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The reactivity of monoclonal antibodies against orf virus with other parapoxviruses and the identification of a 39 kDa immunodominant protein*

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Summary. A panel of 27 mouse monoclonal antibodies (Mabs) was raised against orf virus. Sixteen of these Mabs reacted with a protein with a molecular mass of 65 kDa, 8 reacted with a protein with a molecular mass of 39 kDa and three remain uncharacterised. Reactivity of the Mabs with a library of recombinant vaccinia viruses expressing various regions of the NZ-2 orf virus genome identified the approximate positions of the genes encoding these 2 immunodominant orf virus proteins. The gene encoding the 39 kDa protein was identified and sequenced. The protein was detected in an envelope fraction of orf virus and was shown to be homologous to the envelope protein encoded by the H3L gene of vaccinia virus. The 65 kDa protein has not been fully characterised, but the gene encoding it has been localised to a 10 kbp region of the orf virus genome. The Mabs were used to discriminate 4 parapoxviruses derived from sheep, 2 from cattle and 1 each from a seal and squirrel. Eighteen Mabs reacted with all 4 sheep viruses, 19 Mabs reacted with both cattle viruses, 6 recognised seal parapoxvirus and 2 recognised the squirrel parapoxvirus. Only one of the 27 Mabs reacted with all 8 parapoxviruses suggesting it recognises a conserved epitope within the genus.

*The Genbank accession number of the sequence reported in this paper is AF097215.

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Introduction

Contagious pustular dermatitis is an eruptive skin disease in sheep and goats that is readily transmitted to humans [16]. It is caused by orf virus, the type species of the parapoxvirus genus in the family *Poxviridae* [7]. The lesions of the malady mainly appear around the mouth and nostrils of the affected animals. However, occurrence of the lesions in other sites, such as the foot, udder and tail have also been reported [14, 23].

Mechanisms of immunity to orf virus are not well understood and no protective antigens have been recognised. Nonetheless, in convalescent sera from infected sheep a number of immunogenic proteins have been visualised by western blotting. McKeever et al. [15] studied the antibody response of 21 sheep to orf virus infection. From the whole group, up to 16 orf viral proteins were recognised with considerable variation in the specific antigens recognised by individual sheep. Four proteins were recognised by most of the sheep but only one protein (40 kDa) was clearly recognised by all sera. In another study antibody to five orf virus proteins in convalescent serum from naturally infected sheep was demonstrated [3].

There has been only limited use of monoclonal antibodies (Mabs) to identify and characterise orf virus proteins. Lard et al. [11] produced Mabs against orf virus and showed that two of them recognised distinct proteins of molecular masses of 38–40 kDa and 40–43 kDa. Interestingly, the Mab recognising the 40–43 kDa protein of orf virus was found to react against 45–48 kDa proteins present in 2 other parapoxviruses, pseudocowpox virus and bovine papular stomatitis virus. More recently two polypeptides (molecular masses 39 and 22 kDa) of the highly passaged orf virus strain D1701, have been identified using Mabs [5]. In only one instance has the gene encoding an orf virus antigen recognised by polyclonal or monoclonal antibodies been identified [28].

In this paper we describe the preparation of a panel of Mabs directed against orf virus. We have used the Mabs to identify immunogenic orf virus proteins and in the case of an immunodominant 39 kDa antigen, characterise the gene encoding it. The Mabs were also able to distinguish parapoxvirusus derived from four species.

Materials and methods

Cells

Parapoxviruses were grown in semicontinuous lines of fetal lamb muscle (FLM) or primary bovine testis (BT) cells. Vaccinia viruses were grown in either a human thymidine kinase deficient cell line, TK143B or in CV-1 cells. FLM cells, BT cells, TK143B cells, and CV-1 cells were grown in '199' medium, supplemented Eagles minimal essential medium (MEM), Eagles modified autoclavable MEM and Eagles MEM, respectively. Media were supplemented with 10% fetal bovine serum.

Viruses

Orf-11

Isolated originally from a case of contagious pustular dermatitis in a sheep in Scotland. The virus has been passaged 22 times on sheep thyroid cells, 5 times on FLM cells, twice on fetal lamb skin (FL-Skin) and twice on FLM cells.

NZ-2

Isolated in bovine testis cells from a case of contagious pustular dermatitis in a sheep in New Zealand [24].

D-1701

A German orf virus strain, passaged in lamb kidney cells 135 times, bovine lung (BL) cells 38 times, MA104 cells fifty times and FLM twice [5].

Scabby mouth

An Australian orf virus isolate kindly provided by CSL laboratories and passaged twice on FLM cells [22].

V660

A German isolate of bovine papular stomatitis virus kindly provided by Dr. M. Buttner, Federal Virus Laboratory, Tubingen which had been passaged on BL cells eight times and twice in FLM cells [13].

B074

A German isolate of pseudocowpox virus, kindly provided by Dr. M. Buttner which had been adapted to cell culture and passaged three times on BL and five times on FLM.

Seal parapox

Isolated from a seal in Scotland the virus had been passaged four times on FL-Skin, once on foetal lamb cornea and once on FLM [20].

Squirrel parapox

Isolated from a red squirrel (*Sciurus vulgaris*) in England using red squirrel kidney cells, followed by 15 passages on FLM cells [26]. The virus was kindly provided by Dr. Steven Edwards, MAFF Central Veterinary Laboratory, Weybridge, UK.

Vaccinia virus

The origin of strain Lister (Elstree) has been described [25]

Mab production

Scab-derived orf virus (MRI reference strain) was used for one fusion. This virus was a Scottish field isolate which had been passaged on sheep. Virus for mouse immunisation was purified from scabs, using a published method [15]. For the second fusion clarified, frozen and thawed cell lysates of orf-11-infected FLM cells were used.

For the first inoculation equal volumes of either scab-derived or cell lysate orf virus antigen and Freund's complete adjuvant were homogenised into a stable emulsion. Balb/c mice received 100 μl of vaccine subcutaneously in two sites. Three weeks later, the same antigen was mixed with Freund's incomplete adjuvant and a similar does given as before. Ten weeks after the second vaccination the mice were immunised intravenously with antigen only in the tail vein (100 μl/mouse). Three days after the final immunisation mice were euthanised, blood was collected for serology and the spleens harvested. Fusion of spleen cells with myeloma cells of line NSO/1 derived from NS1/1 Ag 4.1 was carried out as described previously [12]. The medium in all wells containing hybridomas which had

grown to occupy at least half the floor of wells were screened by immunofluorescent test (IFT) (fusion 1) or by enzyme linked immunosorbent assay (ELISA) (fusion 2). Positive hybridoma cells were subjected to limiting dilution 3 times before amplification of stocks. A group of five pristane treated mice was used for ascites production for each cloned Mab. Each mouse received 5×10^6 cells in 0.2 ml of PBS by intraperitoneal injection. Ascitic fluid was collected under terminal halothane anaesthesia, centrifuged at $500\times\mathbf{g}$ for 10 min to remove cell and debris and stored at $-20\,^{\circ}$C. All the Mabs were isotyped using a Sigma isotyping kit.

Immuno assays

Immunofluorescent (IFT) tests, radio immunoprecipitations, western blotting, ELISA and serum neutralisation tests were performed as previously described [4, 15, 19, 20, 28] except that nitrocellulose blots were probed with horseradish peroxidase labelled anti-mouse IgG followed by DAB substrate and in some instances an enhanced chemiluminescence detection system (Amersham) was used. Purified antigen was prepared as described [24]. Lysates of virus-infected cells were prepared as described for the production of Mabs or as previously described [28]. A hyperimmune anti-orf virus sheep serum [1847] was used as a positive control [15]. Viral envelope fractions were prepared as described [1].

VVOV-recombinants

The library of 17 recombinant vaccinia viruses (VVOVs) each expressing defined regions of the NZ-2 orf virus genome has been described previously [17]. A vaccinia virus recombinant expressing orf virus F1L was constructed as follows. A 1.8 kbp *Hpa*I- *Bam*HI fragment derived from the right end of *Bam*HI D was subcloned into the vaccinia virus insertion vector, pUV1 [8]. The orf virus F1L gene was amplified from this plasmid by polymerase chain reaction (PCR) using Pwo DNA polymerase in conjuction with primers PUVL and I-Kpn. PUVL (CGCTAGTCACAATCACCA) corresponds to a region of pUV1 adjacent to the point of insertion of foreign DNA. I-Kpn contained 20 nt complementary to the 3′ end of the F1L gene (nucleotides 1028 to 1047 in Fig. 2) plus a noncomplementary tail which formed a *Kpn*I site when double stranded. The PCR product was digested with *Bam*HI and *Kpn*I and cloned into pUV1. The resulting plasmid was used to generate the recombinant, VVOV436, via homologous recombination at the thymidine kinase locus of the Lister strain of vaccinia virus. In this construct the F1L gene is placed under the control of the P11 promoter and the initiating methionine is replaced by a 9 amino acid sequence derived from pUV1 (Met-Ile-Ser-Cys-Arg-Ser-Thr-Leu-Glu).

DNA sequencing

DNA sequences were determined from plasmid templates using fluorescent dye-labelled dideoxynucleotides and Taq polymerase in a thermocycler. Reaction products were analysed using an Applied Biosystems 373A sequencing system according to the manufacturer's instructions.

Results

Production and characterisation of Mabs

From the first fusion using virus purified from orf scab, up to 25-secreting hybridomas were detected by immunofluorescent test (IFT) of which 10 were successfully cloned and shown to react by western blotting with a 39 kDa protein. Two were

Table 1. Characterisation of 27 monoclonal antibodies

Mab number	Isotype	OD ELISA values	Western blot	RIPA	VVOV
1C7	G1	0.35	–	65 kDa	285, 286
1D9	A	0.14	–	–	–
1G5	G1	0.29	–	65 kDa	285, 286
2B4	G1	0.45	–	65 kDa	285, 286
2B5	G1	0.27	–	–	285, 286
2B6	M	0.59	–	65 kDa	285, 286
2E5 (Orf-11)	G2a	0.38	–	–	–
2E5 (MRI)*	G1	1.79	39 kDa	39 kDa	245, 247, 436
3C7	G2a	0.50	–	65 kDa	285, 286
3F5	A	0.21	65 kDa	–	285, 286
5B5	G2b	0.49	–	65 kDa	285, 286
5C3	G1	0.60	–	39 kDa	–
6C8	G2b	0.30	–	65 kDa	285, 286
6E2	G2b	0.61	–	65 kDa	285, 286
6E8	G2a	1.99	39 kDa	39 kDa	245, 247
6E11*	G2a	0.35	39 kDa	50 kDa	–
7C9	G1	0.68	65 kDa	–	285, 286
8B6	G1	0.70	–	65 kDa	285, 286
8D7	G2b	1.23	39 kDa	39 kDa	245, 247, 436
8G5	G1	0.57	–	65 kDa	285, 286
9C3	G2b	1.40	–	39 kDa	–
10E6	G3	1.02	39 kDa	39 kDa	245, 247, 436
11B6	G1	0.47	–	65 kDa	285, 286
11D7	A	0.39	65 kDa	–	285, 286
11E4	G1	0.38	–	65 kDa	285, 286
13C10	G1	1.31	–	39 kDa	–
13E4	G1	0.52	–	65 kDa	285, 286

The optical density value recorded by ELISA and size of the antigen recognised by each Mab by western blotting or radioimmune precipitation (*RIPA*) with a lysate of orf virus-infected cells are indicated. A dash indicates that no specific antigen was detected by the particular technique. The vaccinia virus recombinants (*VVOV*) recognised by each Mab in immunofluoresence testing is indicated. A dash indicates that none of the vaccinia virus recombinants were detected. Two Mabs derived following immunisation with purified orf virus are marked with an asterisk

selected for further study. From the second fusion using lysate of orf-11 infected cells 293 hybridomas were screened and 41 were shown to be secreting orf virus antibody as detected by ELISA. Twenty-five were cloned and used to produce ascitic fluids.

Isotyping of the 27 Mabs showed that 13 were IgG1, 4 were IgG2a, 5 were IgG2b, one was IgG3 (10E6), one was IgM and 3 (1D9, 3F5, 11D7) were IgA class immunoglobulin (Table 1). There was no correlation between the strength of OD value in the ELISA and the class of isotype of the Mab. None of the 27 Mabs

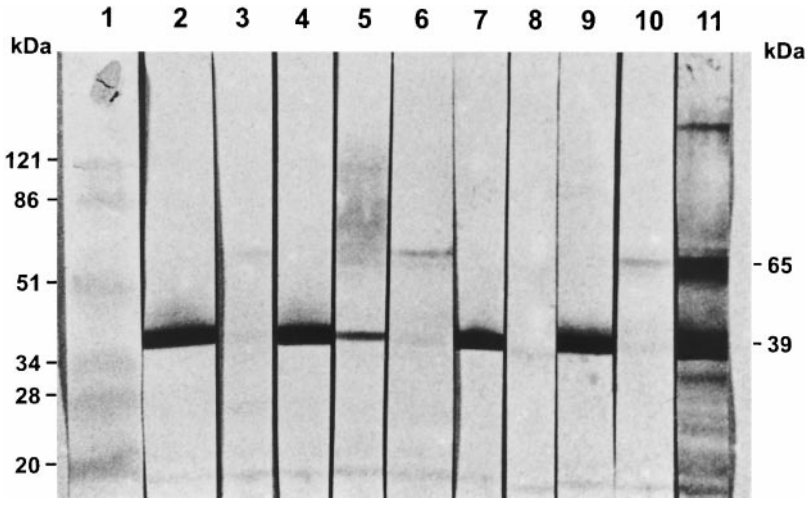

Fig. 1. Western blot of Mabs and hyperimmune serum reacted with a lysate of orf virus-infected cells prepared from freeze-thawed FLM cells four days after infection with Orf-11 virus at an moi of 0.1. *1* Molecular weight markers; *2* Mab 2E5 (MRI); *3* 3F5; *4* 6E8; *5* 6E11; *6* 7C9; *7* 8D7; *8* negative Mab (VPM20); *9* 10E6; *10* 11D7; *11* 1847 hyperimmune anti-orf virus serum

showed any neutralising activity against orf-11 virus. The serum neutralising titre of the sheep hyperimmune serum was 1/16.

In western blot studies using purified orf virus, 5 Mabs recognised a protein of 39 kDa (Table 1). When the Mabs were tested against a lysate of orf virus-infected cells the same 5 Mabs again detected a 39 kDa protein. In addition 3 Mabs detected a protein of 65 kDa (Fig. 1). The other 19 Mabs did not react by western blotting.

Radio immunoprecipitation (RIPA) with 21 Mabs produced bands. Four of the 5 Mabs reacting with a 39 kDa protein by western blotting were found to detect protein of the same molecular mass in RIPA and the fifth Mab reacted with a 50 kDa protein. The 3 Mabs which had been shown to bind to the 65 kDa protein in western blotting did not produce a result using RIPA. In total, 13 of the 21 Mabs reacting in RIPA were against 65 kDa, 7 against 39 kDa and one against the 50 kDa protein (Table 1).

Mab reactivity with a VVOV-recombinant library

Analysis of the reactivity of the Mabs with the 17 VVOV-recombinants by IFT, showed that 17 Mabs reacted with VVOV285 and VVOV286. These 2 viruses contain the same fragment of orf virus DNA recombined into the vaccinia virus genome in opposite orientations. Four Mabs reacted with the 2 over-lapping recombinants VVOV245 and VVOV247 and 6 did not react with any of the recombinants. The IFT profile of the recombinants indicated that the Mabs recognised at least 2 different orf virus proteins. None of the Mabs reacted with a vaccinia virus control recombinant containing the β galactosidase gene but no orf virus DNA.

Identification of the gene encoding a 39 kDa immunodominant orf virus protein

Analysis of the orf virus inserts carried by VVOV recombinants 245 and 247 was undertaken so as to identify the gene encoding the 39 kDa orf virus protein recognised by 4 of the Mabs. These two recombinants carry orf virus DNA fragments of 18.1 and 12.5 kbp, respectively. The locations of these two fragments on the orf virus genome have been determined and shown to overlap by 10.1 kbp [17]. This region of overlap includes three genes, F2L, F3R and F4R which are homologues of vaccinia virus genes H4L, H5R and H6R, respectively [9]. The gene immediately to the left of vaccinia virus H4L is termed H3L and has been shown to encode a dominant antigen of 35 kDa. This raised the possibility that an orf virus homologue of vaccinia virus H3L may encode the orf virus antigen produced by recombinants VVOV245 and 247.

The sequence of 1 153 bp of DNA adjacent to the 3′ end of F2L was determined and is shown in Fig. 2. The sequence of both strands were determined with a redundancy of 4.4. Analysis of the sequence revealed an open reading frame (F1L) of 1,002 bp with the potential to encode a protein with a molecular mass of 36.7 kDa. Comparisons with protein sequence databases revealed significant similarities between orf virus F1L and vaccinia virus H3L. The aligned amino

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     A  I  K  E  L  Y  M  I  N  F  N  N  * [F2L]
                                        F1L>  M  D  P  P  E  I  T  G  Y  I  I  G  V  A  E  G  R  G  T  K
   1 GCCATAAAAGAGTTGTATATGATTAATTTTAATAACTAAATGGATCCACCCGAAATCACGGGCTACATAATCGGGGTTGCCGAAGGCCGCGGGACCAAG

  21  E  V  F  P  T  L  P  Y  L  V  G  L  A  D  D  P  P  K  P  Q  P  A  P  A  P  S  P  A  P  A  P  A  P
 100 GAGGTGTTCCCCACGCTGCCGTACCTGGTGGGCCTCGCCGACGACCCGCCCAAGCCTCAACCCGCGCCTGCTCCCTCTCCTGCCCCAGCCCCCGCACCG

  54  A  P  A  P  A  P  K  P  S  P  P  A  P  H  P  K  G  D  H  V  L  K  A  V  E  W  K  D  V  D  S  K  D
 199 GCCCCCGCGCCGGCACCCAAGCCATCTCCTCCCGCGCCGCACCCCAAGGGCGACCACGTGCTCAAGGCGGTGGAATGGAAAGACGTGGACTCCAAAGAC

  87  Y  P  H  F  F  T  D  M  C  K  S  T  C  P  K  E  M  Q  R  R  A  A  H  H  L  N  L  W  E  S  I  S  A
 298 TACCCGCACTTCTTCACGGACATGTGCAAGTCCACGTGTCCGAAGGAGATGCAGCGCCGCGCAGCGCACCACCTCAACCTCTGGGAGAGCATATCGGCC

 120  G  T  V  P  T  K  Y  S  D  D  D  F  I  L  V  V  D  N  E  M  T  F  R  K  P  E  M  V  K  P  L  I  E
 397 GGCACTGTCCCCACCAAGTACTCCGACGATGACTTCATCCTGGTGGTCGACAACGAAATGACCTTCCGAAAGCCCGAGATGGTGAAGCCGCTCATCGAG

 153  A  M  K  A  N  G  W  Y  M  T  Q  L  K  E  T  Y  M  T  G  A  L  A  T  N  V  P  G  T  G  D  P  E  L
 496 GCGATGAAGGCGAACGGTTGGTACATGACGCAGCTCAAGGAGACCTACATGACCGGGGCGCTGGCCACCAACGTCCCCGGAACCGGGGACCCCGAGCTC

 186  M  V  Y  P  G  G  Y  D  V  S  L  D  A  Y  I  I  S  V  G  G  M  K  K  L  Y  D  A  I  I  K  E  G  G
 595 ATGGTCTACCCCGGGGGGTACGACGTCTCGTTAGACGCCTACATCATCAGCGTCGGCGGCATGAAGAAGCTCTACGACGCGATCATCAAGGAGGGAGGG

 219  L  R  S  G  L  L  T  E  V  F  T  L  E  K  R  L  S  L  A  R  V  V  L  S  G  A  E  Q  V  V  Y  P  E
 694 CTGCGCAGCGGCCTGCTCACCGAGGTGTTCACGCTGGAGAAGCGGCTCTCTCTGGCGCGCGTGGTGCTCTCCGGTGCCGAGCAGGTGGTCTACCCCGAG

 252  Y  Y  I  Q  V  K  T  R  L  S  G  A  P  S  L  W  S  L  L  A  T  W  L  A  R  F  W  P  G  A  I  Y  F
 793 TACTACATACAGGTGAAGACGCGGCTCAGCGGCGCGCCCTCCCTGTGGTCGCTGCTCGCCACGTGGCTGGCGCGCTTCTGGCCCGGCGCCATCTACTTC

 285  L  T  T  P  L  F  S  F  M  G  L  F  D  V  D  V  V  D  I  F  I  L  A  Y  L  L  V  L  V  L  L  L  P
 892 CTCACCACGCCGCTCTTCTCCTTCATGGGGCTCTTCGACGTGGACGTGGTCGACATCTTCATCCTGGCGTACCTGCTGGTGCTCGTGCTGCTGCTGCCG

 318  N  S  R  L  L  W  F  I  A  G  L  L  V  T  A  I  V  *
 991 AACTCGCGGCTGCTGTGGTTCATCGCCGGGCTGCTGGTCACGGCCATCGTGTGATCGTTCACGTGAGCACGCCGGACGTCGCGGCGCACCAGTGGTCGT
                                                [H2R-LIKE] *  T  L  V  G  S  T  A  A  C  W  H  D  D

1090 CCTCGAAGTAGCCGCTGTAGCCCACCAGCCCCATCATCTCTGACCCGCATGTGATGACGTTGTT
       E  F  Y  G  S  Y  G  V  L  G  M  M  E  S  G  C  T  I  V  N  N
```

Fig. 2. Nucleotide and inferred amino acid sequence of the orf virus F1L gene. The inferred amino acid sequences of portions of the adjacent genes are also indicated

acid sequences are 28.4% identical overall but between residues 188 to 300 this figure rises to 42.9% identity and to 62.5% between residues 319 to 334. The F1L protein is ten amino acids larger than its vaccinia virus homologue, H3L.

Further comparisons of the two protein sequences revealed evidence of structural and possibly functional similarities. For example, hydrophilicity plots (Fig. 3) revealed that the amino-terminal halves of each protein contain regularly spaced hydophilic domains and the C-terminal region of each protein consists of a strongly hydrophobic region which the SAPS program identifies as a high scoring transmembrane domain [2]. Both proteins include only two cysteines with the location and spacing of these residues being strongly conserved. Analysis with the program SignalP [21] did not detect a signal peptide in either protein. An unusual proline-alanine repeating motif exists near the amino terminus of the F1L amino acid sequence (amino acids 35–68, Fig. 2). In this region, 55% of the amino acids are prolines and 50% of these are followed by alanine. This motif is not in the vaccinia virus homologue.

At the DNA level the sequence of the two genes are only 34% identical. This is a reflection of the differing G/C contents of the two genes (F1L 65.1%, H3L 34.6%) which is in keeping with the differing G/C content of the two viruses (orf virus 63%, vaccinia virus 34.4%). Similarity was also seen to the 84L gene of molluscum contagiosum virus (Fig. 3) and to the I3L gene of variola virus (data not shown).

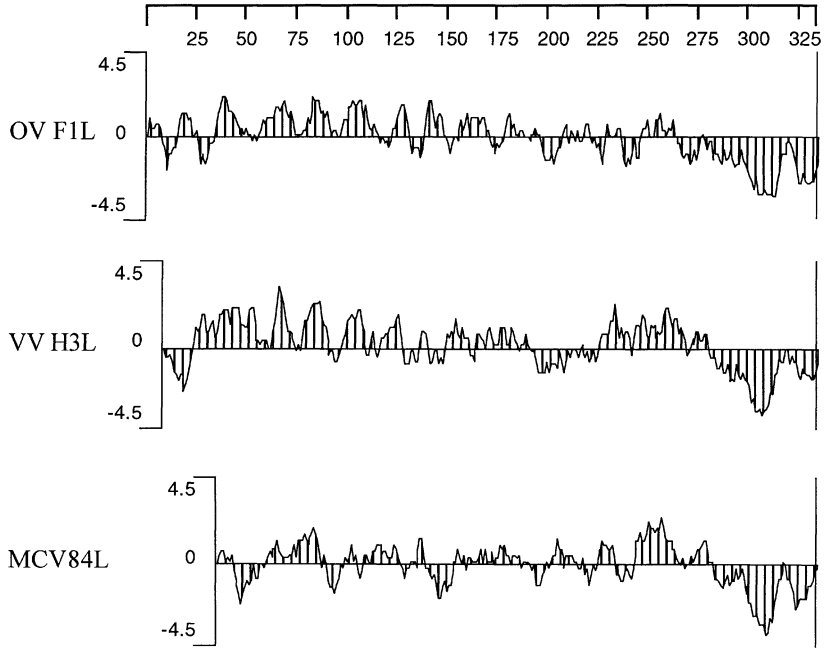

Fig. 3. Structural comparison of F1L and related proteins. Hydrophilicity profiles of the predicted proteins of orf virus F1L (OVF1L), vaccinia virus H3L (VVH3L) and molluscum contagiosum virus 84L (MCV84L) are aligned at the C-termini

The codon for the initiating methionine of F1L lies within a TAAAT(G) motif. This, in conjunction with the A/T-rich nature of the 30 bp immediately 5′ of the initiating codon suggests that F1L is transcribed late in infection.

Within 4 bp 3′ of F1L there is evidence of an adjacent gene. Figure 2 reveals 102 bp of an open reading frame that is orientated in the opposite direction to F1L. Comparisons with sequence databases revealed a significant similarity between this open reading frame and the vaccinia virus H2R protein (60% amino acid identity over 34 amino acids). It is likely that this H2R homologue represents the terminal region of the right most gene initiated in the *Bam*HI D. The relative orientations of this gene and F1L, and the spacing between them is similar to those of vaccinia virus H3L and H2R.

A vaccinia virus recombinant (VVOV436) was constructed in order to analyse the antigenicity of the F1L protein. In this recombinant the F1L open reading frame is under the control of the strong vaccinia virus late promoter, P11, and carries an 8 amino acid, N-terminal fusion which increases the predicted molecular mass from 36 728 Da to 37 419 Da.

Expression of an orf virus-specific protein by VVOV436 was tested initially by IFT. Three of the 4 Mabs which had recognised VVOV245 and 247 also recognised VVOV436 while this single gene recombinant was not recognised by any of those Mabs which recognised a 39 kDa orf virus protein but did not recognise VVOV245 or 247 (Table 1). These results were confirmed by western blotting using anti-orf virus hyperimmune sheep serum (Fig. 4A) and Mab 8D7 (Fig. 4B). It can be seen that an antigen of 39 kDa is detected in lysates of cells infected with orf virus and with recombinants VVOV245 and 247. VVOV436 expresses an antigen of slightly higher molecular weight as a result of the N-terminal fusion generated during its construction. No such antigens were detected in mock-infected lysates

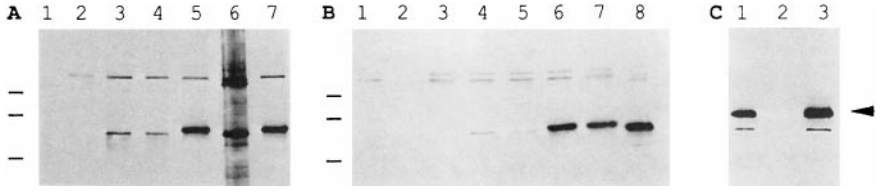

Fig. 4. Western blot analyses of the 39 kDa polypeptide encoded by orf virus gene F1L. Cell lysates were harvested from BT cells infected at an moi of three at 8 h (VVOV) or 18 h (OV) post-infection. Lysates were resolved on a 10% SDS-polyacrylamide gel, reacted with **A** polyclonal hyper-immune anti-orf virus sheep serum or **B, C** monoclonal antibody 8D7 and detected by ECL reagent. **A, B** Lanes contained lysates derived from uninfected BT cells (*A1*, *B1*) and cells infected with VVOVBGal (*A2*, *B3*), VVOV245 (*A3*, *B4*), VVOV247 (*A4*, *B5*), VVOV436 (*A5*, *B7*), VVOV380 (*A7*, *B6*) and NZ2 strain of orf virus (*A6*, *B8*). *B2* contained no sample. VVOV380 is identical to VVOV436 except that it contains an additional 800 bp of orf virus DNA 3′ of the F1L gene. Horizontal bars mark the location of molecular weight markers of 66, 46 and 30 kDa. **C** Gradient-purified NZ2 strain of orf virus (*C1*) was treated with NP-40 and 2-mercaptoethanol and separated into an insoluble fraction (*2*) and a soluble envelope fraction (*3*). Arrow shows location of 39 kDa protein

Table 2. Reactivity of Mabs with different parapoxviruses

Mab	Antigen specificity	Orf-11	D1701	NZ2	Scabby mouth	B074	V660	Seal PPV	Squirrel PPV
1C7	65	+	+	+	+	+	+	+	+
1D9	−	+	−	+	−	+	+	−	−
1G5	65	+	+	+	+	+	+	−	−
2B4	65	+	+	+	+	+	+	−	−
2B5	−	+	+	+	−	+	+	−	−
2B6	65	+	+	+	−	+	+	−	−
2E5 (Orf-11)	−	+	+	+	+	+	−	−	−
2E5 (MRI)	39	+	+	+	+	+	−	−	−
3C7	65	+	+	+	−	+	−	−	−
3F5	65	+	+	+	+	+	+	−	−
5B5	65	+	+	+	+	+	+	−	−
5C3	39	+	+	−	−	−	−	−	−
6C8	65	+	+	+	+	+	+	−	−
6E2	65	+	+	+	−	+	+	−	−
6E8	39	+	+	+	+	+	−	−	−
6E11	39/50	+	+	+	+	+	+	−	+
7C9	65	+	+	+	−	+	+	+	−
8B6	65	+	+	+	+	+	+	−	−
8D7	39	+	+	+	+	+	+	+	−
8G5	65	+	+	+	+	+	+	+	−
9C3	39	+	+	+	−	−	+	−	−
10E6	39	+	+	+	+	+	+	+	−
11B6	65	+	+	+	+	−	+	+	−
11D7	65	+	+	+	+	+	+	−	−
11E4	65	+	+	+	−	+	−	−	−
13C10	39	+	+	+	+	−	−	−	−
13E4	65	+	+	+	+	+	−	−	−
Total		27	26	26	18	23	19	6	2

The reactivity of each Mab with isolates of various parapoxviruses (PPV) was determined by IFT. Significant reactivity is indicated by a plus (+) and the absence of reactivity by a dash. The molecular mass (kDa) of the orf virus antigen detected in western blotting or RIPA is indicated

or from the control recombinant, VVBGal, which contains no orf virus DNA. These data confirmed that the 39 kDa antigen recognised by some of the Mabs and expressed by VVOV245 and 247 is encoded by gene F1L. In recombinant VVOV436 the F1L gene is transcribed from a highly expressed vaccinia virus late promoter (P11), whereas in recombinants VVOV245 and 247 the gene is under the control of its natural orf virus promoter (PF1). The relative amounts of antigen detected are consistent with the expectation, based on sequence comparisons, that P11 would be a more active late promoter than PF1 [6].

Table 3. Serum antibody responses in 8 lambs experimentally infected with orf virus strain NZ2

Serum	Antigen					
	OV	VVOV436	VVOV285	VVOV86	PCR4	VVBGal
1	2200 +	1530 +	600 +	120 −	800 +	70
2	2150 +	1900 +	900 +	200 −	550 +	80
3	2300 +	1800 +	200 −	110 −	1300 +	80
4	570 +	400 +	180 −	200 −	410 +	60
5	1900 +	1400 +	800 +	180 −	1400 +	70
6	480 +	290 +	130 −	110 −	120 −	50
7	1200 +	900 +	500 +	170 −	280 −	70
8	1500 +	1300 +	1200 +	120 −	400 +	70
1 847	2900 +	2200 +	2000 +	400 −	1900 +	170
SPF	<100	<100	<100	<100	<100	<100

Serum was taken from each animal 4 weeks after inoculation with 10^7 plaque forming units of orf virus. Dilution series of the sera were analysed by ELISA and antibody titres calculated as the inverse of the dilution at which the absorbance equalled 1.0. Each response has been summarised as positive (+) or negative (−) by comparison with the values obtained with serum obtained from a specific pathogen free lamb (SPF) or the values obtained using a control antigen consisting of a vaccinia virus recombinant which does not include any orf virus DNA (VVBGal). A positive response was defined as having a titre more than 4 times greater than the appropriate control. The titers of a hyper-immune anti-orf virus sheep serum (1 847) are also included. The antigen used were orf virus (OV) and 4 vaccinia virus recombinants carrying either single orf virus genes (VVOV436 and PCR4) or unrelated, multigene fragments of orf virus DNA (VVOV285 and VVOB86) which do not include either of the single genes (see text for details)

Characterisation of the 39 kDa immunodominant protein

Gradient-purified orf virus (NZ2) was treated with NP-40 and 2-mercaptoethanol, sonicated and centrifuged to separate the insoluble core fraction from the soluble envelope fraction. The fractions were examined by western blotting. Figure 4C shows that Mab 8D7 recognised a 39 kDa protein in the sample of purified virus and that this protein was retained in the soluble fraction but not in the insoluble fraction. These results indicate that the F1L gene product is a virion protein and that it is located within the viral envelope.

ELISA of sera from 8 lambs experimentally infected with orf virus (NZ2) demonstrated that each animal mounted a significant antibody response to the F1L antigen expressed by VVOV436 (Table 3). Also measured was the response of each animal to orf virus antigens expressed by vaccinia virus recombinants VVOV285 (containing 14.0 kbp of orf virus DNA), VVOV86 (containing 16.8 kbp of orf virus DNA) and PCR4 which expresses a 42 kDa orf virus protein able to stimulate both antibody and T cell responses in infected animals [28]. The orf virus DNA fragments carried by VVOV285 and VVOV86 do not include the F1L gene [17]. In contrast to the uniformly positive response of all animals to the F1L antigen, a majority, but not all animals, mounted a significant response to VVOV285 and PCR4, while none of these animals mounted a significant response

to orf virus proteins expressed by VVOV86. Recognition of the F1L antigen by all 8 sera was also confirmed by western blotting (not shown).

Mab reactivity with other parapoxviruses

Reactivity of the Mabs with parapoxviruses from different species by IFT and ELISA showed that it was possible to differentiate the viruses. IFT was more sensitive than ELISA for screening the viruses and revealed 13 distinct reaction patterns (Table 2). Cross-reactivity of the Mabs with the 4 isolates of orf virus demonstrated that scabbymouth contained fewer recognised epitopes than the other 3 viruses. Of the 2 cattle isolates, B074 (pseudocowpox virus) was recognised by more Mabs than V660 (bovine papular stomatitis virus). Reactivity of the Mabs with the seal and squirrel parapoxviruses was limited to 6 and 2, respectively. Seven Mabs recognised all the sheep and cattle viruses. Three (8D7, 8G5, 10E6) reacted with all the sheep and cattle viruses and the seal virus, four (2E5 (orf-11), 2E5 (MRI), 6E8, 13E4) recognised all the orf virus strains and B074 but not V660. Two (5C3, 13C10) reacted with orf viruses only and one Mab (9C3) recognised orf virus strains and V660 only. The 6E11 Mab reacted with all isolates except the seal parapoxvirus. One Mab (11B6) reacted with all the sheep viruses, the seal parapoxvirus and V660 but not with B074 or the squirrel parapoxvirus. One Mab (1C7) reacted with all 8 parapoxviruses suggesting it is recognising a conserved epitope on the 65 kDa antigen.

Discussion

When gradient purified orf virus derived from sheep scabs was used to produce Mabs all 10 hybridomas which were cloned secreted Mabs against a 39 kDa orf virus protein. In an attempt to produce Mabs against a wider range of orf virus proteins a second fusion was performed using a clarified lysate of infected cells as immunogen. The majority of Mabs produced in this way against a 65 kDa protein, but anti-39 kDa Mabs were also produced. The relative preponderance of Mabs against these 2 proteins would suggest they are immunodominant and present in substantial amounts in purified virus preparations and infected cell cultures. Two other reported attempts to produce Mabs against orf virus would tend to support this. When Lard et al. [11] used orf-virus infected cell lysates 2 characterised Mabs were shown to react with either a 40–43 kDa protein or a 38–40 kDa protein from homologous virus. Using gradient purified cell-culture grown orf virus or purified viral envelopes to immunise mice Czerny et al. [5] also succeeded in raising Mabs to a 39 kDa protein.

There is only one report where Mabs have been produced against a wider range of orf virus proteins again using infected cell lysates as inocula [27]. In that report, hybridomas were screened by IIF and 75 Mabs were shown to react by RIPA with 8 proteins of different molecular masses. Interestingly the majority, 33, were against a 66 kDa protein and 18 were against a 41 kDa protein. Other proteins detected had molecular masses of 40 (10 Mabs), 39 (5 Mabs), 42 (3 Mabs), 100 (2 Mabs), 21 (2 Mabs) and 14 kDa (2 Mabs).

A library of vaccinia virus recombinants was used to provide an initial characterisation of the Mabs. Mabs recognising the recombinants either reacted with 2 (VVOV285, 286) that carry the same 14 kbp region of the orf virus genome, or reacted with recombinants VVOV245 and 247 which carry orf virus genome fragments which overlap by 10 kbp. Mabs recognising the 285 and 286 recombinants were shown to react with a 65 kDa protein, while those recognising the 245, 247 recombinants all reacted with a 39 kDa protein.

DNA sequencing and the construction of a further recombinant (VVOV436) allowed the gene encoding the 39 kDa antigen to be identified. The gene was designated F1L, and was shown to be a homologue of the vaccinia virus gene, H3L. The H3L gene product has been shown to be an immunodominant, 35 kDa virion membrane protein [29]. In agreement with this, the F1L gene product was detected in the envelope fraction of purified orf virus. F1L encodes the third major structural protein to be identified in orf virus. The other two being the 10 kDa protein homologous to vaccinia virus A27L [18] and the 42 kDa protein homologous to vaccinia virus F13L [28].

Our observation that F1L encodes a 39 kDa antigen recognised by all sheep sera tested suggests that F1L encodes the dominant antigen of approximately 40 kDa that has been reported in several earlier studies. For example, McKeever et al. [15] detected antibody to a 40 kDa antigen in all post-infection sheep sera and also recorded that this was the first viral component to be recognised by animals undergoing primary exposure to orf virus. However, the failure of some anti-39kDa Mabs to recognise vaccinia virus recombinants expressing orf virus F1L was unexpected since 4 of them reacted with NZ-2 infected cells. The evidence suggests that either the recombinant expressed F1L protein does not contain all the natural epitopes, or that the anti-39 kDa Mabs are against more than one protein. The latter explanation is a possibility since 4 orf virus polypeptides with molecular weights between 38.5 and 41.5 kDa have been described [1].

The identity of the 65 kDa protein remains elusive. The fact that 16 of the 27 Mabs studied were against this protein suggests it is produced in significant quantities in orf virus-infected cells. Failure of all the anti-65 kDa Mabs to react with purified virus suggests that they may not be against a structural protein. All the studies using convalescent sheep serum have, however, provided conclusive evidence that a viral protein of this molecular mass is strongly immunogenic, and in vitro radiolabelling studies have suggested that a 64.5 kDa protein is a major core polypeptide [1]. The importance of the 65 kDa protein in protection against orf virus infection remains to be established.

When the panel of Mabs was tested for its reactivity with 8 different parapoxviruses variable binding was observed. There was no evidence that either the 39 kDa or the 65 kDa protein was more conserved than the other, which coincides with earlier reports [5, 11].

Reactivity of the Mabs with the 4 orf viruses showed the Australian isolate to be different from the other 3. The reactivity of this isolate was more like that of bovine papular stomatitis virus whereas the other cattle virus, pseudocowpox virus, was more reactive and was recognised by almost as many Mabs as 3 of the

orf viruses and may possibly have derived from sheep. No report of cross-species transmission of parapoxviruses between cattle and sheep has however been reported, and DNA-DNA hybridisation has suggested that parapoxviruses from cattle and sheep are clearly distinguishable [10]. Further work will be required to see if this panel of Mabs can be relied on to distinguish sheep and cattle isolates and aid in the classification of ruminant parapoxviruses. When parapoxviruses from seal and squirrel were tested both were shown to be related to the ruminant parapoxviruses with the red squirrel virus showing greatest divergence from orf virus.

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