10 of the cDNA sample. SDS polyacrylamide gel electrophoresis and Western blots were as before.[12]

Cell proliferation assay

B9 cells, a murine IL-6-dependent hybridoma cell line, were used in proliferation assays to measure the biological activity of recombinant ovine IL-6 (rovIL-6). Proliferation was measured according to the method of Aarden *et al*., 1987.[23] 4×10^3 B9 cells were incubated in duplicate wells with test sample in RPMI1640, 10% foetal calf serum 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, in 200 μl total volume for 3 days at 37°C. They were then pulsed with 1 μCi ^{3}H-thymidine for 5 h, prior to harvesting, washing and scintillation counting.

Total immunoglobulin production assay

Washed peripheral blood mononuclear cells (1×10^5) were incubated in 96-well flat-bottomed ELISA plates in 200 μl RPMI1640 containing 2 mM glutamine, 5×10^{-5}M 2-mercaptoethanol, 200 μg/ml gentamycin, 10% fetal calf serum, supplemented with pokeweed mitogen (Gibco product 061-05360B, used at 1 in 10 000) and test samples as stated in figure legends. Assays were performed in triplicate. After 7 days in culture the supernatants were assayed in a capture ELISA for total immunoglobulin. Total Ig was measured using a mouse anti-sheep Ig light chain monoclonal antibody, VPM8,[28] to coat ELISA plates as capture antibody, and a purified rabbit anti-sheep Ig antibody directly conjugated to horse-radish peroxidase to develop (P. Bird, in preparation).

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